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Jochen Maas
Franz J. Hock
Dieter Mayer
Editors

Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays

Springer Reference

Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays

H. Gerhard Vogel • Jochen Maas
Franz J. Hock • Dieter Mayer
Editors

Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays

Second Edition

With 243 Figures and 157 Tables

Springer Reference

Editors
H. Gerhard Vogel
Deceased

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In Memory of Hans Gerhard Vogel 1927–2011

Preface to the Second Edition

Both safety and pharmacokinetic aspects are important factors in the Research & Development of pharmaceuticals: safety pharmacology, toxicology and pharmacokinetics are the cornerstone disciplines of preclinical development, but the scope has widened both towards Research and towards Development over the last few years. In this book, all in-silico approaches, in-vitro, ex-vivo, in-vivo animal and clinical studies had to be taken into consideration. These studies are used to generate a lot of data which must be evaluated carefully. It was therefore necessary to include the conduct of studies but also the technologies to generate these data in a textbook like this. Since toxicology and safety pharmacology depend directly on exposure, it was also mandatory to cover PK aspects and technologies. All these aspects were included in the first edition of *Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays*.

Some of the methods described in the first edition have been introduced into the scientific community over the years and decades – for instance specific chromatography technologies like gas chromatography – and there were no novel tendencies to be observed between the dates of the first and second editions. These chapters were not modified since all the information included can still be regarded as "state of the art".

But there are other topics which have shown considerable development with many new insights and technologies. Transporters and their biology are the best example of these areas: these chapters have been completely modified since the first edition.

A third group of chapters had to consider some new aspects without it being necessary to modify them completely. These were amended by adding new results and experiences.

And last but not least, I want to thank again all the authors who contributed despite their very busy daily lives in the pharmaceuticals industry. And a special thank you to Hans Gerhard Vogel who was the Editor-in-Chief of the first edition and all of the *Drug Discovery and Evaluation* titles at Springer consisting of *Pharmacological Assays, Safety and Pharmacokinetic Assays* and *Methods in Clinical Pharmacology*. Gerhard Vogel passed away in 2011. He initiated the second edition of this title. Without him neither this second edition nor any of the other titles of *Drug Discovery and Evaluation* would ever have been completed.

Frankfurt, Germany 2013 Jochen Maas

Preface to the First Edition

Drug discovery and evaluation has been a sequential process for a long period of time. It started with the selection of the most active compound from a series of newly synthesized compounds with the help of special pharmacological assays. Safety aspects were considered by testing the selected compound in high doses in tests directed to indications other than the intended indication of the new compound. These tests were followed by pharmacokinetic studies, which were mainly aimed at confirmation of a suitable half-life time and of oral activity. Safety relied on acute and subacute toxicity studies, which gave information more on organ structure than on organ function.Toxicological and pharmacokinetic studies were adapted to the progress of studies in clinical pharmacology and clinical trials. This strategy has been changed during the last 15 years for several reasons:

Some negative effects on organ function, e.g., ventricular tachy-arrhythmia were detected too late. On the other hand, negative findings in chronic toxicity studies in animals turned out to be irrelevant for human beings.

New scientific approaches, e.g. combinatorial chemistry, high-throughput screening, in silico models, pharmaco-genomics and pharmaco-proteomics offered new possibilities.

The success rate in the pharmaceuticals industry and the introduction of new chemical entities to the market per year dropped dramatically, whereas the development time for new compounds increased, sometimes exceeding the patent protection. This forced a change of strategy:

- Parallel instead of sequential involvement of the various disciplines.
- The term "Safety Pharmacology" was coined.
- An International Conference on Harmonization (ICH) Safety Pharmacolog working group was founded.
- Easily accessible and most informative tests must be selected.

Exposure of drug to the body by pharmacokinetic studies on absorption, distribution, metabolism and excretion must be investigated at an early stage of development and can contribute to the selection of a compound for development.

Toxicology experienced major improvements by the introduction of new methods, e.g., in silico methods, toxicogenomics and toxicoproteomics.

These aspects stimulated our decision to publish this volume as a counterpart to "Drug Discovery and Evaluation. Pharmacological Assays" (Second Edition Springer-Verlag 2002). The current book contains three sections. Dr. Franz Jakob Hock shares with me the responsibility for the section "Safety Pharmacology"; Dr. Jochen Maas took over the responsibility for the section "Safety Pharmacokinetics" and Prof. Dr. Dieter Mayer for the section "Safety Toxicology".

As with the book on pharmacological assays, this book is intended to aid both scientists and students. The reader can find methods for selecting candidates for drug development at early stages, such as screening methods based on the stage of development, or methods up to advanced stages of development and which are considered necessary for international approval from the scientific and regulatory point of view.

Juni 2006 H. Gerhard Vogel

Acknowledgments

″I want to thank all authors of the PK-part for their tremendous work. It was often not easy to complete their individual chapters in addition to their busy daily activities in their respective companies. A special thanks goes to Dr. Roland Wesch who supported me in an optimal way to collect, review and edit all information received from the different authors″

Jochen Maas

About the Editors

Jochen Maas

Jochen Maas is appointed General Manager, Research & Development (R&D) at Sanofi Deutschland GmbH, as of October 1st, 2010, based in Frankfurt. He is a member of the Global R&D Management Board and of the German Management Board. He was appointed as head of the German hub R&D organization in 2012.

Jochen has huge experience in all phases of the R&D value chain. He started his career in PK, then he expanded his responsibilities to preclinical Development, preclinical and clinical Development and Research & Development. Afterwards, he was responsible for Global Research & Development in the Diabetes Division and acted as Vice President R&D Europe at sanofi-aventis.

Jochen also lectures in pharmacokinetics and administering medication as a professor at Gießen-Friedberg University of Applied Sciences.

He is a biologist and veterinarian. He has a doctorate in veterinary medicine including a specification in Radiology. He studied at the Universities of Zurich, Heidelberg and Munich. After joining the Group in 1992 as head of the pharmacokinetics laboratory, he held various R&D management positions in Germany and France.

Franz J. Hock

Since retiring from Aventis in 2002, Dr. Hock has leveraged his experience as a freelance consultant specializing in Safety Pharmacology. Dr. Hock was a research scientist at Hoechst, Hoechst Marion Roussel and Aventis from 1976 – 2002. He initially worked on methods in general pharmacology and nephrology, before becoming Head of a Laboratory devoted to pharmacological methods for drugs influencing memory and learning. He was ultimately Head of Laboratory for General/Safety Pharmacology at the Frankfurt site of Aventis Pharma Deutschland GmbH. Dr. Hock received his MSc in Neurobiology from the Technical University Darmstadt and his DSc in Zoology form the University Kassel, Department of Biology, Institute of Neuroethology and Biocybernetics.

He received the degree of Fachpharmakologe DGPT ("certified expert pharmacology") in 1981. In 1983 he spent a sabbatical year at the University of California, Irvine, at the Center for the Neurobiology of Learning and Memory (Director Prof. Dr. James L. McGaugh).

He lectured for several years to students in Biology at the University of Kassel und the Technical University of Darmstadt. He has published over 100 original papers on methods in pharmacology and on new compounds.

He is currently a member of the Task Force General/Safety Pharmacology German/Swiss Pharmaceutical Companies. A member of several national and international scientific societies, Dr. Hock is a founding member of “Safety Pharmacology Society”, “Neurowissenschaftliche Gesellschaft e.V.” and “European Behavioural Pharmacology Society”. He served since several years as a member of the program Committee of the Safety Pharmacology Society. He is member of several domestic and international scientific societies.

Dieter Mayer

Dieter Mayer studied veterinary medicine at the University of Munich, Germany. Thereafter, he worked on a thesis on biochemical mechanisms of heavy metal intoxication at the Institute of Pharmacology and Toxicology at the Ludwigs-Maximilian-University of Munich.

In 1975 he joined Hoechst AG Frankfurt, Germany. He worked there at the Institute of Industrial Toxicology and was in charge of the safety assessment of Acesulfam, an artificial sweetener. Further projects consisted of fluorocarbons as replacements for chlorofluoro-carbons. He contributed to the development of several pesticides.

Dieter Mayer was a member of the German MAK (TLV) committee for about 12 years. He worked at the University of Davis, California and at the Centre International de Toxicologie (affiliation of Hoechst AG), Evreux, France, where he held the position of Scientific Director.

In 1986 he became Head of the central toxicology department at Hoechst AG. This entailed responsibility for the entire Hoechst portfolio, and hence also for pharmaceuticals. He was involved in the successful development of antiinfectives, cardiovascular drugs, several insulins, CNS drugs and anti-rheumatics. In 1998 he was promoted to Vice President of Lead Optimization (Toxicology, DMPK and Clin. Pharmacology).

Prof. Mayer has been teaching toxicology at the University of Frankfurt since 1991 and has authored more than 90 scientific articles, abstracts and oral presentations. Today Dieter Mayer is a Consultant for the global pharmaceutical industry and is a member of the Advisary Board of National German Research Associations.

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Part 1

Safety Pharmacology

Safety Pharmacology: Introduction

1

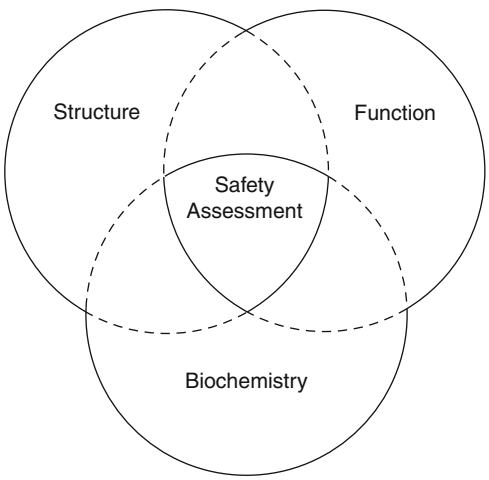
Franz J. Hock

The adverse effects of new drug entities in animals and humans can be manifested by changes in the structural, biochemical, or physiological status of the organism. In the preclinical safety assessment process, toxicological procedures have traditionally focused on the structural and biochemical consequences of drug actions. As a result, there has been a reliance on histopathological evaluation of organs and clinical pathology measures in the toxicological assessment of new drug candidates. Physiological or functional observations of drug action were mostly conducted outside of the safety assessment processes. The last decade has seen a growing awareness of the importance of physiological measures of drug toxicity. This awareness has been prompted by clinical issues of life-threatening effects, e.g., cardiovascular functions. These issues have prompted regulatory action and involvement with respect to "safety pharmacology."

Safety pharmacology refers to the assessment of adverse effects of drugs on functional and physiological systems including central and peripheral nervous system, cardiovascular system, respiratory system, and renal and gastrointestinal functions. The evaluation of functional or physiological toxicities plays a key role in the safety assessment process. It should be viewed as complementary to traditional assessments of toxicity based upon morphological or biochemical lesions. Safety pharmacology is not a new topic, but one with renewed interest in the pharmaceutical industry and the regulatory bodies governing the drug approval process.

F.J. Hock
Business Development Consultant, CorDynamics, Dieburg, Germany

Safety pharmacology can be thought of as one of the key areas to be integrated with structural and biochemical evaluations in the complete safety assessment program (see scheme below).



The authors of this section have assembled information to assist scientists in industry in (1) understanding the recent regulatory status and expectations for safety pharmacology testing and (2) the conduct of safety pharmacology studies in broad. As evidenced by their extensive experience, the authors share a deep commitment to the conduct of safety pharmacology studies and are leaders in this field of drug safety assessment. The emphasis of this section is to provide practical information and approaches to the assessment of central and peripheral nervous system, cardiovascular system, respiratory system, renal and gastrointestinal functions, and of other systems and functions involved in safety issues. Furthermore, in this section, new chapters such as on biologics, antibiotics,

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monoclonics, oncology, stem cells, transgenic animals, etc., are added, showing recent developments in the field of safety pharmacology.

The following sections will detail approaches and options to assessing physiological functions as part of the overall safety assessment of new drug entities, classical and biologics, etc.

Finally, we would like to thank the authors for their contributions to this section.

Status of Safety Pharmacology and Present Guidelines

2

Franz J. Hock

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Ray Lipicky http://spo.escardio.org/eslides/view.aspx?eevtid=40&fp=201 (Director, LIPICKY, LLC, A Consulting Company; retires from FDA in February 2002) started one of his presentations with the following slide:

"Safety" is of Increasing Importance
Although in a round about way is the major theme of my talk; it is among the most important factors shaping the next 10 years of development

WHAT IS SAFETY? (who knows)

Years ago Paul Leber (Neuropharm) said: "Safe" is what I say is "Safe"

Drugs known to kill have been approved if they were more effective than anything else available

Merck and FDA (Viox) said excess 5 Morbid/Mortal events/1,000 patient years requires coming off the market without any regard for benefit

For glucose control, recently an Advisory Committee recommended that prior to approval, for morbid/mortal events a RR >1.3 be rules out –but if you are "old" (or "relatively old") ruling out 1.8 is OK

Gerhard Zbinden (1979) formulated in this way:

> The adverse drug reactions which the standard toxicological test procedures do not aspire to recognize include most of the functional side-effects. Clinical experience indicates, however, that these are much more frequent than the toxic reactions due to morphological and biochemical lesions. . . .

2.1 Origins of Safety Pharmacology

Leber (2002) wrote in his paper: "For over 60 years, the United States has relied primarily on a federal system of premarket drug product clearance to ensure the quality of the nation's drug supply. When the premarket clearance system was first introduced in 1938 in the aftermath of the Elixir of Sulfanilamide tragedy in which over a 100 patients needlessly dies

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because of a drug manufacturer's carelessness (Jackson 1970), federal law required only that new drugs be tested and shown, prior to marketing to be 'safe for use.'"

Serious injury and/or death of volunteers and patients participating in early clinical trials are rare and thus very disturbing when it occurs (Bass et al. 2004a, b; Marshall 2001a, b; Miller 2000). The organ systems and functions most frequently responsible in these events are the central nervous (seizure), cardiovascular (hypotension, hypertension, and arrhythmia), respiratory (asthma/bronchoconstriction), and renal (glomerular filtration) systems, and the result is almost always a critical care emergency (Kinter et al. 1997). The origins of safety pharmacology are grounded upon observations that organ functions (like organ structures) can be toxicological targets in humans exposed to novel therapeutic agents and that drug effects on organ functions (unlike organ structures) are not readily detected by standard toxicological testing (see Mortin et al. 1997; Williams 1990; Zbinden 1984).

Already in the late 1960s, even more in the 1970s, major pharmaceutical companies, alerted by the thalidomide disaster, intensified the preclinical testing of drug candidates. In the USA, since 1962, a law requires that new drug products also be shown to be "effective in use" under the conditions of use recommended in their proposed labeling. Insofar as safety is concerned, the Act demands that a sponsor provide full reports of all tests necessary to establish that the product will be safe for use. On the other hand, Leber (2002) writes that no pharmacologically active drug substance is ever likely to be entirely free of risk. Accordingly, the agency maintains that a regulatory determination that a drug is "safe for use" is, in actually, a favorable "risk-benefit" determination.

For example, in the pharmacological laboratories of the former HOECHST AG, Germany, each compound proposed for development was thoroughly investigated under the term "General Pharmacology." In the specialized laboratories, e.g., for cardiovascular and respiratory function, nephrology, blood coagulation, psychopharmacology, neuropharmacology, analgesic and anti-inflammatory research, gastroenterology, diabetology, atherosclerosis, and endocrinology, the potential new drug was studied according to internal rules for which the head of the department of pharmacology was responsible. For the performance of the tests, the heads of the special laboratories were responsible. Tests rarely being used were performed in a laboratory for general pharmacology.

These rules emphasized:

1. Application in the routes proposed for clinical use (e.g., oral, intravenous).
2. Use of doses considerably higher than those found to be active in pharmacological tests for the proposed indication. If possible, at least ten times higher doses were used.
3. Use of the animal species, which was considered the most sensitive in the special indication; however, several species had to be studied.
4. Multiple dose application, if effects could not be detected after single application, e.g., in atherosclerosis research or in endocrinology.
5. In-depth analysis when unexpected results were found.

Unfortunately, these rules were never published partially because pharmacologists wanted to perform their studies without supervision by GLP. However, the results were published if the investigational drug reached the market (e.g., Omosu et al. 1988). Since only 5% of compounds proposed for development overcome all the hurdles, the majority of data were also not published.

As Kinter et al. (1994) reported, in many pharmacological laboratories prior to 1990, organ function testing was often conducted as an ancillary function of discovery research. The selection of specific studies for a candidate drug was based on concerns raised from its primary (those pharmacodynamic effects related to a drug's targeted indication; special pharmacology) or secondary (those pharmacodynamic effects unrelated to a drug's targeted indication; general pharmacology) pharmacology, or known effects associated with the drug's pharmacological, therapeutic, or chemical class. This ad hoc approach to safety evaluation led to nonsystematic decisions regarding study designs and organ systems studied. Often, the study designs employed were those available for the assessment of efficacy, not safety endpoints (e.g., blood pressure determinations in anesthetized felines). In addition, study designs employed dose levels that exceeded the projected clinical efficacy levels by small multiples, if any. Systemic exposures associated with those dose levels were seldom documented; indeed, investigators were sufficiently

Table 2.1 Comparison of different regulatory guidelines (before 1995)

Deutschland (Germany)	EU	USA	Japan
Allgemeine Verwaltungsvorschrift zur Anwendung der Arzneimittelprüfrichtlinien (Bundesrat Drucksache 580/89; 3. Abschnitt, C 1; Okt. 1989) *Pharmakodynamik* • Wirkungsspektrum eines Stoffes ermitteln • Prospektive erwünschte Wirkungen • Wirkungen auf vitale Funktionen (allgemeine Pharmakodynamik) • Dosis-Wirkungs-Beziehung für erwünschte und unerwünschte Wirkungen sollen ins Verhältnis gesetzt werden	Notice to applicants (Part III F; Jan.1989) *Pharmacodynamics* Studies conducted to establish the pharmacodynamic effects and the mode of action should be evaluated in the following order: • Studies demonstrating desired therapeutic effects (special pharmacodynamics) • Studies demonstrating effects in addition to desired effects (general pharmacodynamics) • Studies to detect drug interaction	Guidelines for the format and content of the nonclinical pharmacology/toxicology section of an application (FDA; Feb. 1987) Part II. D. 1. Pharmacology studies should be presented in the following order, with pharmacodynamic ED_{50} in dose-ranging studies preceding mechanism of action studies • Effects related to the therapeutic indication (primary and secondary activities) • Effects related to possible adverse reactions • Interactions with other drugs	Guidelines for general pharmacology studies (MHW 1995) • Effects on general activity and behavior • Effects on the central nervous system • Effects on the autonomic nervous system and smooth muscle • Effects on the respiratory and cardiovascular systems • Effects on the digestive system • Effects on water and electrolyte metabolism • Other important pharmacological effects • And special additional tests

aware of this criticism that early organ function testing was often conducted using intravenous administration, regardless of the intended clinical route of administration (Kinter et al. 1997). These early organ function assessments were normally disjointed and disconnected from the results of the toxicology program. Attempts to add organ function endpoints to toxicology protocols were frustrated by the fact that data were collected without regard to the physiological status of the subjects and/or pharmacokinetic parameters (Lufy and Bode 2002; Morgan et al. 1994).

Prior to 1990, regulatory guidances on organ function testing were limited. There were some "guidelines," but each country or area had their own regulatory ones (Table 2.1). These guidelines were very unspecific except the Japanese one (see below). The international regulatory documents differ as well (Table 2.2). The US and European regulations provided only general references to evaluations of drug effects on organ system functions (Gad 2004; Kinter et al. 1994; Lumley 1994). Organ function assessments included with investigational new drug applications (INDs) and registrations (NDAs) were inconsistent and often viewed as unimportant (Green 1995; Proakis 1994). However, in Japan, the Ministry of Health and Welfare (now referred to as the Ministry of Health, Labour, and Welfare [MHLW]) had promulgated comprehensive guidances for organ function testing as early as 1975 (see Table 2.3). These guidelines described which organ systems would be evaluated (including central and peripheral nervous systems, cardiovascular, respiratory, gastrointestinal, and renal) as a first tier evaluation (category A studies) and made specific recommendations regarding study designs (including description of models, criteria for dose selection, and which endpoints would be included in the investigation). The guidelines also described a second tier of studies (category B) to be conducted based on the significant findings in the category

Table 2.2 Excerpts from international regulatory documents

- ". . . studies that otherwise define the pharmacological properties of the drug or are pertinent to possible adverse effects." *(**United States of America**, 21 CFR (Section 314:50, paragraph 2)*
- ". . . a general pharmacological characterization of the substance, with special reference to collateral effects." *(**European Economic Community**, Directive 91/507/EEC)*
- "A general pharmacological profile of the substance is required, with specific reference to collateral effects. . .Methods of screening will vary. . .but the aim should be to establish a pattern of pharmacological activity within major physiological systems using a variety of experimental models." *(**United Kingdom**, Medicines Act 1968, Guidance notes on applications for product licences, MAL 2, p. A3F-1)*
- "Secondary actions—studies related to secondary pharmacological actions of the new drug which may be relevant to expected use or to adverse effects of the new drug." "Other studies—pharmacological activities of the drug that may be pertinent to safety and which may or may not be relevant to proposed clinical trials. . ." *(**Canada**, RA5 Exhibit 2, Guidelines for preparing and filing drug submissions, p. 21)*
- "Studies should reveal potentially useful and harmful properties of the drug in a quantitative manner which will permit an assessment of the therapeutic risk. . . Investigations of the general pharmacological profile should be carried out." *(**Australia**, Guidelines for preparation and presentation of applications for investigational drugs and drug products under clinical trial exemption scheme, pp. 12, 15)*
- "New drugs should be studied in a biological screening program so as to define any action over and above that which is desirable for the therapeutic use of the product." *(**Nordic countries**)*
- "The objective of general pharmacological studies is to examine extensively the kind and potency of actions, predict potential adverse effects likely to manifest in clinical practice. . .." *(**Japanese** Guidelines for Safety Pharmacology Studies, 1995)*

A investigations. Because the Japanese guidelines were the most comprehensive of their time, they became the de facto foundation for organ function safety testing throughout the pharmaceutical industry (Kinter and Valentin 2002). The organ function studies included in categories A and B were intertwined with studies whose aim was to catalog additional pharmacological functions and activities (secondary or general pharmacology) in addition to the primary pharmacological function/activity. Kinter et al. (1994) first distinguished two subgroups of objectives embedded in the Japanese studies as safety and pharmacological profiling. This concept was enlarged upon by the International Conference on Harmonization (ICH) safety pharmacology expert working group to define three categories of pharmacological characterizations: primary and secondary pharmacodynamic, and safety pharmacology (see ICH S7A, Table 2.3).

During the same period, European, US, and Japanese regulatory agencies prepared positions on general pharmacology/safety pharmacology in the form of guidance and concept papers (Bass and Williams 2003; Kurata et al. 1997; Table 2.3). Draft documents appeared from Japan, Europe, and USA by 1998. Later that year, the Ministry of Health and Welfare and the Japanese Pharmaceutical Manufacturer's Association (JPMA) proposed to the ICH Steering Committee the adoption of an initiative on safety pharmacology. This proposal was accepted and given the designation of Topic S7.

The origin of the term safety pharmacology is obscure. It first appeared in drafts of the ICH guidelines "Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals," Topic M3, and "Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals," Topic S6 (see Table 2.3). ICH S6 stated that ". . .The aim of the safety pharmacology studies should be to reveal functional effects on major physiological systems (e.g., cardiovascular, respiratory, renal, and central nervous system). . .." The ICH Topic S7 Expert Working Group began their work in the first quarter of 1999, and a harmonized safety pharmacology guideline was finalized and adopted by the regional regulatory authorities over 2000–2001 (ICH S7A). The guidelines describe the objectives and principles of safety pharmacology, differentiate tiers of investigations ("safety pharmacology core battery," "follow-up," and "supplemental" studies), establish the timing of these investigations in relationship to the clinical development program, and embrace GLP procedures (when applicable).

A significant issue that was extensively debated by the ICH Topic S7 Expert Working Group was how to evaluate the potential of new drugs to produce a rare but potentially life-threatening ventricular tachyarrhythmia (torsade de pointes) in susceptible individuals (Ackerman 1998; Anderson et al. 2002; De Ponti et al. 2001; Haverkamp et al. 2000). The incidence of torsades de pointes with drugs that are targeted at noncardiac indications can be very low, for example, 1 in 120,000, and, hence, the imperative to find nonclinical surrogates to identify those drugs with the potential to elicit this serious cardiac arrhythmia (Malik and Camm 2001; Moss 1999; Thomas 1994;

Table 2.3 Current international guidelines and draft documents on safety pharmacology

Year	Document
1975	Notes on application for approval to manufacture (import) new drugs, issued in 1975 (MHW Japan)
1995	Japanese guidelines for nonclinical studies of drugs manual 1995. Yakuji Nippo Tokyo, 1995
1998	Guideline for safety pharmacology study (draft 3.17, 1998; Japan; personal communication, Dr. K. Fujimori)
	Committee for proprietary medicinal products (EU). Note for guidance on safety pharmacology studies in medicinal product development (Draft 1998) FDA DRAFT concept paper on safety pharmacology
	Committee for proprietary medicinal products (EU). Points to consider: the assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products
1997	ICH S6: preclinical safety evaluation of biotechnology-derived pharmaceuticals, EU: adopted September 1997 MHLW: adopted February 2000 FDA: adopted November 1997
	ICH M3: nonclinical safety studies for the conduct of human clinical trials for pharmaceuticals, July 1997
2000	S7A: safety pharmacology studies for human pharmaceuticals EU: adopted November 2000
2001	Guidance for industry. S7A safety pharmacology studies for human pharmaceuticals. US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, ICH. July 2001 MHLW: adopted June 2001
	Therapeutic products directorate guidance document (Canada). Assessment of the QT prolongation potential of non-antiarrhythmic drugs (2001)
2002	The clinical evaluation of QT/QTc interval prolongation and proarrhythmic potential for non-antiarrhythmic drugs. FDA DRAFT preliminary concept paper. Nov. 15, 2002
2003	ICH guideline on safety pharmacology studies for assessing the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals (S7B). Sept, 2, 2003
	The clinical evaluation of QT/QTc interval prolongation and proarrhythmic potential for non-antiarrhythmic drugs. ICH E14 Step 1 Draft 2 (July 17, 2003)
	FDA (draft) guidance for industry: nonclinical studies for development of pharmaceutical excipients. February, 2003
	FDA (draft) guidance for industry: nonclinical studies for development of medical imaging agents. February, 2003
	CPMP position paper on nonclinical safety studies to support clinical trials with a single microdose. July 2003
	FDA (draft) guidance for industry: nonclinical safety evaluation of pediatric drug products. February, 2003
2004	S7B revised: the nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. Step 3, June 2004
	E14: principles for clinical evaluation of new antihypertensive drugs. Step 3, June 2004
2005	S7B (Step 5; May 2005): EU: adopted by CHMP May 2005, issued as CHMP/ICH/423/02. EU: adopted May 2005 MHLW: adopted October 2009 FDA: adopted October 2005
	E14 (Step 5; May 2005): EU: adopted by CHMP May 2005, issued as CHMP/ICH/2/04. Date for coming into operation: November 2005. MHLW: adopted October 2009 FDA: adopted October 2005
2009	S9: nonclinical evaluation for anticancer pharmaceuticals (Step 5, October 2009) EU: adopted November 2009 MHLW: adopted June 2010 FDA: adopted March 2010
	M3(R2): guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (Step 5, June 2009) EU: adopted June 2009 MHLW: adopted February 2010 FDA: adopted January 2010

Viskin 1999; Webster et al. 2002). The surrogates of cardiac ventricular repolarization prolongation have included in vitro assessment of drug effects on repolarizing cardiac ion currents (e.g., sodium current, I_{Na}, calcium current, I_{Ca}, rapid, delayed potassium rectifying current, I_{Kr}, slow, delayed potassium

rectifying current, I_{Ks}, and inward rectifying potassium current, I_{K1}) and cardiac cell action potential waveforms (Hammond et al. 2001; Redfern et al. 2003), and in vivo electrocardiography assessments of QT Interval Prolongation (with heart rate correction, QTc), monophasic action potentials, and effective refractory periods (Batey and Doe 2003; Champeroux et al. 2009, 2010; Hammond et al. 2001; Holzgrefe et al. 2007a, b; Spence et al. 1998; Vargas et al. 2008). The controversial issue is the accuracy of these models to identify problematic drugs, and how these data may be assimilated into an assessment of human risk (Kinter and Valentin 2002). Recognizing that resolution would not be easily forthcoming, the ICH S7 Expert Working Group proposed to the ICH Steering Committee that a new initiative be accepted to generate guidelines on the assessment of drugs for effects on cardiac ventricular repolarization. This proposal was accepted in November 2000 and was designated ICH Topic S7B, "Guideline on Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals." The guidelines on safety pharmacology finalized at the same ICH meeting was redesigned Topic S7A, "Safety Pharmacology Studies for Human Pharmaceuticals." Recently, it was found that some compounds show a QT shortening. This finding is recognized as an emerging potential safety issue with a clear business impact (Del Rio et al. 2001; Deurinck and Traebert 2008; Himmel and Hoffmann 2010; Holbrook et al. 2009; Lu et al. 2008a, b; Pugsley and Curtis 2007; Regan et al. 2008). According to Lu et al. (2008a, b), drug-induced QT shortening is associated with a potential risk for ventricular tachycardia (VT) and ventricular fibrillation (VF), which is invariably fatal, whereas the QT prolongation–associated torsades de pointes has only an approximately 20% incidence of death. Lu et al. (2008a) concluded in their paper, that the current S7B regulatory guideline may be useful for predicting drug-induced QT prolongation; amendments to this guideline may be warranted in the future to address the potential liability for drug-induced short QT and the associated fatal arrhythmias (Shah 2010).

Shortly after the adoption of the ICH Topic S7B, the US Food and Drug Administration and the Pharmaceutical Research and Manufacturers of America proposed to the ICH Steering Committee the adoption of a parallel initiative to prepare guidelines on clinical testing of new therapeutics for their potential to prolong ventricular repolarization. This proposal was accepted as ICH Topic E14 entitled, "The Clinical Evaluation of QT/QTc Interval Prolongation and Pro-Arrhythmic Potential for Non-Antiarrhythmic Drugs." In November 2003, the ICH Steering Committee directed the ICH Topic E14 and S7B expert working groups to align their respective guidelines, in particular, the role that nonclinical findings will serve in the design of the clinical study to assess a drug's effect on ventricular repolarization (QT interval). Both guidelines were recommended for adoption at step 5 of the ICH process in May 2005 by the ICH Steering Committee. The Committee for Medicinal Product for Human Use (CHMP) of the EMEA has adopted both guidelines in May 2005 (Shah 2005a, b) and issued as CHMP/ICH/423/02 (S7B) and CHMP/ICH/2/04 (E14). The date for coming into operation of the S7B was November 2005, and in the Federal Register (FDA), it was published in Vol. 70, N 202, pages 61133–61134; October 20, 2005. The date for coming into operation of the E14 was November 2005, and it was published in the Federal Register, Vol. 70, N 202, pages 61134–61135, October 20, 2005.

2.2 Practice of Safety Pharmacology (ICH S7A)

Safety pharmacology is ". . .those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relationship to exposure in the therapeutic range and above. . ." (ICH S7A, Table 2.3). Three primary objectives are encompassed in these investigations.

1. To provide a perspective of the potential pharmacodynamic risk posed to humans by exposure to a new therapeutic agent. This is accomplished through the pharmacodynamic characterization of the new drug on central (peripheral) nervous (Haggerty 1991; Mattsson et al. 1996; Moser 1991; Porsolt et al. 2002; Ross et al. 1998), cardiovascular (Bunting and Siegl 1994; Lacroix and Provost 2000; Kinter and Johnson 2003), and respiratory (Murphy 1994, 2002; Sarlo and Clark 1995) systems (safety pharmacology core battery studies), and other major organ systems (supplemental studies; e.g., gastrointestinal [Baldrick et al. 1998; Kinter 2003; Mojaverian 1996] and

renal [Chiu 1994; Kinter 2003]) as appropriately based on concern for human safety.

2. To investigate the underlying mechanism(s) of observed effects to refine and improve upon the integrated assessment of the risk posed by the drug when adverse findings have been noted in nonclinical or clinical investigations. These may be follow-up studies of the safety pharmacology core battery or the study of other major organ systems (supplemental studies) based on a potential clinical concern (Gad 2004; Kinter and Dixon 1995; Williams and Bass 2003).
3. To determine the temporal relationship between the pharmacodynamic responses noted with the test substance and the peak blood levels of parent drug and any major metabolites. This information will be used to identify the peak drug levels at the low-observed-effect level (minimal dose level tested that produces an effect; LOEL) and no-observed-effect level (maximum dose level tested without an effect; NOEL), the relationship between parent drug and/or major metabolite and the pharmacodynamic response, and whether the pharmacodynamic changes noted with the test material may be related to animal-specific metabolites. These data are critical to defining a margin of safety between the NOEL and the projection of plasma levels needed to achieve clinical efficacy. They also serve to define the human risk posed by exposure to the new drug (e.g., little risk if the response can be attributed to an animal-specific metabolite) and the possible timing of onset and recovery from any observed effects (Williams and Bass 2003).

The safety pharmacology core battery and any supplemental studies deemed to be necessary to assure that human safety are to be conducted in advance of initial clinical trials ("first in human" studies) so that a new drug can progress safely into the clinical phases with an appropriate level of monitoring. In situations where the adverse effects are judged to be potentially serious, or when unexpected pharmacodynamic effects occur in humans, the next tier of testing, investigational safety pharmacology studies (e.g., follow-up or supplemental studies), may be appropriate (ICH S7A; see Table 2.3).

The ICH S7A guideline has brought uniformity to the evaluations of new drugs for effects on organ functions, mandating with few exceptions, that all drug candidates will be evaluated in the safety pharmacology core battery studies and in follow-up and supplemental as appropriate to assure human safety (Pugsley et al. 2008). The cardiovascular, respiratory, and central (peripheral) nervous system functions were selected for the safety pharmacology core battery based on the concern that an acute failure of these systems would pose an immediate hazard to human life. The examination of additional organ systems (gastrointestinal, renal, etc.) may also be appropriate based on a cause for concern for human safety. The experimental models, endpoints, and study designs chosen should be relevant to the prediction of the potential human response. Preference is given to studying animals in the conscious versus the anesthetized states and in the unstressed/unrestrained versus stressed/restrained conditions, to the extent possible within animal welfare guidelines. The clinical route is the preferred route, unless otherwise justified, for example, intravenous rather than oral route to achieve higher blood levels of the parent drug, where oral bioavailability in the test species may be low. Data are collected for a period that has the potential to define the onset, duration, and recovery from possible pharmacodynamic effects. This data collection period would be initially based on the pharmacokinetic (or toxicokinetic) properties of a drug in the selected species and, at a minimum, encompass the time at which the maximum plasma concentrations of the parent drug and any major metabolites are achieved. The demonstration of reversibility/recovery from pharmacodynamic effects may be accomplished by waiting five or more half-lives before terminating the data collection. In the event that human-specific metabolites are detected in the early clinical phases, consideration would be given to nonclinical pharmacodynamic studies that would be appropriate to assure continued human safety.

2.3 Institutional Strategies

The impact of the implementation of the ICH S7A guideline on the organization, philosophy, and practices in safety pharmacology in the pharmaceutical industry shows some controversies and challenges.

In a survey, Valentin and Bass (2004, personal communication) showed the impact in various pharmaceutical companies. The types of safety

pharmacology studies conducted are different. During the precandidate drug selection phase, primarily in vitro and in vivo vascular studies were conducted. The focus in these studies was on cardiac repolarization. CNS studies were of lesser extent. In the postcandidate phase, the "core battery" of S7A, as well as "follow-up" and "supplementary" studies was performed. In late phases, during clinical development, studies followed on a case-by-case basis.

Should early safety pharmacology studies replicated in order to claim GLP compliance? This question was answered differently.

YES for those who think: it increases the statistical power, shows higher exposure of the test article, slightly different focus, GLP, "box-ticking" reasons.

NO for those who mentioned: studies sometimes acceptable by regulatory agencies, animal welfare (3Rs), limited resources, cost/time versus benefit, limited resources, avoid generating conflicting results (Wakefield et al. 2002).

The design and the execution of safety pharmacology studies are focused upon the safety of human volunteers and patients in clinical trials. ICH S7A and S7B strive for effective integration of safety pharmacology results with those of the nonclinical (toxicology) and clinical safety databases.

Overall, the ICH S7A and S7B guidelines are successfully implemented in the pharmaceutical world. The "core battery" is in general performed prior to "First in Man." These guidelines increase the visibility of safety pharmacology within companies and increase focus by regulatory agencies.

2.4 Future of Safety Pharmacology

The future of safety pharmacology will depend, in part, upon the scientific and technological advances and regulatory challenges that envelop pharmaceutical development (Cavero and Crumb 2005; Porsolt et al. 2005). With advances in molecular biology and biotechnology and cancer (see guidelines S6(R1) (1997) and S9 (2009)), which allow for the identification of new clinical targets, newer pharmaceutical agents are being identified that act at these novel molecular sites in an attempt to ameliorate the disease condition. Inherent in the novelty of new targets is the risk of unwanted effects that may or may not be detected with current techniques. The scientific challenge facing safety pharmacology is to keep pace, to adapt, and to incorporate new technologies in the evaluation of new drugs in nonclinical models and identifying the effects that pose a risk to human volunteers and patients.

Recent examples include safety pharmacology's embracement of modern electrophysiological techniques to evaluate the effects of new drugs on the ionic components of the cardiac action potential (Redfern et al. 2003) and telemetry techniques to permit the chronic monitoring of physiological functions in unstressed animals (Kinter and Johnson 1999; Kramer and Kinter 2003; Kramer et al. 1998). Efforts continue to construct databases relating the similarities and differences between animal and human responses to pharmaceutical agents (Igarashi et al. 1995; Olsen et al. 2000). As an example, nonclinical safety studies, including safety pharmacology studies, are typically conducted in normal, healthy, young adult or adult animals. However, these tests may not appropriately detect specific responses in humans at other ages (e.g., neonates, adolescents, and geriatrics) or those with underlying chronic diseases (e.g., heart failure, renal failure, and type II diabetes), conditions which may alter the pharmacodynamic response to a drug. In some cases, animal models that overexpress or are deficient in the unique targets, or are otherwise manipulated to model the human pathophysiological conditions, may provide additional focus and sensitivity to detect and interpret the potential unwanted effects of new drugs in terms of human risk (Hondeghem et al. 2001).

The future of safety pharmacology is also intertwined with international regulatory guidelines such as ICH Topics S6(R1) (1997), S7A (2000), S7B (2005), S9 (2009), and E14 (2005). The discipline is considered integral to the evolving regulatory strategies for safely accelerating the introduction of these drugs into the clinical phases (e.g., EMEA (CPMP), "Position Paper on Non-clinical Safety Studies to Support Clinical Trials with a Single Microdose" and the US Food and Drug Administration, Screening Investigational New Drug Application). Additionally, safety pharmacology is also considered important to newly emerging regulatory guidelines from US Food and Drug Administration, such as the "Safety Evaluation of Pediatric Drug Products and Non-clinical Studies for Development of Pharmaceutical Excipients." The ICH M3(R2) (2009) guideline further

expanded the exploratory clinical trial approach with international consensus (Bass et al. 2011).

The introduction of pharmaceutics into the environment is gaining the attention of both regulators and pharmaceutical industry (Calamari 2003; Huggett et al. 2003; Kopin et al. 2002). While this is not currently the subject of any international environmental guideline, the use of organ function endpoints may become an important component in bridging safety data collected in mammalian vertebrates (including humans) to aquatic species for purposes of the identification of relevant target species and organ functions and the design of specific environmental toxicology studies.

Regulatory interventions in drug safety have become more and more stringent as evidenced continuous promulgation of novel guidelines (Table 2.3). Another ongoing challenge facing drug developers, regulators, and physicians is a need to find the correct balance between introducing new drugs to the market for unmet medical needs and assuring the safety of the clinical trial subjects and patients (Anon 2008).

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Central Nervous System (CNS) Safety Pharmacology Studies

3

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3.1 General Considerations

3.1.1 Definition of Safety Pharmacology

Safety pharmacology studies are "those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic dose-range and above" (Anonymous 2000). Safety pharmacology is an essential stage in drug development and includes studies evaluating the potential effects of a test substance on vital systems, including the cardiovascular and central nervous systems (CNS) (Lindgren et al. 2008; Pugsley et al. 2008). There has, unfortunately, been considerable confusion about the term "safety pharmacology," and many other kinds of study have also been included. This is no doubt explained by the multitude of terms which have been used to describe the area: general pharmacology, ancillary pharmacology, secondary pharmacology, high-dose pharmacology, and regulatory pharmacology (Porsolt 1997).

3.1.2 Scope of CNS Safety Pharmacology

What kinds of studies come within the scope of CNS safety pharmacology? The *European Agency for the Evaluation of Medicinal Products* has proposed a set of guidelines ICH S7A (Anonymous 2000) that came into effect in June 2001 and have since been adopted in the USA and Japan. The European guidelines include as core battery CNS studies "motor activity, behavioral changes, coordination, sensory/motor reflex responses, and body temperature" with the remark that "the central nervous system should be assessed appropriately."

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Follow-up studies should include "behavioral pharmacology, learning and memory, ligand-specific binding, neurochemistry, visual, auditory, and/or electrophysiology examinations, etc." In general, core battery studies should be carried out prior to first administration in humans, whereas the follow-up studies should be carried out prior to product approval. A final recommendation is that core battery studies should be carried out in full accordance with GLP, whereas follow-up studies, because of their unique characteristics, require only assurances of "data quality and integrity."

ICH S7A guidelines suggest a clear intent by the European and US authorities to free safety pharmacology from the constraints of a cookbook approach. On the other hand, their vagueness does not provide any clear idea of what could or should be done. In particular, only passing mention is made of drug abuse/dependence assessment, despite this evident safety concern for a large variety of pharmacological agents. For more recent discussions on safety pharmacology, see Porsolt et al. 2002, Bass et al. 2009.

3.1.3 In Vivo Versus In Vitro

CNS safety pharmacology makes use mainly of in vivo methods in conscious animals. The primary reason is that CNS function is best evaluated in an intact and freely moving animal. In contrast to cardiovascular safety pharmacology, where important aspects of function can be evaluated in anesthetized animals or even isolated organs or cells, behavior is the global result of multiple mechanisms, many unknown, and by definition occurs only in the conscious animal or person. Possible exceptions are drug effects on CNS electrophysiology, where drug effects on cerebral cells or structures can be investigated under anesthesia (Easter et al. 2009). On the other hand, CNS electrophysiology can also be investigated using EEG techniques in conscious animals with higher predictability for real-life situations.

3.1.4 Core Battery CNS Safety Pharmacology Studies

ICH S7A distinguishes between core battery studies and supplementary or follow-up studies (Bass et al. 2009). Core battery CNS procedures are typically simple tests, using traditional techniques, which can be carried out rapidly in a routine fashion. They are the first techniques to be employed in safety assessment and are frequently applied at the very beginning of the discovery process as a screen to eliminate substances with a potential for CNS risk. Because of their use early in the safety evaluation process, such studies are conducted almost exclusively in the rodent. Another requirement is that such studies be carried out according to GLP. Nevertheless, it is now recognized that it may be advantageous to identify potential CNS risks earlier in drug development using exploratory studies performed outside the constraints of GLP (Cavero 2009). ICH S7A recommends that core battery studies should include measures of drug-induced observable signs, measures of spontaneous locomotion, and motor coordination. Three other kinds of measure, originally recommended by the Japanese guidelines Category A but dropped from the ICH S7A core battery, are the convulsive threshold, interaction with hypnotics, and the pain threshold. In contrast to ICH S7A, it seems to us that such measures could usefully be included in a core battery of CNS safety pharmacology procedures. Decreases in the convulsive threshold are by no means negligible in the assessment of CNS safety. Several substances, including antipsychotics like clozapine, do not induce frank convulsions at any dose but clearly decrease the convulsive threshold. Even anticonvulsive activity, which in itself is not a risk factor, could be a useful predictor of cognition-impairing effects. Several anticonvulsants, such as benzodiazepines and NMDA antagonists, are known to impair cognition in addition to their anticonvulsant activity. Thus, the presence of anticonvulsant activity could represent a useful first screen for potential cognition-impairing effects (Porsolt et al. 2002). In a similar fashion, sleep-inducing or sleep-attenuating activity could be unmasked by a barbiturate interaction procedure. Benzodiazepines, for example, do not by themselves induce sleep, but their sleep-enhancing activity can be readily detected in interaction with barbiturates. The same is true for psychostimulants which may or may not induce signs of excitation in a primary observation procedure, but clearly block barbiturate-induced sleep. Finally, a drug-induced decrease in pain sensitivity would certainly constitute a CNS risk factor which could be assessed fairly simply using a nociception procedure. It has even been suggested that the presence of analgesic activity constitutes a predictor of abuse liability (Franklin 1998). Compared with current cardiovascular and respiratory procedures,

inclusion of such tests in a core battery would not represent a major expense, but would considerably improve the assessment of CNS risk. Protocols for such tests are included below (Sect. 3.2).

3.1.5 Supplementary CNS Safety Pharmacology Studies

Supplementary or follow-up studies are more wide ranging and cover cognitive function (dependence/abuse potential, learning, memory and attention, and brain function (EEG)). Because of their complexity, there exist no standard protocols, and there is no formal requirement that such studies be carried out in compliance with GLP. There is nonetheless a clear preference by regulatory authorities that even supplementary studies carried out in compliance with GLP as far as possible. A stringent requirement is that such procedures be carried out according to internationally accepted scientific standards of excellence. Protocols which we have found useful for evaluating such effects are also included below (Sect. 3.3).

3.1.6 Choice of Animal Species

Most of the core battery studies can be performed in the mouse or the rat. The protocols described below provide the rat version and have been standardized in our own laboratory using male Wistar rats weighing between 150 and 250 g at the beginning of the experiments, depending on the tests. Apart from general observation procedures, where the behavioral repertoire in the rat is richer than that in the mouse, there is no theoretical reason for preferring the rat. On the other hand, the rat is the species of choice for many other areas of drug development (chronic treatment studies, toxicology, biochemistry, pharmacokinetics). It is therefore preferable to use the rat for CNS core battery studies to ensure a maximum of coherence with other available data. Although the mouse is clearly more economical both in terms of cost and the quantity of test substance required for carrying out the experiments, such considerations are less relevant later in the drug development process (just before phase I), where larger quantities of test substance are available and the costs of such experiments are minor in comparison with other development costs.

For supplementary studies, the rat remains the species of choice. A possible exception is the use of primates which can be used under certain conditions as discussed in Sect. 3.3 for assessment of abuse potential (Moser et al. 2011b). The reason is not that drug effects clearly differ between the species, but more because primates are closer to man in terms of active doses and pharmacokinetics, thereby increasing predictability in areas of more complex CNS function.

3.1.7 Route of Administration

Test substances can be administered by different routes of administration. ICH S7A guidelines suggest that safety pharmacology studies should be performed using the same route of administration as that intended in man. Because in most cases drugs are administered by the oral (p.o.) route, the p.o. route is used most frequently for CNS core battery studies. On the other hand, there is no guarantee that rats have similar absorption and metabolism to man via the oral route. Indeed, some drugs, for example, the antipsychotics haloperidol or sulpiride, are poorly absorbed in the rat after oral administration but are given orally in man. The risk of poor absorption in the rat might indeed provide a justification for using a more effective route, intravenous (i.v.), intraperitoneal (i.p.), or subcutaneous (s.c.), for safety pharmacology studies, where the aim is to establish potential risk. Thus, showing an absence of adverse effect by a pharmacologically sensitive route should in principle provide the best estimate of safety.

3.1.8 Statistical Analyses

The aim of safety pharmacology is the detection of risk. It is therefore essential not to miss elements which could compromise the safety of a potential therapeutic agent. In other words, the number of false negatives should be kept to a minimum. False positives (erroneous detections of possible risk), although troublesome, are less serious and can usually be corrected by supplementary testing.

Translated into statistics, this implies that for safety pharmacology, the risk of type 2 errors (false negatives) should be decreased as much as possible, even if there is an increase in the risk of type 1 errors (false positives). In other words, the statistical tests

employed in safety pharmacology should err in the direction of oversensitivity rather than the reverse. A test substance found not to have significant safety risks based on preclinical studies, even after the use of oversensitive statistics, is more likely to be truly devoid of risk. As a consequence, the statistical analyses proposed for the CNS safety procedures described below (mainly two-by-two comparisons with control using Student's *t* tests) have been selected for maximal sensitivity to possible effects per dose at the acknowledged risk of making more type 1 errors. More conventional statistical procedures such as use of analysis of variance (ANOVA) followed by post hoc tests for two-by-two comparisons can also be used. Nevertheless, use of a too strict statistical procedure may generate false-negative results which are to be avoided in studies designed to detect risk.

3.1.9 Ethical and Animal Welfare Issues

As with all procedures involving living animals, important considerations in the choice of method are the ethical issues surrounding it. Most regulatory bodies have made pronouncements on this subject, and the reader should consult these documents for more detailed information. However, the guiding principles are to use as few animals as necessary and to avoid stressful procedures as much as possible.

In the field of safety pharmacology, where the aim is to assess the risk of inducing unwanted effects, the possibility of causing animal distress is potentially higher than in other areas of pharmacology. This is particularly true for CNS safety pharmacology studies which use primarily intact and conscious animals. The experimenter must therefore maintain awareness of these issues, not only in planning and devising the protocols but also during the experiments, where procedures for stopping the experiment in the event of well-defined events (e.g., pain or death) should be in place. Conditions should be fixed in advance such that these events are absent or exceptional and allow decisions to be made to stop the experiment if they occur.

Safety pharmacology generally uses larger group sizes than efficacy pharmacology, and it is important that the results are sufficiently reliable for regulatory decisions to be made. However, in some circumstances, methods exist to reduce animal use while at the same time maintaining scientific validity. One example is the supplanting of the traditional LD50 acute toxicity test, which uses a large number of animals to obtain very limited data. Similar data, with fewer animals and considerably more information, can be obtained using the Irwin procedure.

The test procedures described below have been selected and conceived to comply with the ethical requirements outlined above.

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3.2 CNS Core Battery Studies

This section describes basic protocols satisfying ICH S7A recommendations for core battery CNS studies. Included are protocols for measuring general behavioral signs induced by test substances (Irwin test), effects on spontaneous locomotion (activity meter

test), effects on neuromuscular coordination (rotarod test), effects on the convulsive threshold (electroconvulsive shock (ECS) threshold and PTZ seizure tests), interaction with hypnotics (barbital interaction test), and effects on the pain threshold (hot plate test).

3.2.1 Irwin Test

PURPOSE/RATIONALE

The primary aim of the Irwin test, first described in the mouse by Irwin (1968) but easily adapted to the rat, is to evaluate the qualitative effects of the test substance on behavior and physiological function, from the first doses that have observable effects up to doses which induce clear behavioral toxicity or even death. The Irwin test also permits a reasonable estimate of the test substance's duration of action on the different parameters observed.

Because most measures involve subjective assessment of different aspects of the animal's behavior, the test must be performed in a highly standardized manner by well-trained observers to ensure reproducible findings on different occasions or at different observation times on the same day. Indeed, because of systematic changes in the behavior and reactions of test animals over a test day, drug-treated animals are always evaluated with reference to a simultaneously treated group of vehicle controls under nonblind conditions to gauge whether an observed effect is truly a consequence of treatment with the test substance.

PROCEDURE

Rats are administered the test substance and are observed simultaneously with a control group given vehicle. Although the test treatment can be blinded, the observer is always aware of which group received vehicle. All treated groups are compared with the same control simultaneously, with two or three animals within a treatment group observed at any one time.

Behavioral modifications, physiological and neurotoxicity symptoms, rectal temperature, and pupil diameter are recorded according to a standardized observation grid derived from that of Irwin. The grid contains the following items: death, convulsions, tremor, Straub tail, decrease in general activity, increase in general activity, jumping, abnormal gait (rolling, tiptoe), motor incoordination, altered muscle tone, loss of grasping, akinesia, catalepsy, loss of traction, loss of balance, forepaw treading, writhing, piloerection, stereotypies (sniffing, chewing, head movements), head twitches, scratching, altered respiration, aggression, altered fear, altered reactivity to touch, ptosis, exophthalmia, loss of righting reflex, loss of corneal reflex, analgesia, defecation/diarrhea, salivation, lacrimation, rectal temperature (hypothermia/hyperthermia), and pupil diameter (myosis/mydriasis). Further details of the evaluation of the signs/symptoms are provided in the following table (Table 3.1).

Observations are performed 15, 30, 60, 120, and 180 min after administration of the test substance and also 24 and 48 h later. The symptoms marked (*) are observed continuously from 0 to 15 min after administration. Depending on the pharmacokinetic properties of the test substance, observations can be conducted at shorter or longer times after administration.

Six rats are studied per group.

The test substance is usually evaluated at four doses, administered p.o. immediately before the test. For safety pharmacology studies, the lowest dose is close to the therapeutic dose as estimated in tests predictive of the indication, and the highest dose can be 100 or 300 times this dose if the substance's toxicity or physical characteristics permit administration of such a dose.

EVALUATION

Being mainly a qualitative assessment procedure, no formal statistical analysis is conducted. Most symptoms are evaluated by their presence or absence. Some (increased or decreased activity) are rated on a 3-point scale. Others (respiration, fear/startle, reactivity to touch) are scored in two directions. Finally, certain parameters (rectal temperature, pupil diameter) are measured quantitatively.

CRITICAL ASSESSMENT OF THE METHOD

The Irwin test serves at both ends of the drug discovery/development spectrum.

In the exploratory phase of drug development, it is frequently the first test employed in vivo. During this phase, the purpose of the test is to provide a rapid detection of the test substance's toxicity, active dose range, and principal effects on behavior and physiological function. A first estimate of safety is provided by the difference between the first doses inducing observable effects and those inducing frank behavioral

Table 3.1 Symptoms observed during the Irwin test in the rat

Symptoms	Observations
*Death**	Presence
	Indicate the time of death if <15 min
*Convulsions**	Presence
	Indicate the time of appearance if <15 min
*Tremor**	Presence
	Whole or part of body trembling
*Straub tail**	Presence
	Rigid tail held upright and tending to curve over the back of the animal
Decreased activity	Three intensities: +: After removing the cage cover and manipulation, the animal moves more slowly than controls ++: Under the same conditions, the animal moves very slowly +++: Under the same conditions, the animal does not move at all
Increased activity	Three intensities: +: After removing the cage cover and manipulation, the animal is slightly more active than controls ++: Under the same conditions, the animal moves rapidly (frequently stopping) +++: Under the same conditions, the animal moves very rapidly (occasionally stopping)
*Jumping**	Presence
*Abnormal gait**	Presence
	For example, Rolling gait. Motor coordination preserved, occasional staggering
	Locomotion on tiptoe
*Motor incoordination**	Presence
	Motor coordination impaired. Disorganized locomotion (e.g., crossing of paws, etc.)
Altered abdominal muscle tone	Increase (↑) or decrease (↓)
	Hardness or softness of the abdomen when pressed laterally between forefinger and thumb is compared with controls.
Loss of grasping	Presence
	When placed on a horizontal wire grid, the animal does not grasp grid when pulled backward by the tail
Akinesia	Presence
	When placed on a wire grid, the animal does not move when the grid is progressively moved toward a vertical position (about 30°)
Catalepsy	Presence
	When placed in Buddha position (upright posture on its hind paws), the animal does not move
Loss of traction	Presence
	When placed on a horizontal bar by the forepaws, the animal fails to bring its hind paws up onto a second bar placed below the first one (three trials maximum)
*Loss of balance**	Presence
	Motor coordination impaired. The animal falls on its side when it moves
*Forepaw treading**	Presence
	Repeated stamping of forepaws without displacement
*Writhing**	Presence
	Coordinated contraction of abdominal muscles usually resulting in hollow flanks and stretching of hind limbs
*Piloerection**	Presence
	Fur standing on end
*Stereotypy**	Presence
	Abnormal repeated movements: Sniffing Chewing Head movements
*Head twitches**	Presence
	Rapid saccadic side-to-side axial movement of the head
*Scratching**	Scratching with fore- or hind paws anywhere on the body
*Altered respiration**	Increase (↑) or decrease (↓)
	Respiration rate is compared with controls
*Aggression**	Presence
	Biting attempts when approached toward head with a ballpoint pen
	Biting attempts toward the other animals or toward the experimenter
Altered fear/startle	Increase (↑) or decrease (↓)
	Animal's reaction (flinch, jump. . .) when fingers are snapped above cage is compared with controls
Altered reactivity to touch	Increase (↑) or decrease (↓)
	Animal's flight reaction to downward finger pressure on the hindquarters is compared with controls
Ptosis	Presence
	Eyelid partially or completely closed
Exophthalmia	Presence
	Protrusion of the eyeballs

Table 3.1 (continued)

Symptoms	Observations
Loss of righting reflex	Presence
	When placed on its back, the animal does not right itself
Loss of corneal reflex	Presence
	When the corneal surface is lightly touched with the tip of a pen, the animal does not close eye
Analgesia	Presence
	When pinched at base of tail with forceps, the animal does not react (turning toward forceps, vocalization)
Defecation/ diarrhea	Presence
Salivation	Presence
	Dampness visible around mouth
Lacrimation	Presence
	Dampness visible around eyes
Hypothermia	Three levels based on mean temperature measured in treated and control animals: +: Decrease >1°C ++: Decrease >2°C +++: Decrease >3°C
Hyperthermia	Three levels based on mean temperature measured in treated and control animals: +: Increase >1°C ++: Increase >2°C +++: Increase >3°C
Myosis	Three levels based on mean pupil diameter measured in treated and control animals (1 unit = 1/30 mm): +: Decrease >10 units ++: Decrease >20 units +++: Decrease >30 units
Mydriasis	Three levels based on mean pupil diameter measured in treated and control animals: (1 unit = 1/30 mm) +: Increase >10 units ++: Increase >20 units +++: Increase >30 units

toxicity or lethality. Even when the test substance is intended for a nonpsychotropic indication, the Irwin test permits the choice of doses for subsequent tests. Furthermore, the Irwin test can frequently permit identification of the kind of activity exerted by the test substance in the psychotropic domain. As is illustrated in Figs. 3.1 and 3.2, clearly distinct and identifiable profiles are observed with drugs such as the antipsychotic haloperidol and the analgesic morphine.

Data obtained in the Irwin test can be grouped into major categories related to effects on general activity (decrease/increase), motor behavior/coordination, modification of pain threshold, autonomic signs, or other effects. Figure 3.1 provides an example of such a representation comparing the effects of amphetamine with the effects of diazepam. The modified presentation gives a more visual representation of the main effects of each substance and their duration of action, thereby facilitating comparison between substances.

At later stages of drug development (before phase I), when the test substance has been more fully characterized, the Irwin test provides a clear overall index of the test substance's margin of safety. Furthermore, although the test is mainly behavioral, it can give global indications of drug effects on vital functions such as respiration or intestinal motility. On the other hand, the test provides little information about effects which are not visible from direct observation, including the whole range of cardiovascular, pulmonary, and gastrointestinal parameters.

MODIFICATIONS TO THE METHOD

The Irwin test, although originally described in the mouse, was later applied to the rat (Esteve et al. 1988). Indeed, the principle of systematic observation of drug-induced symptoms using standardized observation criteria can be applied to a wide range of animal species and even constitutes the basis of symptom checklists in man. Every laboratory has its own manner of doing the Irwin test. That described above is what we have been practicing for over 20 years. ICH S7A simply requires that animals be observed on a systematic basis over a wide dose range. Alongside variants of the Irwin procedure, ICH S7A mentions another systematic observation procedure, the functional observation battery (FOB) more recently described by Mattson et al. (1996) as being functionally equivalent. This test, derived from classical toxicology, is more specifically used for testing neurotoxicity.

References and Further Readings

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Irwin S (1968) Comprehensive behavioral assessment: 1a A systematic quantitative procedure for assessing the

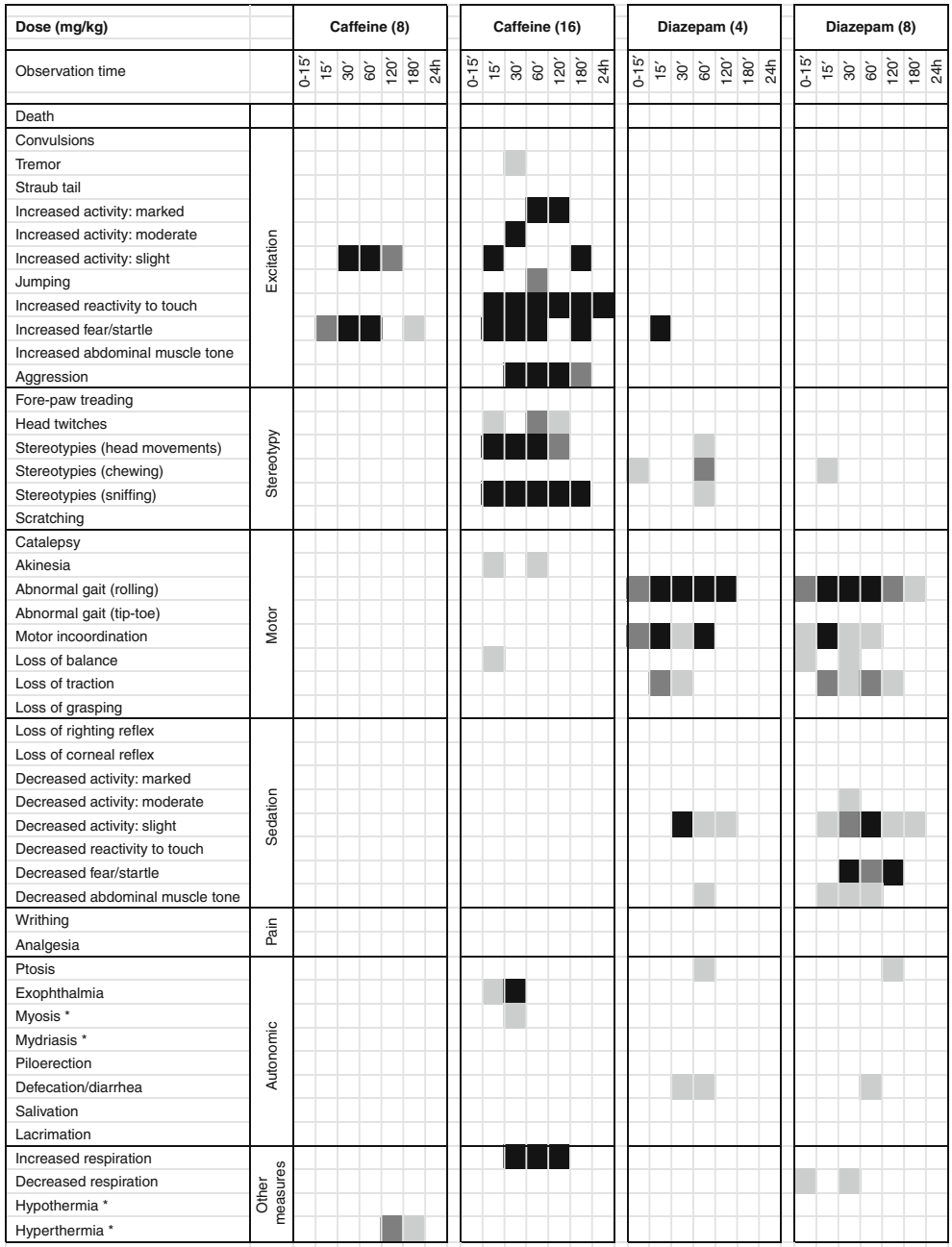

Fig. 3.1 Synoptic presentation of effects of caffeine and diazepam in the Irwin test in the rat. The clear differentiation observed between stimulating and inhibitory effects on general activity of the two substances clearly appears in this modified presentation of data

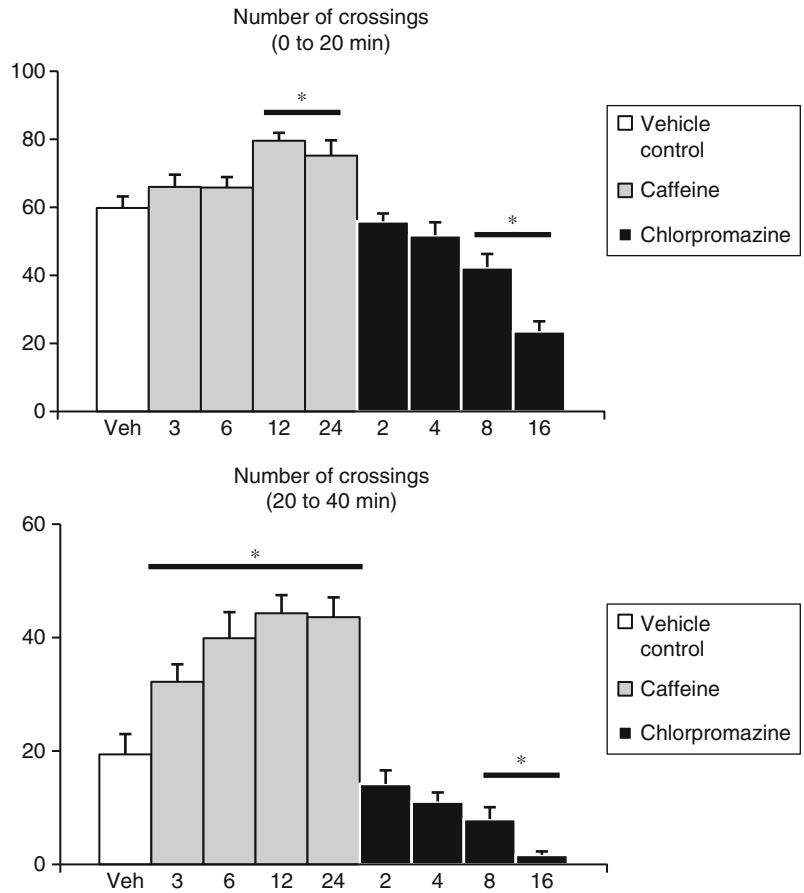

Fig. 3.2 Effects of caffeine and chlorpromazine (p.o.) on locomotion in the rat. Administrations were performed 60 min before the test. *Different from vehicle control

behavioral and physiologic state of the mouse. Psychopharmacologia 13:222–257

Mattson JL, Spencer PJ, Albee RR (1996) A performance standard for clinical and Functional Observational Battery examination of rats. J Am Coll Toxicol 15:239–250

Example. Table 3.2 The profile of effects of diazepam (p.o.) in the Irwin test in the rat. Note the dose-dependent increase in decrease in general activity and decreases in reactivity to touch, traction, and muscle tone accompanied by abnormal gait

Observations were performed at 15, 30, 60, 120, and 180 min and at 24 h after administration. The symptoms which did not necessitate handling were also observed up to 15 min immediately following administration. Pupil diameter and rectal temperature modifications were evaluated by comparison of the mean scores obtained in treated and control animals.

Table 3.3 The profile of effects of caffeine (i.p.) in the Irwin test in the rat. Note the dose-dependent occurrence of increase in general activity followed by stereotypies (sniffing, head movements, head twitches), increased fear/startle, and reactivity to touch.

3.2.2 Activity Meter Test

PURPOSE/RATIONALE

Locomotor activity can be quantified in rodents by a variety of means. The method described below uses interruptions of photoelectric beams and is based on studies from Boissier and Simon (1965). The difference between Irwin estimations of drug effects on spontaneous activity and activity meter tests is mainly a question of quantification. Activity meter tests are generally carried out using automated apparatus with a larger number of animals and are, therefore, less labor intensive but permit more precise statistical analyses. The quantitative data obtained enable the generation of

Table 3.2 Effects of diazepam in the Irwin test in the rat

2 (mg/kg p.o.)	4 (mg/kg p.o.)	8 (mg/kg p.o.)	16 (mg/kg p.o.)
Abnormal gait (rolling)	↓ *Activity*	↓ *Activity*	↓ *Activity*
(1/3) at 15’	+ (3/3) at 30’	+ (1/3) at 15’	+ (2/3) at 15’ → 60’
	+ (1/3) at 60’ → 120’	+ (2/3) at 30’	+ (3/3) at 180’
		+ (3/3) at 60’	++ (1/3) at 15’ → 60’
		+ (1/3) at 120’ → 180’	++ (2/3) at 120’
		++ (1/3) at 30’	
	Abnormal gait (rolling)	*Abnormal gait (rolling)*	*Abnormal gait (rolling)*
	(2/3) at 0’ → 15’	(2/3) at 0’ → 15’	(3/3) at 0’ → 120’
	(3/3) at 15’ → 120’	(3/3) at 15’ → 60’	
		(2/3) at 120”	
		(1/3) at 180’	
	Motor incoordination	*Motor incoordination*	*Motor incoordination*
	(2/3) at 0’ → 15’	(1/3) at 0’ → 15’	(1/3) at 0’ → 15’
	(3/3) at 15’	(3/3) at 15’	(3/3) at 15’ → 60’
	(1/3) at 30’	(1/3) at 30’ → 120’	(1/3) at 120’
	(3/3) at 60’		
	Stereotypies (sniffing)	*Loss of balance*	*Stereotypies (chewing)*
	(1/3) at 60’	(1/3) at 0 → 15’	(3/3) at 0’ → 15’
		(1/3) at 30’	
	Stereotypies (chewing)	*Stereotypies (chewing)*	↓ *Altered respiration*
	(1/3) at 0’ → 15’	(1/3) at 15’	(3/3) at 60’
	(2/3) at 60’		
	Head movements	↑ *Altered respiration*	↓ *Fear/Startle*
	(1/3) at 60’	(1/3) at 0’ → 15’	(3/3) at 30’
		(1/3) at 30’	
	↑ *Fear/startle*	↓ *Fear/startle*	↓ *Abdominal muscle tone*
	(3/3) at 15’	(3/3) at 30’	(3/3) at 15’ → 60’
		(2/3) at 60’	
		(3/3) at 120’	
	↓ *Abdominal muscle tone*	↓ *Abdominal muscle tone*	*Ptosis*
	(1/3) at 60’	(1/3) at 15’ → 60’	(3/3) at 60’
	Ptosis	*Ptosis*	*Loss of traction*
	(1/3) at 60’	(1/3) at 120’	(2/3) at 15’
			(1/3) at 30’→ 60’
	Loss of traction	*Loss of traction*	*Defecation/diarrhea*
	(2/3) at 15’	(2/3) at 15’	(2/3) at 15’
	(1/3) at 30’	(1/3) at 30’	(1/3) at 60’
		(2/3) at 60’	
		(1/3) at 120’	
	Defecation/diarrhea	*Defecation/diarrhea*	*Mydriasis*
	(1/3) at 30’ → 60’	(1/3) at 60’	+ at 15’

$N = 3$ for each treatment group; + = slight; ++ = moderate; +++ = marked; (’) = minutes

dose-response curves and more precise estimations of the minimal effective dose (MED) or the dose which increases or decreases locomotion by 50% (ED50).

PROCEDURE

The activity meter we use (Imetronic, Bordeaux, France) consists of 16 covered Plexiglas cages (40 × 25 × 25 cm)

Table 3.3 Effects of caffeine in the Irwin test in the rat

2 (mg/kg i.p.)	4 (mg/kg i.p.)	8 (mg/kg i.p.)	16 (mg/kg i.p.)	32 (mg/kg i.p.)
No change	↑ *Activity*	↑ *Activity*	*Tremor*	*Straub tail*
	+ (3/3) at 15’	+ (3/3) at 30’ → 60’	(1/3) at 30’	(3/3) at 0’ → 15’
	+ (1/3) at 30’	+ (2/3) at 120’		
	Stereotypies (sniffing)	↑ *Fear/startle*	↑ *Activity*	↑ *Activity*
	(3/3) at 15’	(2/3) at 15’	+ (3/3) at 15’	+ (3/3) at 15’
	(2/3) at 60’	(3/3) at 30’ → 60’	+ (3/3) at 180’	+ (3/3) at 60’ → 120’
		(1/3) at 180’	++ (3/3) at 30’	++ (3/3) at 30’
			+++ (3/3) at 60’	
			+++ (3/3) at 120’	
	↑ *Fear/startle*	*Hyperthermia*	*Jumping*	*Stereotypies (writhing)*
	(3/3) at 15’	++ at 120’	(2/3) at 60’	(1/3) at 0’ → 15’
	↑ *Reactivity to touch*	+ at 180’	*Loss of balance*	*Stereotypies (sniffing)*
	(1/3) at 15’		(1/3) at 15’	(2/3) at 30’
	(1/3) at 60’			
				(1/3) at 60’ → 120’
			Stereotypies (sniffing)	*Head twitches*
			(3/3) at 15’ → 180’	(1/3) at 30’
			Stereotypies (head movements)	↑ *Fear/Startle*
			(3/3) at 15’ → 60’	(3/3) at 15’
			(2/3) at 120’	(2/3) at 30’ → 120’
			Head twitches	↑ *Reactivity to touch*
			(1/3) at 15’	(2/3) at 15’
			(2/3) at 60’	
			(1/3) at 120’	
			↑ *Respiration*	↑ *Muscle tone*
			(3/3) at 30’ → 120’	(2/3) at 30’
				(1/3) at 60’
			Aggression	*Exophthalmos*
			(3/3) at 30’ → 120’	(3/3) at 15’
			(2/3) at 180’	
			↑ *Fear/Startle*	*Loss of grasping*
			(3/3) at 15’ → 60’	(1/3) at 15’
			(3/3) at 180’	(3/3) at 30’
			↑ *Reactivity to touch*	*Akinesia*
			(3/3) at 15’ → 24 h	(3/3) at 30’
			Exophthalmos	*Loss of traction*
			(1/3) at 15’	(1/3) at 15’
			(3/3) at 30’	
			Akinesia	
			(1/3) at 15’	
			(1/3) at 60’	
			Myosis	
			+ at 30’	

contained within a darkened enclosure and connected to silent electronic counters. Each cage is equipped with four photocell assemblies (two at each end of the cage) 3 cm above the floor to measure the number of movements by each animal (one per cage) in the horizontal plane. Ten additional photocell assemblies are placed at even intervals 20 cm above the floor along the long wall to record rearing. The number of (horizontal)

crossings by each animal (one per cage) from one pair of photocells to the other at the extremities of the cage is recorded by computer at 10-min intervals for 40 min. A similar procedure is utilized for recording of rearing, except that individual photobeam breaks are recorded. The scores are cumulated over the (1) 0–20-min period, (2) 20–40-min period, and (3) entire 40-min observation period.

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at four doses, administered p.o. 60 min before the test, and compared with a vehicle control group.

Caffeine (24 mg/kg p.o.) and chlorpromazine (16 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes seven groups.

EVALUATION

The basic measure taken is the global effect of the test substance over the whole measurement period. More detailed information can be obtained by analyzing the drug effects over sequential time slots, for example, every 10 min. For example, some drugs (diazepam, nicotine) attenuate high activity levels (first 10 min) but increase low levels (last 10 min). Further analyses can compare the effects of the test substance on rearing with its effects on locomotion within the enclosure. Finally, comparison of single photobeam interruptions with paired interruptions of the beams at each end of the enclosure can permit distinctions between drug effects on small and large movements.

The data are usually analyzed using Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

Locomotion tests estimate whether a test substance possesses psychostimulant or sedative activity, but the data obtained must be interpreted with caution. Locomotion can be decreased not only because the animals are sedated but also because the animals have drug-induced motor impairment or are otherwise debilitated by the test substance. Even substances with marked psychostimulant properties can decrease activity meter scores because the animals are rotating rapidly in a small space or are showing other stereotyped behaviors. It is therefore unwise to interpret activity meter scores in isolation from direct observation (Irwin test above) or measures of neuromuscular coordination (rotarod test below).

Another complication with activity meter tests is the phenomenon of habituation. All animals placed in an unknown environment will tend to explore it more at the beginning of the exposure with a decline in exploration with time. This can be clearly seen in most activity meters despite the apparent simplicity of the test environment. Thus, when locomotion is followed over sequential time slots, an apparent increase or decrease in drug effect over time cannot simply be interpreted as a change in its pharmacological activity. The substance may be interacting with the process of habituation. It is for this reason that activity meters are not the ideal means of measuring the duration of drug action.

Because the activity meter evaluates spontaneous behavior, the behavioral baseline is intrinsically variable and subject to many kinds of influence, including lighting, apparatus cleanliness, ambient temperature, noise level, and even time of day. As a consequence, particular care has to be taken to ensure constant experimental conditions to obtain reproducible results. Furthermore, when comparing different drug treatments, it is important to take advantage of the fact that several animals can be evaluated simultaneously, by distributing the different treatments in a balanced fashion over the test period, and even in the positions within the experimental apparatus or of the observation chambers in the experimental room.

MODIFICATIONS TO THE METHOD

Locomotor activity can be quantified in rodents by a variety of means, including interruptions of photoelectric beams, activity wheels, changes in electromagnetic fields, Doppler effects, video image analysis, telemetry, or detection of vibrations generated by the animals (Reiter and McPhail 1979; Lynch et al. 2011). As has been suggested above, it is not very important how locomotion is measured because the main outcome measure is whether a test substance increases or decreases spontaneous locomotion.

More important is the time at which a test substance is administered in relation to placing the animal in the activity meter. Because of potential interactions of the test substance with the process of habituation, we think it is important to start testing when the drug effect has had time to reach its maximum, i.e., a certain interval after drug administration. If the animal is treated and

immediately placed in the activity meter, the kind of drug effect observed may critically depend on the interaction of the onset of drug action with the habituation process.

Another procedural variant, aimed to minimize the role of habituation, is to prehabituate the animal to the environment before administering the test substance. In this way, it is hoped to assess drug action on "baseline" behavior in already habituated animals. This approach appears to be a reasonable one but considerably increases the time and costs for conducting the test. Furthermore, there are cases where drug effects are dramatically attenuated in prehabituated animals. In particular, this approach is much less sensitive to drug-induced decreases in motor activity.

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Lynch JJ III, Castagné V, Moser PC, Mittelstadt SW (2011) Comparison of methods for the assessment of locomotor activity in rodent safety pharmacology studies. J Pharmacol Toxicol Methods 64:74–80

Reiter LR, McPhail RC (1979) Motor activity: a survey of methods with potential use in toxicity testing. Neurobehav Toxicol 1:53–66

Example (See Fig. 3.2).

3.2.3 Rotarod Test

PURPOSE/RATIONALE

Locomotor coordination is most commonly assessed using a rotarod (Dunham and Miya 1957). The rotarod consists of a circular rod turning at a constant or increasing speed. Animals placed on the rod will naturally try to remain on the rod rather than fall onto a platform some 30 cm below. This test therefore provides an estimate of the animal's level of neuromuscular coordination. Drugs which are known to perturb neuromuscular coordination (e.g., benzodiazepines) clearly reduce the time the animals stay on the rod.

This test lends itself readily to automation with several animals being tested simultaneously on the same rod, each animal being separated by vertical barriers.

PROCEDURE

3.2.3.1 Habituation

Rats are placed on a rod (diameter 7 cm) rotating at a speed of 12 revolutions per minute for a 2-min period. If they fall off during this period, they are replaced on the rod. At the end of the habituation period, rats are placed on the rod under the same conditions as used during the test session (i.e., for a maximum of 3 min) and are not replaced on the rotarod if they fall off.

3.2.3.2 Test

The test session is performed at least 2 h after the habituation period. Rats are placed on the rotarod for a maximum period of 3 min. The number of animals which fall off before the end of this period is counted, and the latency to fall off is recorded (maximum 3 min).

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at four doses, administered p.o. 60 min before the test, and compared with a vehicle control group.

Diazepam (8 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes six groups.

EVALUATION

Two basic measures are taken, the number of animals per group which fall off the rod within the 3 min test and the drop-off times. The quantal data (number of animals falling) are analyzed using Fisher's exact probability test. The quantitative data (drop-off times) are analyzed using Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

The rotarod test is one of the oldest used in the behavioral assessment of drug action (Rustay et al. 2003). It provides a simple first estimation of whether a test substance has any effect on neuromuscular coordination and has been found sensitive to a variety of agents which are known to disturb it, for example, the benzodiazepines.

When preceded by the habituation procedure (described above), the test is very reliable and reproducible. Generally speaking, the doses impairing rotarod performance are higher than those affecting locomotor activity, except for drugs having particular effects on

motor coordination (notably, the benzodiazepines). Furthermore, in contrast to the activity meter test, the rotarod test is not particularly sensitive to environmental factors such as lighting, ambient temperature, or noise. This is no doubt due to the fact that the test imposes a behavior on the animal (staying on the rod), where the consequences of falling are mildly aversive.

On the other hand, the rotarod test can be considered only as an initial screen for neuromuscular impairment. More complex tests, for example, analyses of gait, are required to further understand the motor aspects, whereas electrophysiological procedures, for example, the electromyogram (EMG), are required for better understanding the neuromuscular aspects.

In contrast to most of the other core battery CNS tests, the rotarod is mainly unidirectional, detecting principally the capacity of substances to decrease neuromuscular coordination. On the other hand, this is not a serious limitation in that the risk factor evaluated is whether the test substance causes impairment. When used in conjunction with locomotor activity tests, the rotarod test provides a useful quantification of the margin of safety between doses of test substances which alter spontaneous activity and those which disturb motor function.

MODIFICATIONS TO THE METHOD

The sensitivity of the rotarod test can be increased by placing animals on a rod turning at an accelerating speed to measure the latency to fall (Bohlen et al. 2009). This procedure has been shown to provide a more graduated assessment of the motor-impairing effects of various anticonvulsants and benzodiazepines (Capacio et al. 1992).

References and Further Readings

Bohlen M, Cameron A, Metten P, Crabbe JC, Wahlsten D (2009) Calibration of rotational acceleration for the rotarod test of rodent motor coordination. J Neurosci Methods 178:10–14

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Dunham NW, Miya TS (1957) A note on a simple apparatus for detecting neurological deficit in mice and in rats. J Am Pharm Ass 46:208–209

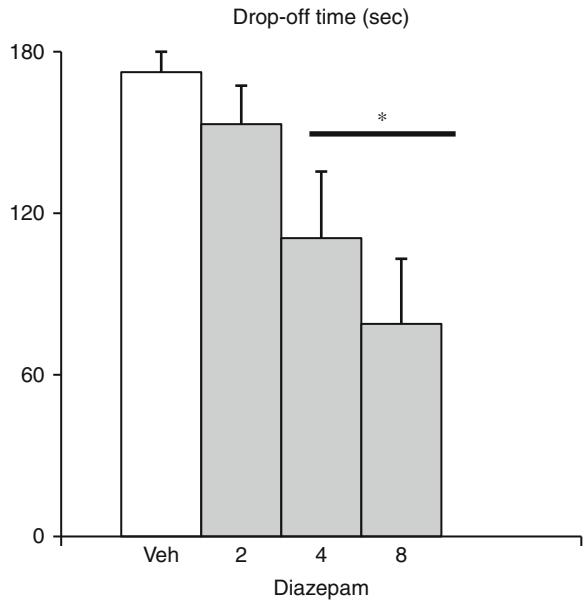

Fig. 3.3 Effects of diazepam (p.o.) on rotarod performance in the rat. Administrations were performed 60 min before the test. *Different from vehicle control

Rustay NR, Wahlsten D, Crabbe JC (2003) Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. Behav Brain Res 141:237–249

Example (See Fig. 3.3).

3.2.4 Convulsive Threshold Tests

PURPOSE/RATIONALE

Tests for the convulsive threshold estimate whether a test substance induces changes in the probability of convulsions occurring either spontaneously or, more importantly, in association with other treatments (Lynch et al. 2010). Although overt convulsions can usually be detected using the Irwin procedure, proconvulsant activity can occur in the absence of overt convulsions and therefore needs to be evaluated in specific tests. The existence of anticonvulsive activity is less dramatic as a risk factor. Nonetheless, as suggested in the "General Considerations" above, such activity might be an indicator of potential cognitive disturbance. There is therefore a need, in CNS safety pharmacology, to include procedures which assess changes in the convulsive threshold in both directions.

Described below are two procedures whereby convulsions are induced either electrically (ECS threshold test), following Swinyard et al. (1952), or chemically (PTZ seizure test), following Krall et al. (1978). We recommend using two methods for inducing convulsions because of considerable past experience showing that congruent findings are not always obtained between the two methods. In terms of predictability, it seems important to obtain coherent findings between different means of inducing convulsions before concluding as to the presence or absence of risk.

PROCEDURES

3.2.4.1 ECS Threshold Test

Rats are administered ECS (rectangular current, 1.5 s, 200 Hz) via earclip electrodes connected to a constant current shock generator (Ugo Basile Type 7801).

Treatment groups of 20 rats are exposed to ECS as follows:

Animal n°1 is exposed to 30 mA of ECS. If animal n°1 does not convulse (tonic convulsions) within 5 s maximum, animal n°2 is exposed to 35 mA, with increases of 5 mA until the first tonic convulsion is observed. Once the first tonic convulsion is observed, the intensity of ECS is decreased by 2 mA for the next animal, and then the intensity is decreased or increased by 2 mA from animal to animal, depending on whether the previous animal convulses or not. If animal n°1 does convulse (tonic convulsions) within 5 s maximum, animal n°2 is exposed to 25 mA, with decreases of 5 mA until the absence of tonic convulsions is observed. At this point, the intensity of ECS is increased by 2 mA for the next animal, and then the intensity is decreased or increased by 2 mA from animal to animal, depending on whether the previous animal convulses or not. The minimum intensity given is 5 mA, and the maximum intensity given is 95 mA.

The first five animals, which serve to identify the threshold current, are not included in the analysis.

The results are represented as the mean current intensity administered to the final 15 animals of a group and as the percent change from control values.

The test is performed blind.

A positive percent change indicates an anticonvulsant effect. A negative percent change indicates a proconvulsant effect.

The test substance is usually evaluated at four doses, administered p.o. 60 min before ECS, and compared with a vehicle control group.

Theophylline (128 mg/kg p.o.) and diazepam (16 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes seven groups.

3.2.4.2 PTZ Seizure Test

Rats, placed in individual Makrolon cages (25 × 19 × 13 cm), are injected with PTZ (75 or 100 mg/kg s.c.). The occurrence and latency of clonic convulsions, tonic convulsions, and deaths are noted over a 30-min period.

The results for the number of convulsions and deaths are also represented as percent change from control.

Twenty rats are studied per group. The test is performed blind.

The test substance is usually evaluated at four doses, administered p.o. 60 min before PTZ, and compared with a vehicle control group.

Theophylline (128 mg/kg p.o.) or diazepam (16 mg/kg p.o.), administered under the same experimental conditions, is used as the reference substance.

The basic experiment therefore includes six groups.

EVALUATION

ECS, if sufficiently intense, induces immediate full clonic-tonic convulsions but only occasionally death. In contrast, with PTZ, clonic convulsions can be observed without necessarily being followed by tonic convulsions. On the other hand, when tonic convulsions occur, they are almost invariably followed by death.

With the ECS procedure, the primary measure is the mean electroshock intensity administered per group of 20 animals. If less current is required to induce a clonic-tonic convulsion after a particular treatment, this is reflected by a decrease in the mean intensity administered (proconvulsant effect). Conversely, if more current is required to induce a clonic-tonic convulsion, this is reflected by an increase in the mean intensity (anticonvulsant effect). In other words, the mean intensity administered is a direct representation of the electroconvulsive threshold.

With the PTZ procedure, pro- or anticonvulsant activity is indicated both by the frequency of the convulsant events (clonic convulsions, tonic convulsions,

deaths) and by their latency of occurrence. The cut-off time is indicated when a test substance completely blocks convulsions.

The ECS threshold procedure permits the assessment of both pro- and anticonvulsant activity within a single experiment. To evaluate proconvulsant risk in the PTZ test, a moderate dose of PTZ (75 mg/kg) is used to induce clonic and tonic convulsions in half the control animals and is validated by showing a significant difference with animals treated with theophylline. Conversely, for evaluating anticonvulsant activity, a higher dose of PTZ (100 mg/kg) is used to induce convulsions in all control animals. The experiment is validated with diazepam which completely suppresses convulsions.

In both procedures, quantitative data (mean shock intensity, latencies) are analyzed using ANOVA followed by planned post hoc comparisons, as appropriate. In the PTZ procedure, quantal data (number of occurrences) are analyzed using Fisher's exact probability test.

CRITICAL ASSESSMENT OF THE METHOD

It is generally accepted that there is a good correlation between potentiation or antagonism of experimentally induced convulsions in animals and effects observed in man (Kupferberg 2001). Most substances with antiepileptic properties in man antagonize experimental convulsions, whereas many substances which either induce convulsions or lower the convulsive threshold in man (certain CNS stimulants, some antipsychotics) show similar effects in animals. There are nonetheless differences in the efficacy of certain substances in antagonizing experimental convulsions. Drugs, identified by ECS generally act by modulating voltage-dependent sodium channels, whereas drugs identified by PTZ generally have a benzodiazepine-like mode of action, potentiating the inhibitory effects of GABA (Löscher 2002).

The existence of such differences is an argument for employing at least two different methods in a core battery for evaluating drug effects on the convulsive threshold. If signs of risk are apparent, further models can be employed to establish either the mechanism or the anatomical locus in the brain. To establish mechanisms, use can be made of different convulsive agents (strychnine, bicuculline, picrotoxin, NMDA). As far as anatomical locus is concerned, the kindling model can be used with recordings taken from different brain regions (amygdala, temporal lobe, hippocampus, prefrontal cortex) to establish the development and regional distribution of the convulsive phenomena (Ebert et al. 1997). The simple EEG trace recording from the cortex is also useful for identifying the existence of convulsive brain phenomena at doses inducing no overt behavioral effects. Signals from the cortex are sometimes recorded in parallel with the electromyographic techniques or by observation of the behavior of animals by video EEG to compare the changes in brain activity with the intensity of the overt seizure.

MODIFICATIONS TO THE METHOD

The ECS method described above involves titration of the shock level between successive test animals to determine the mean intensity required to induce convulsions after different treatments. This procedure represents a variant of the more habitual procedure where a set level of ECS is selected to induce convulsions in 100% of the animals (maximal electroshock). Another variant would be to select a low electroshock level (subconvulsant) to detect proconvulsant activity. In addition to evaluating both pro- and anticonvulsant activity within a single test, the present method possesses the advantage of generating quantitative data (mean intensity) as opposed to quantal data (frequencies), permitting more powerful statistical analyses.

Another ECS method evaluates the intensity needed to induce tonic convulsions in different groups of animals challenged with electroshocks of preselected intensities inducing seizures in 10–30%, 30–50%, 50–70%, and 70–90% of the animals. The median current strength (CS50) represents the current intensity required to induce tonic hind limb extension in 50% of animals.

The intravenous PTZ infusion seizure test is a modified version of the PTZ test and can be used to assess proconvulsant drug activity (Löscher 2009). The dose of PTZ needed to induce convulsions is determined for each animal. This test provides a sensitive parametric method for assessing seizure threshold in individual animals (White et al. 2008).

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Swinyard EA, Brown WC, Goodman LS (1952) Comparative assays of antiepileptic drugs in mice and rats. J Pharmacol Exp Ther 106:319–330
White HS, Porter RJ, Kupferberg HJ (2008) Screening of new compounds and the role of the pharmaceutical industry. In: Engel JJ, Pedley TA (eds) Epilepsy. A comprehensive textbook, 2nd edn. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, pp 1469–1485

Examples (See Figs. 3.4, 3.5).

3.2.5 Barbital Interaction Test

PURPOSE/RATIONALE

Although not included in the ICH S7A core battery, simple tests evaluating the interaction between test substances and hypnotics can usefully be included in a CNS core battery. The additional expense is minimal, whereas such procedures provide useful information not provided by the other tests. For example, several substances with psychostimulant activity (caffeine, modafinil) induce frank signs of increase in general activity at higher doses, whereas this kind of activity can be detected at lower doses when tested by interaction with a barbiturate. Similarly, sleep-enhancing effects can be readily detected in barbiturate interaction procedures by many substances, for example, benzodiazepines and antipsychotics, which do not induce sleep even at very high doses when administered alone. Thus, interaction tests with barbiturates can serve to unmask stimulant or sedative activity not otherwise readily apparent. Furthermore, there is a high correlation between the effects observed in such procedures and those observed in more complex tests and in man.

A further consideration is which barbiturate to employ. We recommend the use of barbital because this substance, in contrast to many other barbiturates, undergoes no metabolism (Remmer 1972; Simon et al. 1992). For this reason, results obtained can be more readily interpreted in terms of a pharmacological interaction without confounding by pharmacokinetic or metabolic factors.

PROCEDURE

Rats, placed in individual Makrolon cages (25 × 19 × 13 cm), are injected with barbital sodium (150 mg/kg i.p.). The latency to sleep and the duration of sleep (maximum 6 h after barbital injection) are then recorded. Sleep is indicated by loss of the righting

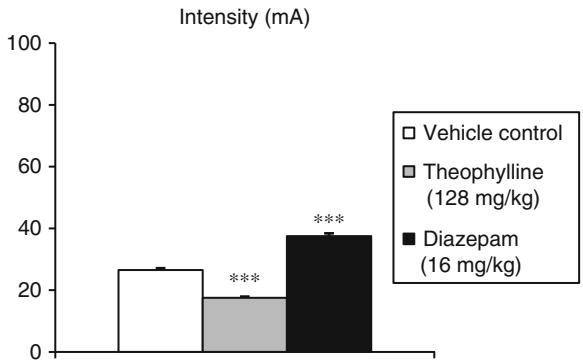

Fig. 3.4 Effects of theophylline and diazepam (p.o.) on the mean shock intensity required to induce tonic convulsions following ECS in the rat

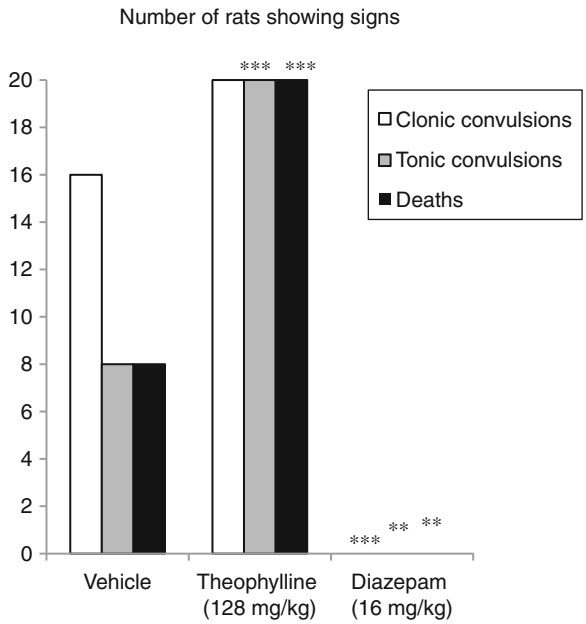

Fig. 3.5 Effects of theophylline and diazepam (p.o.) on the number of clonic convulsions, the number of tonic convulsions, and the number of deaths induced by PTZ in the rat

reflex. To avoid disturbing the experiment, no righting reflex test is performed before 50 min has elapsed after barbital injection (i.e., minimum sleep latency = 50 min).

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at four doses, administered p.o. 60 min before barbital, and compared with a vehicle control group.

Caffeine (16 mg/kg p.o.) and diazepam (8 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes seven groups.

EVALUATION

Barbital at 150 mg/kg i.p. induces sleep in 100% of the animals. The two principal parameters measured are the latency to sleep and the duration of sleep which are analyzed for statistical significance using Student's *t* tests. In addition, the number of animals sleeping is counted, and differences from control are then analyzed using Fisher's exact probability test.

A statistical problem arises when the test substance completely blocks sleep in some of the animals. In such cases, we recommend that parametric statistics be applied to the latency and duration data as long as a minimum of four animals show sleep, to avoid score bias by inclusion of floor and ceiling effects.

CRITICAL ASSESSMENT OF THE METHOD

For the reasons given in the "Purpose/Rationale" section above, barbiturate interaction tests yield useful information not readily provided by the other procedures in the core battery. Furthermore, the data obtained are highly correlated with results that could be expected in man. To our knowledge, no sleep-enhancing agent used in man has been found inactive in a barbiturate interaction procedure. Conversely, no clinically known enhancer of wakefulness, for example, caffeine, amphetamine, and modafinil, has failed to reduce or abolish barbiturate-induced sleep. Potential to cause drowsiness, as is observed with many antihistamines, is also clearly picked up in barbiturate interaction procedures as are the potential sedative effects of a variety of antipsychotics. The procedure therefore possesses high predictive validity.

On the other hand, this procedure does not provide information about drug effects on the components of sleep, for example, paradoxical versus slow-wave sleep, or on the cyclical changes which can occur during natural sleep over longer periods (24 h).

Although procedurally simple, barbiturate interaction procedures are extremely subject to small environmental changes (lighting, ambient noise and temperature, time of day) and therefore have to be performed under particularly highly controlled conditions to yield reproducible results.

MODIFICATIONS TO THE METHOD

Being one of the long-time standards in basic psychopharmacology, there are few major variants to the procedure apart from the kind of barbiturate employed. Several undergo clear hepatic metabolism, for example, pentobarbital and phenobarbital, thereby confounding interpretations because of pharmacokinetic and metabolic factors. Indeed, one modification of the method has been specifically employed to estimate enzyme induction in the liver, as indicated by more rapid barbiturate metabolism. Animals given a preexposure to a test substance are then exposed to a standard dose of phenobarbital (80 mg/kg i.p.) 24 h later and assessed for sleep duration. The presence or absence of a decrease in sleep duration is taken as an index of the hepatic enzyme induction produced by the test substance (Kushikata et al. 2003).

References and Further Readings

Kushikata T, Hirota K, Yoshida H, Kudo M, Lambert DG, Smart D, Jerman JC, Matsuki A (2003) Orexinergic neurons and barbiturate anesthesia. Neuroscience 121:855–863

Remmer H (1972) Induction of drug metabolizing enzyme system in the liver. Eur J Clin Pharmacol 5:116–136

Simon P, Chermat R, Doaré L, Bourin M, Farinotti R (1992) Interactions imprévues de divers psychotropes avec les effets du barbital et du pentobarbital chez la souris. J Pharmacol (Paris) 13:241–252

Example (See Fig. 3.6).

3.2.6 Hot Plate Test

PURPOSE/RATIONALE

The hot plate test (Eddy and Leimbach 1953) is a simple behavioral screen for estimating the effects of test substances on the threshold for pain sensitivity. It is based on the principle that rodents, placed onto a hot surface, will demonstrate the aversive effects of the stimulation

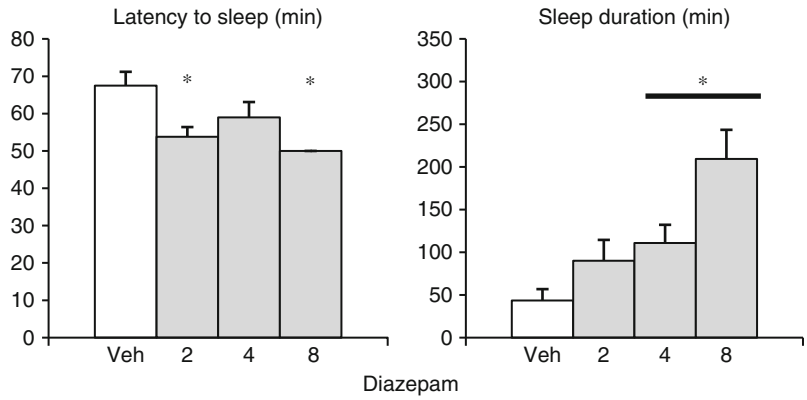

Fig. 3.6 Effects of diazepam (p.o.) on the duration of sleep induced by barbital in the rat. Administrations were performed 60 min before the test. *Different from vehicle control

first by licking their paws and subsequently by clear attempts to escape the situation (jumping). Substances changing the nociceptive threshold will either increase the latency to licking/jumping (analgesic effect) or decrease it (hyperalgesic effect).

PROCEDURE

Rats are placed onto a hot metal plate maintained at 52°C surrounded by a Plexiglas cylinder (height 26 cm; diameter 19 cm) (Apelex Model DS37). The latency to the first foot lick is measured (maximum 30 s).

Ten rats are studied per group. The test is performed blind.

The test substance is generally evaluated at four doses, administered p.o. 60 min before the test, and compared with a vehicle control group.

Morphine (128 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes six groups.

EVALUATION

The principal parameter assessed in the hot plate test is the latency to the first paw-lick response.

Data are analyzed for statistical significance using Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

The hot plate test is one of the most frequently employed screens for evaluating the nociceptive threshold. It is reliable and reproducible and remains robust in the face of moderate environmental variations. Most known analgesic substances show analgesia in the method which can therefore be considered as possessing a reasonable predictive validity. On the other hand, moderate analgesics, in particular those with a primarily anti-inflammatory profile (aspirin, paracetamol, ibuprofen), show less marked effects than major analgesics such as the opioids.

Of the two parameters measured (foot licking, jumping), foot licking appears more sensitive to the analgesic properties of test substances, whereas decreases in jumping can often reflect their locomotor effects. Other screening tests, for example, chemically induced writhing (phenyl benzoquinone, acetic acid), although revealing analgesic activity at lower doses, are sensitive to a wider range of substances and therefore yield more false positives.

The search for hyperalgesic effects is more delicate because standard drugs which cause hyperalgesia are rare and more difficult to demonstrate because of the particular conditions of administration required, for example, topical administration of capsaicin (Yoshimura et al. 2000).

The hot plate procedure possesses an advantage over other methods of thermal stimulation, for example, the tail-flick procedure (d'Amour and Smith 1941), in that it can be applied repeatedly in the same animals over a short period of time (2–3 h) without causing tissue injury, particularly if the maximum observation duration is 30 s. The hot plate procedure also constitutes a more global estimate of nociceptive reactivity because it represents a complex willed behavior rather than a simple reflex like the tail flick.

MODIFICATIONS TO THE METHOD

Different hot plate temperatures have been used, varying from 48°C to 58°C. Lower temperatures are in

principle more useful for detecting hyperalgesic activity. Although consistent with ethical requirements for decreasing pain, lower temperatures produce more variable responding and therefore require more animals to obtain statistically reliable data. A better way of satisfying the ethical requirement is to reduce the total possible duration of the test session. We have found that 30 s is largely sufficient to obtain measurable changes in the foot-licking latency in both directions.

Higher temperatures, although producing less variable data, reduce the range of analgesics which can be detected. The analgesic activity of most opioids can almost always be detected, but not milder analgesic activity of agents such as salicylates.

In addition to foot-licking latency, another parameter frequently measured is the latency to jumping attempts to escape from the apparatus. Jumping is less selective as analgesic parameter because it can be influenced by substances without known analgesic activity, but which cause either locomotor stimulation or inhibition, for example, antipsychotics. In our own procedure, with a 30-s cut-off, jumping is not reliably observed during the observation period under control conditions and is, therefore, not a useful parameter.

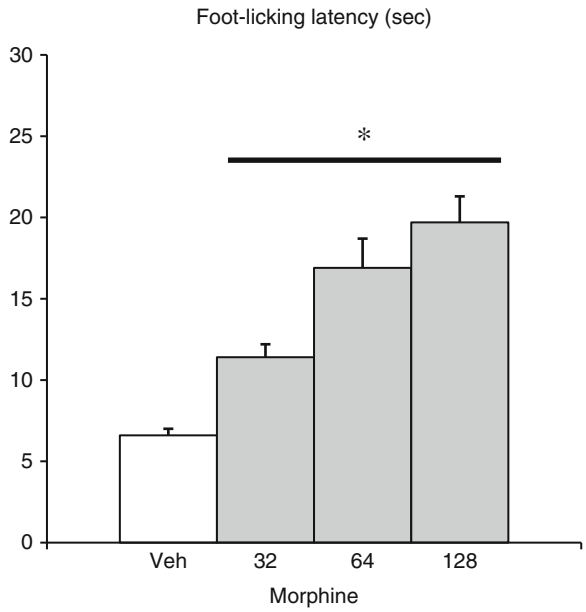

Fig. 3.7 Effects of morphine p.o. in the hot plate test in the rat. Administrations were performed 60 min before the test. *Different from vehicle control

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Example (See Fig. 3.7).

3.3 Supplementary CNS Studies

Supplementary CNS safety studies are more complex procedures, investigating test substance effects on their potential to cause drug dependence/abuse, cognitive processes, or electrophysiological brain activity. The following section is therefore divided into three subsections dealing with these different areas. As for the core battery studies described above, an essential aspect of CNS supplementary studies is that they are all performed in vivo, in the intact freely moving animal.

3.3.1 Drug Dependence and Abuse

Another subject, clearly within the notion of safety, is the assessment of a novel substance's potential to be abused or to induce dependence (Moser et al. 2011b).

Although dependence and abuse frequently occur together, they are not synonymous (Balster 1991). Drug dependence, as its name implies, refers to the inability of the dependent individual to function normally in the absence of drug. Dependence can be both physical and psychological and is defined by the emergence of withdrawal either upon administration of an antagonist or upon discontinuation of drug treatment. Psychological dependence is indicated by drug-seeking behavior (e.g., craving) that can occur even after long periods of abstinence, whereas physical dependence is demonstrated more objectively by signs ranging from changes in body temperature to life-threatening conditions such as status epilepticus or delirium tremens. Abuse, on the other hand, refers to misuse or overuse of a drug and is indicated

behaviorally by drug seeking and drug taking, many times in the absence of any evidence for physical dependence. The difference between dependence and abuse can be illustrated pharmacologically. For example, drugs such as heroin and alcohol are widely abused, and often their abuse is associated with marked physical and psychological dependence. On the other hand, marijuana and LSD are commonly abused, but typically under conditions where neither physical nor psychological dependence is apparent.

Studies assessing dependence or abuse liability are, in principle, required only for drugs acting on the CNS. Candidates for particular attention are psychostimulants, nicotinics, certain kinds of antidepressants, anxiolytics, sedative-hypnotics, and analgesics. Drugs with psychotomimetic potential also require evaluation, whereas antipsychotic agents are virtually never abused. Safety studies for abuse/dependence liability are rare for substances without CNS activity, although peripherally acting analgesics might also have to come under scrutiny.

The following section will describe protocols for evaluating both dependence and abuse. For reasons of homogeneity with the other procedures presented above, only protocols in the rat will be presented. Although results in the rat are generally similar to those obtained in nonhuman primates, the regulatory authorities, in particular the FDA, prefer primate studies for abuse evaluation because of a presumed increased predictability to man. The active doses and pharmacokinetics are likely to be closer between human and nonhuman primates, as are the kinds of overt behavioral effects observed.

References and Further Readings

Balster RL (1991) Drug abuse potential evaluation in animals. Brit J Addiction 86:1549–1558

Moser P, Wolinsky T, Duxon M, Porsolt RD (2011b) How good are current approaches to nonclinical evaluation of abuse and dependence? J Pharmacol Exp Therap 336:588–595

3.3.1.1 Drug Dependence

Tests for drug dependence consist mainly of repeated treatment studies, followed by drug withdrawal, where withdrawal symptoms occur either spontaneously (nonprecipitated withdrawal) or after administration of specific antagonists, for example, naloxone (opioids) or flumazenil (benzodiazepines). Precipitated withdrawal tests are usually the most sensitive and require only very short periods of drug administration for demonstrating withdrawal phenomena. Clear jumping can be induced in mice by administration of the μ-opioid antagonist naloxone after as few as five pretest administrations of low doses of morphine (Saelens et al. 1971). Similarly, a decrease in the convulsive threshold can be induced by administration of the benzodiazepine antagonist flumazenil after as few as two benzodiazepine administrations (von Voigtlander and Lewis 1991). The relationship between these measures of "acute dependence and withdrawal" and the dependence and withdrawal that emerge after extended periods of drug treatment followed by discontinuation of treatment are far from clear. Because such acute tests might be less indicative of dependence potential than of a particular mechanism of action or affinity for a receptor, they will not be discussed further here.

References and Further Readings

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Von Voigtlander PF, Lewis RA (1991) A rapid screening method for the assessment of benzodiazepine receptor-related physical dependence in mice. Evaluation of benzodiazepine-related agonists and partial agonists. J Pharmacol Methods 26:1–5

Nonprecipitated Withdrawal Test

PURPOSE/RATIONALE

The aim of nonprecipitated withdrawal procedures is to evaluate whether sudden cessation of drug treatment is associated with the occurrence of identifiable withdrawal symptoms. The kinds of symptoms examined are changes in food intake, body weight gain, body temperature, and the occurrence of one or more overt behavioral and other symptoms, for example, tremor, teeth chattering, wet dog shakes, diarrhea, or piloerection. The occurrence of such signs is a first indication that the test substance induces drug dependence after repeated administration. An important advantage of nonprecipitated withdrawal procedures is that they can be used to evaluate a wide variety of substances, including those for which specific antagonists are not available.

More specific procedures can be employed to assess whether drug withdrawal induces changes in fearfulness, pain sensitivity, convulsive threshold, or even memory. On the other hand, it is frequently difficult to demonstrate effects on these parameters and the tests involved are particularly time-consuming. The procedure described below represents an initial screen which has been shown to be sensitive to several dependence-inducing drugs such as opioids and benzodiazepines (Goudie et al. 1993).

Whereas many CNS safety tests evaluate multiple doses, we recommend the inclusion of just two doses for nonprecipitated withdrawal studies. The low dose should be close to those inducing clear effects in the test predictive of the substance's therapeutic indication. The high dose should be the maximally tolerated dose as determined, for example, from an Irwin test. If it can be shown that the test substance can be repeatedly administered at a maximally tolerated dose under conditions where similar treatment with an appropriately chosen reference substance induces clear withdrawal signs, a reasonable conclusion would be that the test substance is unlikely to cause physical dependence.

PROCEDURE

Rats receive twice daily administrations of the test substance for 20 days (with the last administration on day 20 at about 10:00) and are then subjected to an 8-day observation period without drug treatment during which they are observed for changes in food consumption, body weight, and rectal temperature. In addition, they are observed for behavioral and physiological manifestations (e.g., jumping, tremor, hyperactivity, excessive grooming, and diarrhea). During the pretreatment phase, different groups of animals receive the test substance at two doses and are compared with a vehicle-treated control group.

Twelve animals are studied per group. The test is performed blind.

Morphine (32 mg/kg i.p. or 64 mg/kg p.o.), cocaine (32 mg/kg i.p.), chlordiazepoxide (64 mg/kg p.o.), or diazepam (16 mg/kg i.p.) can be used as the reference substance.

The experiment includes a control group pretreated with the vehicle twice daily for 20 days.

EVALUATION

Before assessing withdrawal effects in the absence of the test substance, it is important to assess the intrinsic effects of the test substance, to see whether repeated treatment itself influences the parameters observed. Body weight, food consumption, body temperature, and the occurrence of behavioral and physiological manifestations are therefore observed during the last 3 days of drug treatment. Changes occurring after cessation of drug treatment can thus be more accurately assessed in relation to the drug effects themselves. Although we recommend doing most behavioral tests blind, this is particularly important for the present procedure where many of the behavioral parameters are assessed subjectively.

Differences from control are evaluated on a day-by-day basis using nonpaired Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

The nonprecipitated withdrawal procedure represents a first screen for possible induction of drug dependence and has been shown to be sensitive to withdrawal effects with a variety of dependence-inducing agents including amphetamines, cocaine, opioids, and benzodiazepines. It therefore possesses face validity. On the other hand, it is remarkably difficult, under the conditions of the protocol described, to show signs of withdrawal after treatment with agents such as nicotine or 9-tetrahydrocannabinol (THC) (unpublished data from our laboratory). While absence of withdrawal effects with THC may reflect the absence of proven drug dependence with cannabinoids in man, the human drug dependence data with nicotine suggest that nicotine dependence does occur.

A principal weakness of the procedure using one, two, or three daily administrations is that the behavioral and physical signs induced are modest and very short-lived (2–3 days). Indeed, under conditions of oral, i.p., or s.c. administration, there are no dramatic manifestations as have sometimes been described in the literature, particularly with opioids (Grasing et al. 1996). Administration by the i.v. route produces more rapid onset of drug action and mirrors more closely human drug abuse behavior, but it is difficult to ensure repeated i.v. administrations or continuous i.v. slow infusion over longer periods (weeks) in rats to obtain the exposure necessary to induce signs of dependence. Certain authors recommend use of osmotic minipumps to ensure a more constant exposure to the test substance and a more abrupt discontinuation by surgical removal at the end of the exposure period (Kalinichev and Holtzman 2003). This has been reported with

nicotine (Semba et al. 2004), although we have not been able to reproduce this data in our own laboratory. A major problem with osmotic minipumps is that they require the test substances to be very soluble, and this is not always the case.

There is no easy way of ensuring that withdrawal from reference substances will induce dramatic behavioral changes. The experimenter is obliged to retain more subtle effects as indices of the dependence potential of the test substance. The method does possess the advantage that it is not mechanism bound and, therefore, may detect dependence-like phenomena with agents having no common neurobiological substrates. Absence of any signs of withdrawal after repeated treatment with maximally tolerated doses can be taken as a reasonable indicator of the absence of dependence liability.

MODIFICATIONS TO THE METHOD

The nonprecipitated withdrawal procedure described above represents an open-ended screen for dependence liability. The procedure can be rendered more discriminating, by preexposing the animals to the same administration schedule and then testing them in procedures with more specific indications. For example, withdrawal-induced fearfulness could be assessed by using a plus maze or a light-enhanced startle test appropriately calibrated to detect anxiogenic activity. Similarly, a withdrawal-induced decrease in the convulsive threshold could be evaluated using either an ECS or PTZ procedure.

More complex procedures for unmasking drug dependence liability could involve assessment of acquired positive reinforcing effects as a result of repeated treatment. Induction of conditioned place preference (see below) has been reported with different substances, for example, nicotine (Shoaib et al. 1994) and alcohol (Meert 1994), after the animals have been preexposed to repeated treatment with the substance, whereas no conditioned place preference can be induced with the same substances in the absence of pretreatment.

Use of telemetry for assessment of withdrawal is advantageous as compared with more traditional approaches as it can detect changes that would be missed if only intermittent observations were made. In addition, telemetry allows a measure of withdrawal effects without complications that might arise from interactions with the experimenter. This general approach, using a range of parameters that are not modified by the process of repeat testing, and which can be affected indirectly by general feelings of malaise brought on by discontinuation, is applicable to all classes of test substance without the need to make assumptions about their specific effects or their time course (Froger-Colléaux et al. 2011). Data collected by telemetry following treatment with chlordiazepoxide and morphine and ensuing withdrawal are presented in the examples below.

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Froger-Colléaux C, Rompion S, Guillaume P, Porsolt RD, Castagné V, Moser P (2011) Continuous evaluation of drug withdrawal in the rat using telemetry: effects of morphine and chlordiazepoxide. J Pharmacol Toxicol Methods 64:81–88

Goudie AJ, Harrison AA, Leathley MJ (1993) Evidence for a dissociation between benzodiazepine withdrawal signs. Neuroreport 4:295–299

Grasing K, Wang A, Schlussman S (1996) Behavioral measures of anxiety during opiate withdrawal. Behav Brain Res 80:195–201

Kalinichev M, Holtzman SG (2003) Changes in urination/defecation, auditory startle response, and startle-induced ultrasonic vocalizations in rats undergoing morphine withdrawal: similarities and differences between acute and chronic dependence. J Pharmacol Exp Ther 304:603–609

Meert TF (1994) Pharmacological evaluation of alcohol withdrawal-induced inhibition of exploratory behaviour and supersensitivity to harmine-induced tremor. Alcohol Alcohol 29:91–102

Semba J, Wakuta M, Maeda J, Suhara T (2004) Nicotine withdrawal induces subsensitivity of hypothalamic-pituitary-adrenal axis to stress in rats: implications for precipitation of depression during smoking cessation. Psychoneuroendocrinology 29:215–226

Shoaib M, Stolerman IP, Kumar RC (1994) Nicotine-induced place preferences following prior nicotine exposure in rats. Psychopharmacology (Berl) 113:445–452

Examples (See Figs. 3.8, 3.9, 3.10, 3.11).

3.3.1.2 Drug Abuse

Drug abuse represents a more serious social problem than drug dependence because it involves a much greater variety of substances and because it can and does occur in the absence of dependence. Tests for abuse measure various aspects of drug-taking and drug-seeking behavior with the aim of establishing whether the test substance possesses positive reinforcing effects (Moser et al. 2011a).

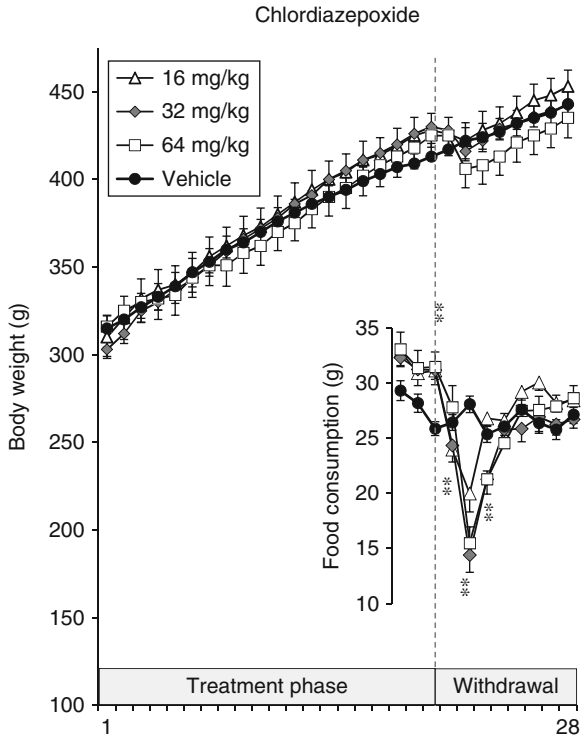

Fig. 3.8 Body weight gain and food consumption during treatment (days 1–20) and discontinuation (days 21–28) of chlordiazepoxide. Body weight was recorded during each day of treatment and discontinuation prior to the first administration each day. Food consumption was recorded over each 24-h period starting on day 18 of treatment. ** = $p < 0.01$ versus vehicle controls; ANOVA for each day followed by post hoc Student's t tests. Values are mean ± s.e.m. for $n = 8$

Indirect tests can assess whether the animal prefers to be in an environment associated with a substance with known positive reinforcing properties (conditioned place preference procedures) or whether the test substance has stimulus effects that resemble a drug with known positive reinforcing properties (drug discrimination procedures). The most direct test of drug abuse liability, however, is to assess whether animals will work to receive administrations of the test substance (self-administration procedures) (Moser et al. 2011b). Examples of the three approaches are given below.

Conditioned Place Preference Test

PURPOSE/RATIONALE

The principle of conditioned place preference is that an animal, repeatedly exposed to a distinct environment in the presence of a substance with abuse potential, will show preference for that environment when later given a choice because the environment has become associated with the rewarding properties of the test substance (Schechter and Calcagnetti 1993). If conditioned place preference can be established with a test substance, this suggests that the test substance possesses positive reinforcing effects and is therefore likely to be abused. If, on the other hand, the test substance has no such effects or even induces a conditioned place aversion, where the animal avoids the environment previously associated with the test substance, this suggests that the test substance is devoid of positive reinforcing effects or may even be aversive and is, therefore, unlikely to be abused.

PROCEDURE

Rats are given one session per day (a.m.) over 8 days. During sessions, which last 30 min, they are allowed to explore one side of a two-compartment box (35 × 35 × 70 cm). The two compartments are tactually and visually distinct (black/white striped walls with smooth floors versus gray walls with corrugated floors). The opening between the two compartments (12 × 12 cm) is closed by a guillotine door. Before each session, the animals receive a p.o. administration of either the test substance or vehicle. The test substance is always associated with the gray compartment. Thus, after 8 days, each animal will have been exposed to four pairings of test substance with the gray compartment and four pairings of vehicle with the striped compartment.

On the ninth day, in the absence of any treatment, the animals have free access to both compartments via the guillotine doorway which is left open, and the session is monitored and recorded on videotape. The time spent by the animal in each of the two compartments is scored from the video records and is broken down into 5-min segments over the 20-min test period. The number of crossings from one compartment to the other is also recorded.

Twelve animals are studied per group. The experiment is performed blind.

The test substance is usually evaluated at two doses and compared with a vehicle-treated control group.

Morphine (16 mg/kg i.p. or 64 mg/kg p.o.) can be used as reference substance.

EVALUATION

Before commencing conditioning, animals are generally given a 20-min pretest in the two compartments

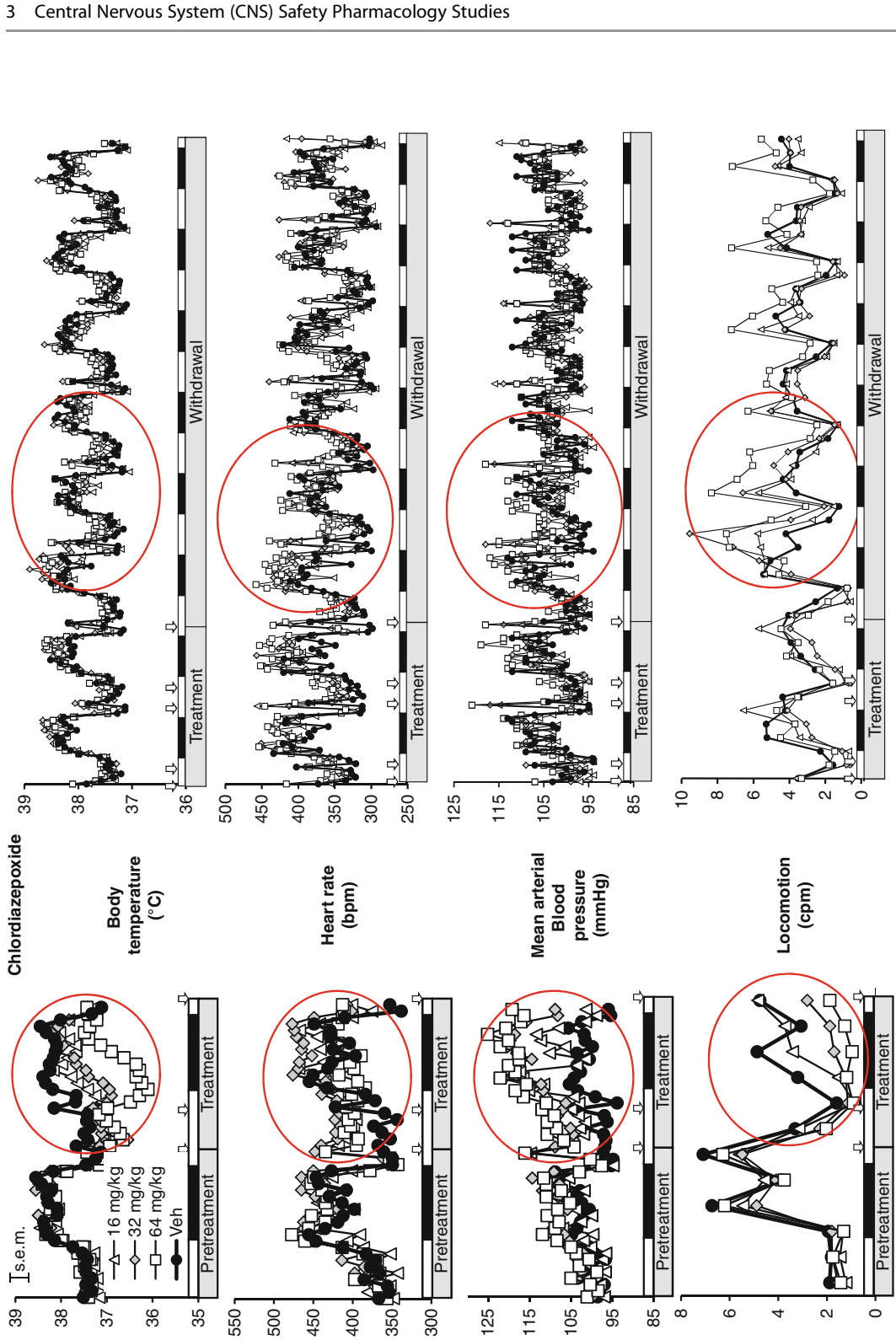

Fig. 3.9 The effect of chlordiazepoxide on body temperature, locomotion, heart rate and mean arterial blood pressure during the first and the last 3 days of treatment and the first 5 days of discontinuation. Error bars have been omitted for clarity, but for information, a value corresponding to the 75th percentile of all s.e.m. values is indicated for each graph. The *light* and *dark* segments indicate the light and dark phases of the 24-h cycle and the *arrows* indicate the times of drug administration

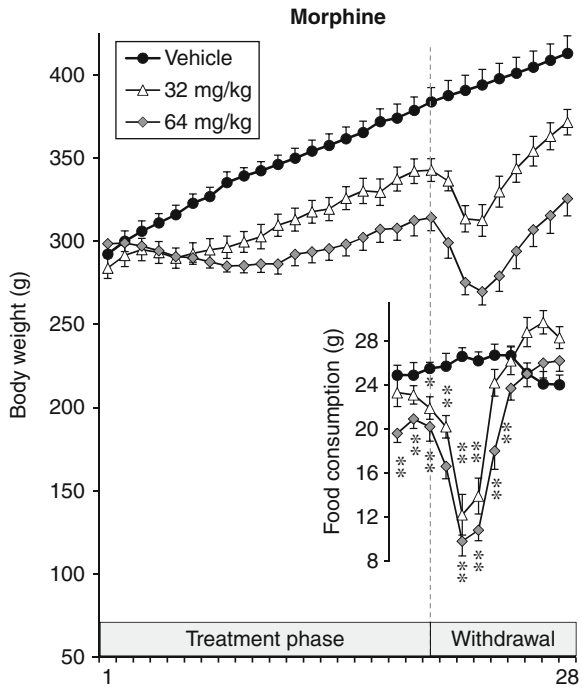

Fig. 3.10 Body weight gain and food consumption during treatment (Days 1–20) and discontinuation (Days 21–28) of morphine. Body weight was recorded during each day of treatment and discontinuation prior to the first administration each day. Food consumption was recorded over each 24-h period starting on Day 18 of treatment. For body weight, both treatment groups were significantly different from vehicle for each day of treatment from day 5 onward (pb0.01; ANOVA for each day followed by post hoc Student's *t* tests). * = $p < 0.05$, ** = $p <$ 0.01 versus vehicle controls; ANOVA for each day followed by post hoc Student's *t* tests. Values are mean ± s.e.m. for $n = 8$

with the guillotine door open, and the time spent in each compartment is measured. These data provide an initial assessment of any natural preference for either compartment in the absence of drugs and allow assignment of animals to treatment groups such that initial place preference is matched between the groups.

During the conditioning phase itself, where the animal in confined to one or the other compartment, no behavioral monitoring is undertaken.

The measure of time spent in each compartment on the test day (day 9) in the absence of the test substance is an indication of the animal's preference for each compartment. Measures of the number of crossings provide an indication of the animal's spontaneous level of activity under the conditions of the test. It should be noted that crossings are not an indication of the effects of the test substance on locomotion, because the animal is not under the influence of the test substance during the test. Clear decreases in the number of crossings are, nonetheless, systematically observed on the test day after previous conditioning with substances such as morphine and, therefore, appear to be part of the conditioned place preference process. On the other hand, no ready interpretation can be offered for these changes.

CRITICAL ASSESSMENT OF THE METHOD

Conditioned place preference appears to provide a fairly simple indication of whether a test substance exerts positive reinforcing effects. The existence of conditioned place preference with a novel substance therefore represents a clear first sign of abuse potential.

On the other hand, absence of conditioned place preference cannot be taken to indicate that the test substance is devoid of abuse potential. Indeed, the main weakness of the conditioned place preference paradigm is that several known substances of abuse (amphetamine, THC, nicotine, alcohol) do not readily induce conditioned place preference, if at all. Moreover, even with substances such as cocaine, the conditioned place preference which occurs is considerably less robust than that observed with morphine, for example. Cocaine place preference is generally observed only during the first 5–10 min of the test, in contrast to morphine where the effect is more clearly present over the whole test period. Even with morphine, the effect is observed only over a narrow dose range, 8–16 mg/kg i.p. or 64–128 mg/kg p.o., and is not clearly dose dependent (our unpublished data).

Further problems are encountered with substances such as THC, where place preference can be observed over a narrow range of low doses (0.5–1 mg/kg i.p.), but where place aversion is observed at higher doses (Maldonado and Rodriguez de Fonseca 2002). Conditioned place preference has been reported with nicotine, but only after a short pretreatment with nicotine before commencing place preference conditioning (Shoaib et al. 1994). We have been unable to replicate this finding in our own laboratory (our unpublished data).

Taken together, existing data with conditioned place preference suggest that it is rather insensitive to several known drugs of abuse (false negatives). On the other hand, the demonstration of conditioned place preference with a particular substance represents a danger sign which should not be ignored.

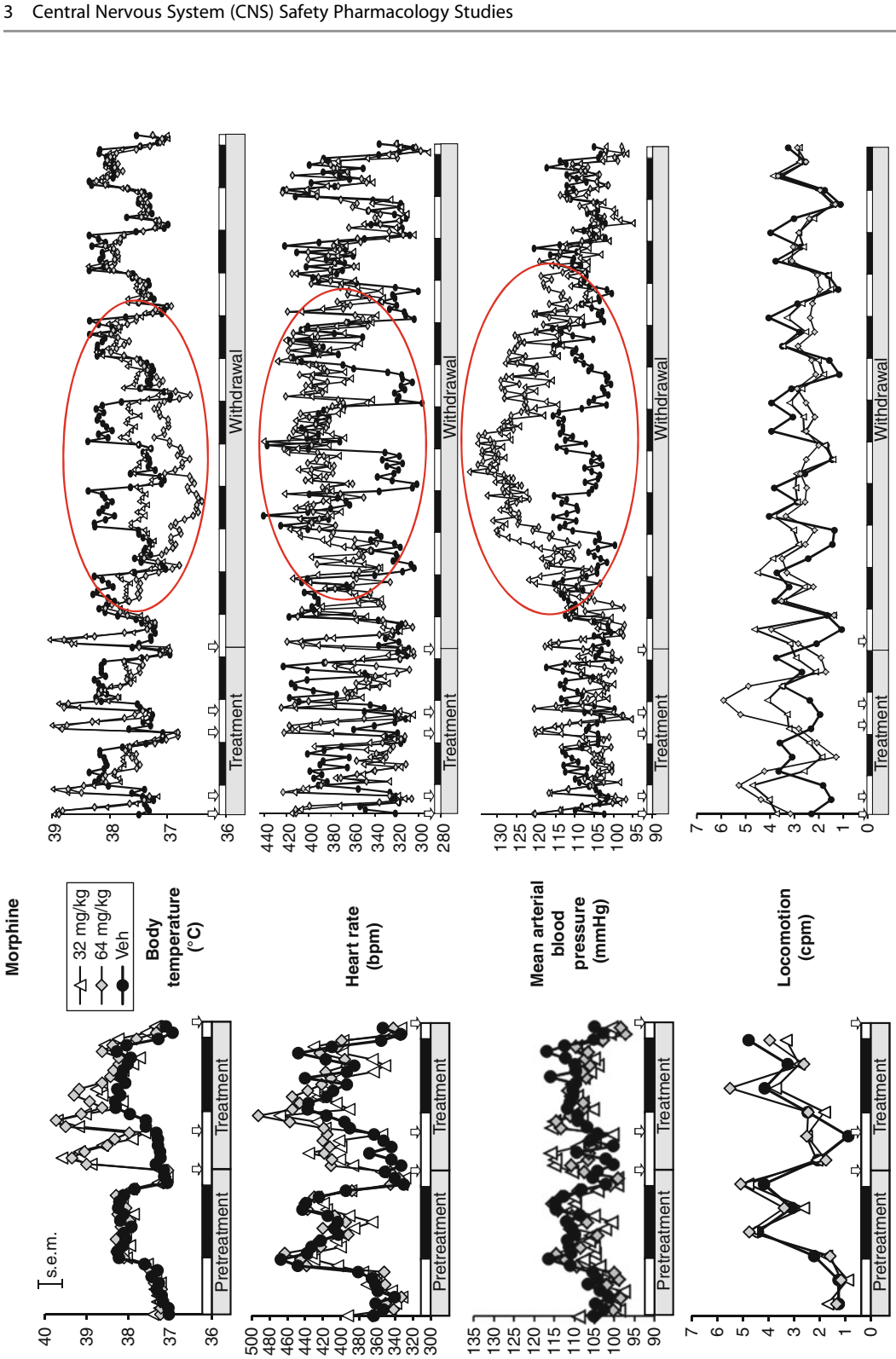

Fig. 3.11 The effect of morphine on body temperature, locomotion, heart rate, and mean arterial blood pressure during the first and the last 3 days of treatment and the first 5 days of discontinuation. Error bars have been omitted for clarity, but for information, a value corresponding to the 75th percentile of all s.e.m. values is indicated for each graph. The *light* and *dark* segments indicate the light and dark phases of the 24-h cycle, and the *arrows* indicate the times of drug administration

MODIFICATIONS TO THE METHOD

The procedure described above gives individual conditioning sessions on separate days. This procedure, while time-consuming, ensures that the animals enter each conditioning session in the absence of drug effects from a preceding session.

Shorter procedures are available where the animals are given two conditioning sessions within the same day (mornings and afternoons). With drugs such as morphine administered parenterally (i.v. or i.p.), the 5-h interval between the two sessions is sufficient to enable adequate place preference conditioning to occur, presumably because the drug effect from the morning session is no longer present in the afternoon. This assumption cannot be made when the test substance has a long duration of action or even when the test substance is administered by the p.o. or s.c. routes, where the drug effects typically last longer. If there is no clear pharmacological differentiation between two successive conditioning sessions, the absence of conditioned place preference could represent a "false negative" in that the animal is unable to discriminate the two compartments in terms of the presence and absence of the test substance. For test substances with a demonstrably short duration of action, the 2-sessions-per-day procedure represents a considerable economy in experimental time and, therefore, cost.

Another time-consuming aspect of the paradigm described above is the off-line visual analysis of the video recordings conducted after test completion. Considerable economies can be realized by use of commercially available automatic video image analysis systems. Once correctly validated, these systems offer a viable alternative to visual analysis, providing cost-effective and objective data evaluations compatible with the requirements of GLP.

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Example (See Fig. 3.12).

Drug Discrimination

PURPOSE/RATIONALE

Another indirect procedure for evaluating abuse liability is drug discrimination where animals, by pressing on one of two levers in a Skinner box, can show that they can discriminate the presence of a particular drug of abuse (training drug) from vehicle. They are then given the new substance and can indicate by their choice of lever whether the new substance resembles the training drug (Colpaert 1987; Solinas et al. 2006).

The basic assumption of drug discrimination procedures is that if a test substance is perceived as being subjectively similar to a drug of abuse, it is also likely to be abused. Many drugs of abuse are capable of being discriminated in a drug discrimination paradigm in rats and include cocaine, amphetamine, morphine, phencyclidine, mescaline, THC, nicotine, and benzodiazepines (Colpaert and Slangen 1982). It is important to note that the ability of a drug to serve as a discriminative stimulus does not necessarily indicate any potential for abuse since certain drugs that are not abused,

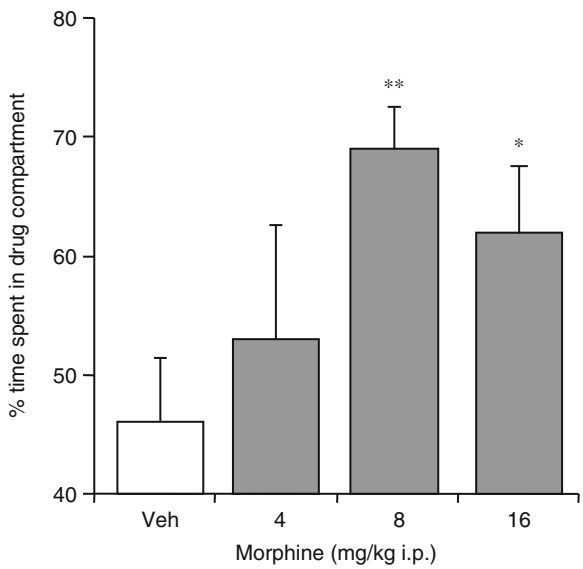

Fig. 3.12 Effects of morphine (i.p.) in the place preference test in the rat

for example, flumazenil (Gerak and France 1999), can be established as a reliable discriminative stimulus in animals.

PROCEDURE

Training

Sessions are given in commercially available operant chambers equipped with two response levers, and a food hopper. Initially, rats are submitted to lever-pressing acquisition sessions where a response on either the right or the left lever resulted in the delivery of a food pellet (i.e., FR1 schedule of reinforcement). The levers are inserted in the chamber at the beginning of the session and are withdrawn at the end of the session. The house light comes on at the beginning of the session and is extinguished at the end of the session. Sessions terminate after 45 min or after the animals completed 50 responses, whichever occurred first. At the end of this phase, animals which fail to acquire the lever-press response are discarded from the experiments. No administration is given during the acquisition of the lever pressing.

When animals receive 50 pellets in a session, the response requirement is increased to FR2 and discrimination training commences whereby only responding on one of the levers is reinforced. For a particular day, the active lever depends on the treatment administered (i.e., saline or drug.). The treatment is administered 15 min before the sessions. The allocation of the right or the left lever to the training substance (drug) is balanced out between animals. The response requirement is increased progressively across days to a final fixed ratio (FR) value of 10. Animals must make consecutive responses (1 FR) on a given lever in order to receive a food pellet, the FR value being reset to zero if an animal responds on the other lever prior to completion of the FR.

Saline and drug are administered across sessions according to alternating sequences of single and double alternation (e.g., drug, saline, saline, drug, drug, saline, saline, drug, saline, drug), with training sessions taking place on weekdays only. Two sequences are used so that the rats do not systematically all receive the same treatment each day. During the pretreatment period, animals return to their home cage prior to placement in the chamber. The session ends after the delivery of 50 food pellets or 15 min, whichever occurs first. At the end of this phase, animals which fail to discriminate within 75 sessions are discarded from the experiment. Training continues until the following criteria are satisfied:

(a) Eight of ten consecutive training sessions (this performance must thereafter be maintained for at least a further five sessions) (i.e., 80% of correct training sessions over 3 weeks)
(b) The two sessions (one saline and one drug session) just prior to a test session must be correct.
(c) The above criteria must be maintained between test sessions which are carried out with at least two intervening successful training sessions of which at least one must be a saline session and one an cocaine session.

The test sessions are identical to training sessions except that rats receive different doses of the test substance in a pseudorandom order, administered 15 min before the session. During test sessions, 10 responses on either lever result in delivery of a food pellet, with the FR value being reset to zero if an animal responds on the other lever prior to completion of FR10 (i.e., rats must make 10 consecutive responses on a given lever in order to receive a food pellet). Morphine (5 mg/kg i.p.), cocaine (8 mg/kg i.p.), diazepam (2 mg/kg i.p.), D-amphetamine (0.6 mg/kg i.p.), DOI (0.63 mg/kg i.p.), or THC (3 mg/kg i.p.) can be used, among others, as comparator drugs.

To ensure that 8–10 animals can be retained for drug testing, training is commenced with 12 animals per drug.

Drug Testing

Test sessions are identical to training sessions except that rats receive different doses of the test substance in a pseudorandom order, administered 15 min before the session. During test sessions, 10 responses on either lever result in delivery of a food pellet, with the FR value being reset to zero if an animal responds on the other lever prior to completion of FR10 (i.e., rats must make 10 consecutive responses on a given lever in order to receive a food pellet). The training dose of the training drug and saline are tested initially to confirm adequate stimulus control for testing. Thereafter, three different doses of the test substance (and the vehicle used for the preparation of the test substance, if needed) are studied using a Latin square design. On concluding test substance evaluation, the training drug and saline are tested again.

EVALUATION

Two basic measures are taken during drug discrimination procedures, the percent responses on the drug-associated lever and the response rate on both levers (in response per second).

The first parameter (percent responses on the drug lever) provides an estimate of the degree of generalization between the test substance and the comparator (training drug). No responding on the drug lever (i.e., 100% responding on the saline lever) would indicate that the test substance does not generalize to the training drug and that the test substance is therefore not recognized as being similar. Exclusive (100%) responding on the drug lever would indicate complete generalization to the training drug and that the test substance is therefore recognized as resembling the training drug. In cases where clear generalization occurs, a dose-response effect is observed and the data lend themselves to calculations of ED50s which represent the dose levels at which the test substance generalizes 50% to the training drug.

The second parameter (response rate on both levers) provides an estimate of the effects of the test substance on operant performance. If the test substance exerts marked sedative effects, the number of responses would normally be decreased. Response rate could even be increased if the test substance possessed psychostimulant effects. Interpretation of drug effects on operant performance is, however, not simple, because other factors can contribute to effects of the test substance on response rate. In the present procedure, where the effect of the reinforcement schedule (FR10) is to produce a high rate of baseline responding, test substances with either sedative or psychostimulant effects will generally decrease the rate of responding.

Although the test substance may generalize partially or fully to the training drug up to some dose, further increasing the dose will eventually decrease or completely suppress responding. Partial generalization, accompanied by a suppression of responding at higher doses, could suggest that the test substance does not fully share discriminative stimulus effects with the training drug. Alternatively, such a result could indicate that rate-decreasing effects occur at comparatively smaller doses and, thereby, preclude measures of discriminative stimulus effects. Partial effects of this type must be evaluated with great caution. However, if complete generalization occurs before the test substance suppresses responding, this suggests a clear similarity between the test substance and the training drug, at least within that particular dose range.

Discrimination results from animals that have had a low response rate (fewer than 50 responses) were not used for calculating the percentage of responding on the drug-associated lever. However, all data from all tests were used to calculate response rate.

No formal statistical analysis was performed on percentage of the total responses on the drug-associated lever. Response rate was analyzed, comparing the different treatments with saline (or vehicle) using paired Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures provide a unique opportunity for assessing subjectively perceived drug effects using purely behavioral criteria and quantitative measures. Furthermore, under appropriate training conditions, the method has been shown to be exquisitely sensitive even to minor changes in pharmacological activity, for example, between different benzodiazepines (Ator and Griffiths 1989) or even between different doses of the same drug (Walker et al. 2001). At the same time, the numerous published data show a high degree of selectivity within different substance classes. The perceived similarities between different psychotomimetics, for example, PCP and MK-801 (France et al. 1991) or opioids (France et al. 1995), provide cogent behavioral evidence for a common mechanism of action or even action at the same receptor subtype, whereas dissimilar discriminative stimulus effects provide suggestive evidence for action at different receptors or different actions at the same receptor. Thus, the greatest strength of drug discrimination is its pharmacological selectivity.

The interplay between generalization curves and drug effects on response rate can often provide behavioral evidence for the degree of similarity between a selected training drug and the test substance, or the dose range in which such similarity exists, or of the kind of action occurring at a target receptor including agonism, antagonism, inverse agonism, and the relative efficacy of those effects.

A further advantage of drug discrimination paradigms is that similar methodology can be used in a variety of species from mice (Shelton et al. 2004) to pigeons (Walker et al. 2001), to rats (Ator and Griffiths 1989), to monkeys (Gerak and France 1999), and even

to humans (Foltin and Fischman 1992), thereby permitting direct extrapolations across species.

The primary disadvantage of drug discrimination procedures is that they are time-consuming. This becomes most apparent when the procedure is used to characterize the perceived pharmacological profile of a new test substance, where it may have to be evaluated in numerous independent groups trained to recognize a variety of known drugs of abuse. Thus, characterization of test substances solely on the basis of drug discrimination procedures is likely to take considerable time.

The principal weakness of drug discrimination procedures for assessing abuse liability is that they provide only indirect evidence regarding abuse. If a test substance is discriminated as being similar to a known drug of abuse, this is taken to indicate that the test substance is likely to be abused in a similar manner. On the other hand, if a test substance does not share discriminative stimulus effects with any known drug of abuse, drug discrimination procedures alone provide no indication of whether the test substance is likely to be abused.

MODIFICATIONS TO THE METHOD

There are many variants in drug discrimination methodology whereby small procedural differences (fixed or variable interval instead of fixed or variable ratio reinforcement, probe trials with or without reinforcement to test for generalization, test sessions where either the chosen lever, i.e., that on which the animal emits say its first 10 responses, or both levers are reinforced) are practiced in different laboratories without marked differences in the results obtained.

A more important difference is whether to score the level of generalization by the percentage responding on the drug-associated lever or by the initial lever choice of the animal when exposed to the test substance. The former in theory permits a more quantified and graded estimate of the degree of generalization at the different doses and should, thereby, permit a more powerful statistical analysis. The latter might provide a more unbiased measure of discriminative stimulus effects since responding prior to food delivery, unlike responding after food delivery, cannot be influenced by contingencies of reinforcement. However, simple lever choice measures permit only quantal measures (proportion of animals choosing the drug-associated lever) at each dose investigated, with correspondingly less powerful statistical analysis. Again, there are no marked differences in the kinds of results reported by laboratories using the two approaches. One reason is that the distribution of scores between the two levers is essentially bimodal, with little variation at the two extremes and considerable variation around the 50% mark.

Another potentially useful drug discrimination approach is to establish whether the test substance itself can exert discriminative stimulus control as a training drug. If the test substance is poorly discriminable, more sessions will be required before achieving an adequate level of stimulus control or abandoning training. Indeed, failure to establish discriminable control over many training sessions constitutes suggestive evidence that the substance has few CNS effects, assuming that it has been administered in an appropriate dose and at an appropriate time prior to training sessions. While inability to establish discrimination with a test substance provides presumptive evidence for a lack of CNS effects that could predict little or no abuse potential, the opposite result is not true. That is, rapid acquisition of discriminative control with a test substance simply indicates that the substance has stimulus effects, perhaps mediated in the CNS. Because many CNS-acting drugs that are not abused are reliably discriminated by nonhumans (e.g., kappa opioid agonists; France et al. 1994), no predictions can be made regarding abuse potential based solely on ease of discrimination training.

Despite the time taken to train an animal to recognize a test substance, once the animals are sufficiently trained, they can be used repeatedly with a variety of drugs of abuse with the aim of identifying which of these substances generalize to the training substance (in this case, the test substance). In this fashion, it would be possible to establish the profile of perceived drug effects using the same animals tested with different drugs of abuse. This kind of procedure is rarely reported, but could represent a considerable economy in the number of animals required.

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Examples (See Figs. 3.13, 3.14, 3.15).

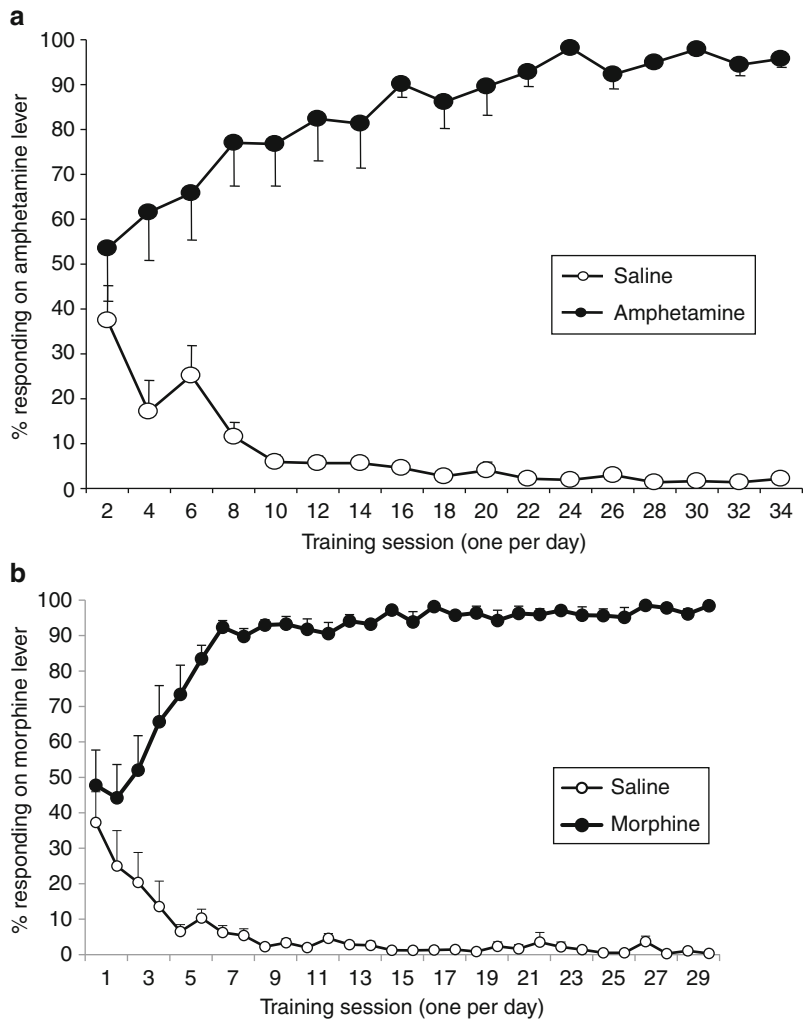

Fig. 3.13 (**a**, **b**) Acquisition of D-amphetamine (0.6 mg/kg i.p.) and morphine (5 mg/kg i.p.), drug discrimination in rats

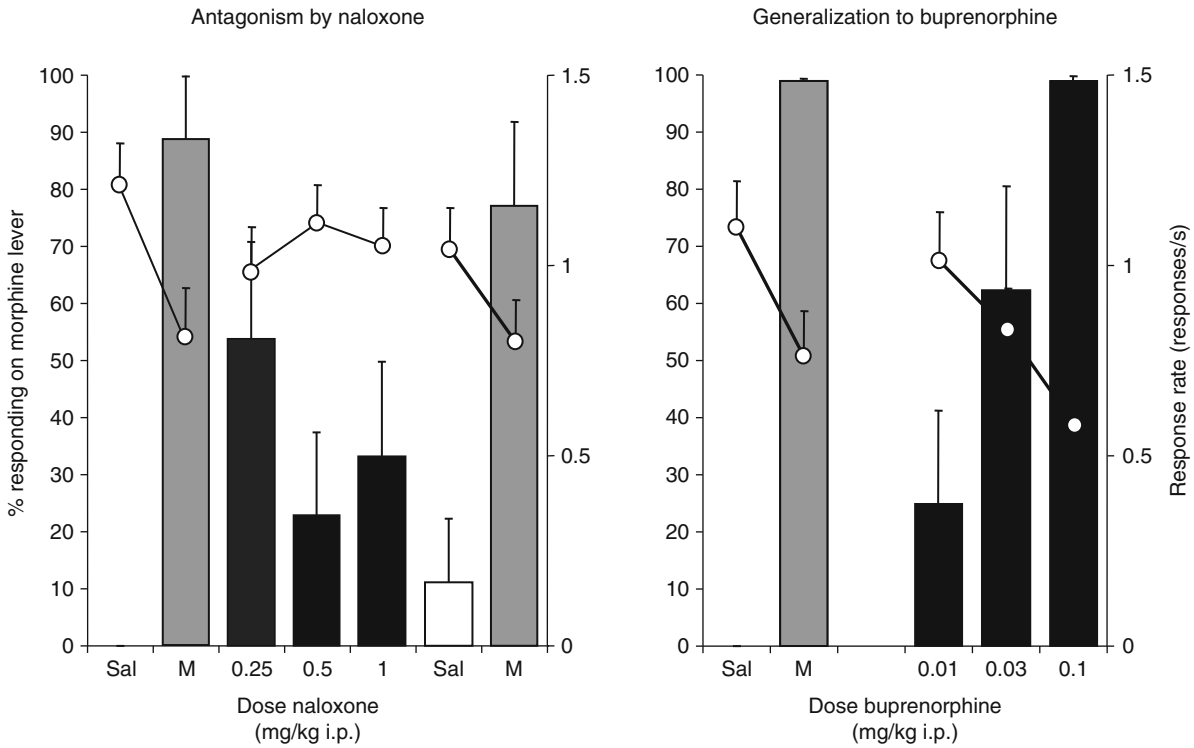

Fig. 3.14 Effects of naloxone on drug discrimination in rats trained to discriminate morphine from saline and generalization to buprenorphine. Note the antagonism of responding on the lever associated with morphine by naloxone. There is a dose-dependent generalization to buprenorphine toward responding to the lever associated with morphine

Self-administration

PURPOSE/RATIONALE

The most direct tests of abuse potential are self-administration procedures, where the animals can, by pressing on a lever, receive an i.v. infusion of the test substance via a pump system connected to an indwelling catheter (Brady and Griffiths 1976). Parallel methods have been used widely with rats and monkeys and provide substantially similar findings. More recently, self-administration procedures have also been developed using mice (Caine et al. 2002; Thomsen and Caine 2005). Virtually, all drugs which are abused in humans will induce or sustain self-administration behavior in animals. The self-administration paradigm is a clearly homologous animal model of human drug abuse with a consequently high level of face validity, construct validity, and predictive validity.

PROCEDURE

Experiments are conducted in ventilated, sound-attenuating operant chambers. Each chamber is equipped with a single lever located to the right of a 5 × 5 cm opening for food pellet delivery from a food hopper. A translucent stimulus light is located above the food hopper. Experimental events are controlled and monitored, and data are collected and stored, by a microcomputer and associated interface. Male rats (240–320 g) are housed individually and maintained under a noninverted 12/12-h light/dark cycle. Upon arrival, rats have restricted access to food for 5–7 days (15 g/rat/day then 18 g/rat/day after the surgery), after which, lever training begins under a continuous reinforcement (CRF) schedule of food presentation. Daily sessions comprise 45 min or 20 food presentations, whichever occurs first. Subsequently, the response requirement is increased to a fixed ratio (FR) 10. Once rats receive 20 food pellets in a single session under the FR10 schedule (typically within 5–10 days), food training is suspended and a chronic indwelling i.v. jugular catheter is implanted. Beginning at least 3 days after surgery, daily sessions (typical maximum duration of 120 min) are conducted during which rats can receive

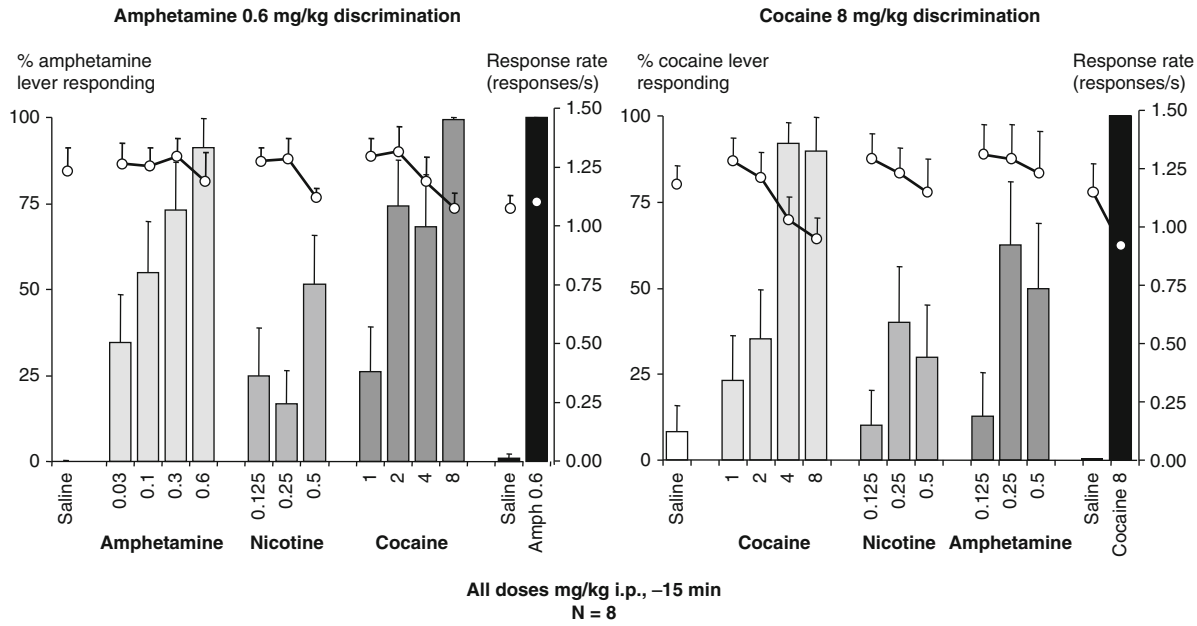

Fig. 3.15 Effects of nicotine and cocaine on drug discrimination in rats trained to discriminate amphetamine from saline and effects of nicotine and amphetamine in rats trained to discriminate cocaine from saline. There is a dose-dependent generalization to cocaine to the lever associated with amphetamine, whereas the converse is partially true. There is only partial generalization to nicotine in rats trained to discriminate amphetamine or cocaine from saline

a maximum of 50 i.v. infusions of the baseline drug (cocaine, 0.5 mg/kg/infusion) under the FR10 schedule. Immediately prior to the beginning of daily sessions, rats receive 1 noncontingent (i.e., "priming") infusion of the same solution that is delivered after appropriate responding during the session (baseline drug, saline, or test substance). A distinctive visual stimulus is displayed during periods when i.v. infusions are available. Each infusion is followed by a 30-s timeout, during which the chamber is dark and lever presses have no programmed consequence. The infusion duration is 4–6 s, and the infusion volume is approximately 138–210 µl (infusion rate 2.075 ml/min), depending on the weight of the subject and the solubility of the test substance.

Self-administration Testing

After responding for the baseline drug stabilizes (i.e., when the variability in the number of infusions delivered per session does not exceed ± 20% over three consecutive sessions), saline (the cocaine vehicle) is substituted for the baseline drug until the number of infusions per session is less than 8% or 40% of cocaine responding for three consecutive days. Following this training phase, rats are tested with 0.25 mg/kg/infusion of cocaine for at least 3 days to verify that lever pressing is contingent on a rewarding substance being available and that rats rapidly increase their lever-pressing behavior (i.e., within one or two sessions) and to a high level (>20 infusions per session) as soon as such a substance is available. This is followed by a test substance vehicle phase for at least 3 days, where lever pressing must rapidly drop to low levels (less 40% of cocaine responding). Once these four experimental phases are completed, a specified dose of the test substance is substituted for saline (or test substance vehicle) (for at least five sessions) until there are fewer than eight infusions over three consecutive sessions or until the number of infusions per session does not vary by more than ±20% over three consecutive sessions. Next, saline is substituted for the test substance until the criteria listed above are satisfied. Finally, the baseline drug (cocaine, 0.25 mg/kg/infusion) is substituted for saline until the criteria listed above are satisfied. Testing starts with a minimum of 10 rats per group with the objective of obtaining data from 8 rats that complete all phases of the experiment. Each subject is used to study only a single dose of test substance. A total of three dose levels of the test article are administered.

EVALUATION

The primary behavioral measure recorded is the number of infusions received during the session.

CRITICAL ASSESSMENT OF THE METHOD

The model described above is a so-called substitution procedure where the animal is first trained to respond for i.v. infusion of a known drug of abuse, in the present case cocaine. Once reliable self-administration is established, the test substance is substituted for cocaine to see whether the test substance continues to maintain self-administration behavior.

This procedure had several advantages. The first is that drug testing commences in an animal already shown to administer the reference drug. As such, the model possesses a high level of face validity in that the test substance evaluation is conducted in a drug-experienced animal. Moreover, even in the rat, there can be individual differences in the rate at which animals self-administer the reference drug, even after extensive training. Thus, the substitution procedure provides an assessment of the rate at which the test substance is self-administered compared with the reference drug, for individuals and for groups of subjects. Furthermore, evaluating the test substance in animals already shown to self-administer a reference drug of abuse decreases the possibility that a negative finding with the test substance is due either to the animal's low intrinsic tendency to self-administer or to other factors which might have prevented the animal from initiating self-administration. Thus, the substitution model represents a highly sensitive procedure for detecting positive reinforcing effects of test compounds which, in turn, have high predictive validity for abuse potential in humans. No self-administration behavior under these conditions suggests the absence of abuse potential.

A critical element in all self-administration procedures is the choice of the doses to be evaluated. If the doses are too high, it is possible that no self-administration will be observed because the high doses directly interfere with operant responding. Studying inappropriately high doses could therefore lead to the false conclusion that the test substance is devoid of abuse liability, whereas lower doses might clearly maintain self-administration. The best way to avoid overdosing is to conduct prior dose-finding experiments, using either an operant behavior schedule or a more simple measure of spontaneous locomotion, like the activity meter test described above. The highest dose chosen for the subsequent self-administration experiment should be one just below that inducing a clear effect on operant or spontaneous behavior. The choice of the lowest dose represents less of a problem in that it should be close to that found active in procedures used to evaluate the potential therapeutic activity of the test substance.

Use of an appropriate self-administration paradigm, such as the substitution procedure described above, is likely to be sensitive to a large variety of known drugs of abuse (Griffiths and Balster 1979; Griffiths et al. 1980), although certain substance classes, for example, nicotine (Corrigall 1999) and benzodiazepines (Broadbear et al. 2005), while clearly positive reinforcers under a limited range of conditions, are not as readily self-administered as opioids or cocaine. Indeed, the different self-administration effects of these diverse substance classes probably reflect their real-life abuse liability as indicated by "street use." On the other hand, the experimental literature describes self-administration in animals with several substances, for example, modafinil (Gold and Balster 1996), nomifensine (Aspen and Winger 1997), and bupropion (Bergman et al. 1989), which have never been associated with significant problems of abuse in humans. Thus, self-administration procedures in nonhumans can generate a certain number of apparent "false positives." Reasons for the absence of abuse in humans with some substances that maintain self-administration responding in animals could be related to the ease at which these substances can be put into an injectable formulation, pharmacokinetic factors such as their rapidity of absorption, their availability compared with other drugs, or to important differences between the self-administration doses and those which are active in the therapeutic indication.

MODIFICATIONS TO THE METHOD

The major variants of the self-administration paradigm, for the purposes of abuse potential studies, are substitution procedures and initiation procedures. With substitution procedures, animals are first trained to self-administer a reference drug and are then administered the test substance to see whether the test substance can substitute for the reference drug in maintaining self-administration behavior. The advantages of substitution procedures in CNS safety evaluation have been described above. Another advantage

of substitution procedures is that they permit reuse of the same animals for assessing different test substances or different doses of the same test substance. This becomes highly important when primates are used because of the cost of the animals themselves and of ethical issues surrounding the use of primates. Reuse is less of an advantage with rats because the practical lifespan of the assay (i.e., catheter patency) is significantly less with rodents.

Initiation procedures ask whether a drug-naïve animal will come to self-administer the test substance without any prior self-administration training. If a test substance induces clear self-administration in a drug-naïve animal, this constitutes even more compelling evidence of its abuse potential. On the other hand, initiation procedures for the same reason can be considered less sensitive, i.e., less likely to detect self-administration, and are therefore less appropriate for CNS safety pharmacology. Because there is typically no positive control in this type of initiation study, the failure to establish self-administration responding in naïve animals does not constitute adequate evidence for concluding there is no abuse potential.

Another important variant in self-administration procedures is the animal species used. The most frequently used animals are rats and nonhuman primates, usually macaques but also new world monkeys (capuchin, squirrel, marmosets) and baboons. More recent publications have described rapid self-administration procedures in the mouse (Caine et al. 2002) with consequent reductions in cost. There is no evidence that the data obtained between different species differ qualitatively in terms of the kinds of drugs showing positive reinforcing properties. On the other hand, primates, and in particular the macaque, are the species preferred by the drug registration authorities mainly because of their greater similarity to humans in terms of active doses and pharmacokinetics and because of the extensive literature on the behavioral effects of CNS-acting drugs in this species.

Analysis of self-administration behavior is mainly qualitative, indicating whether or not a test substance is a positive reinforcer in self-administration studies. Although there are clear differences between drug classes and even between individual animals, in the number of infusions taken during a test session, the number of infusions per session might not provide a clear reflection of the reinforcing effectiveness of a test substance because ongoing behavior can be affected by repeated drug delivery, potentially leading to suppression of responding or even lethality. The pharmacokinetic profile of a test substance is a major determinant of whether or not reinforcing effects are observed (i.e., in general, rapid-onset drugs are more reinforcing than slow onset drugs) and whether the number of reinforcers received throughout a test session reflects overall reinforcing effectiveness (i.e., in general, long-duration drugs can accumulate through the session, suppress responding, and, thereby, appear to be less reinforcing than might be the case). One alternative to the typical repeated dosing self-administration procedure is a second-order schedule of self-administration (e.g., Negus and Mello 2004). Under this schedule, nondrug stimuli (e.g., lights) that have been paired with drug delivery are presented under a schedule of reinforcement throughout the session and only a single drug injection is administered at the end of the session. Thus, behavior throughout a session is not affected directly by drug delivery since drug is administered only once, at the end of the session. Second-order schedules have not been used widely in abuse potential studies, perhaps because they require extensive training and because behavior under these schedules tends to respond less rapidly to changes in dosing conditions. Another indicator of the reinforcing effectiveness of a test substance can be the amount of work an animal will perform to obtain the test substance. This can be investigated with a progressive ratio schedule (e.g., Solinas et al. 2004) whereby the response requirement increases for every infusion or after a fixed period of time (Wilcox et al. 2000) until the animal ceases to lever press for a predetermined interval, defined as the breaking point. The presumption of progressive ratio studies is that the higher the breaking point, the greater the reinforcing effectiveness, although the same potential complications regarding pharmacokinetics that were noted above for simple (FR) schedule of self-administration also apply to progressive ratio schedules. Breaking point studies are usually undertaken as a supplementary investigation after a test substance has demonstrated positive reinforcing effects under other conditions.

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Examples (See Figs. 3.16, 3.17).

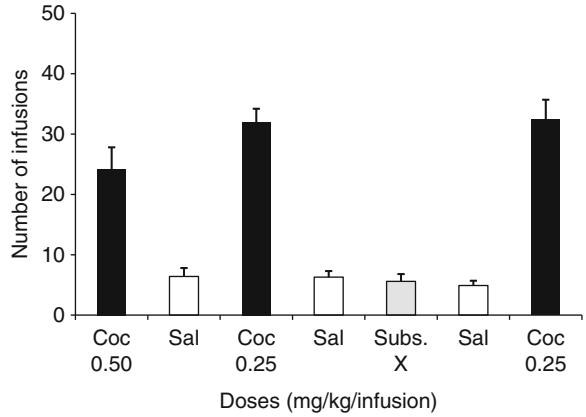

Fig. 3.16 Effects of cocaine in a self-administration procedure in the rat. Cocaine induces clear self-administration at the beginning and the end of the experiment, in contrast to saline which induces a low level of self-administration

3.3.2 Cognitive Processes

Tests of higher cognitive function find their place during the later phases of CNS safety assessment because they are more time-consuming to perform and cannot therefore be performed on a routine screening basis. Included under the term cognitive function are learning, memory, and attention. It is important that drugs be devoid of impairing effects on these functions, whatever their indication. It is thus essential that CNS safety pharmacology provide procedures for evaluating these effects in animal studies. There is no standardized set of procedures in this area. The following procedures provide an indication of the kinds of protocols that can be applied. The list is by no means exhaustive.

3.3.2.1 Passive Avoidance Test

PURPOSE/RATIONALE

One of the simplest procedures for looking for adverse effects on learning/memory is the so-called one-trial passive avoidance task (Bammer 1982). A mouse or a rat receives an aversive stimulation in a recognizable environment and on a later occasion shows it has remembered by not going there (passive avoidance). Amnesia-inducing drugs (benzodiazepines, anticholinergics, NMDA antagonists), administered before the first exposure, attenuate the animal's memory for the aversive stimulus as shown by a decreased avoidance of the environment in which it was previously received.

PROCEDURE

The passive avoidance apparatus we use consists of two compartments, one (30 × 30 × 30 cm) brightly lit and the other (20 × 20 × 12.5) dark, connected by a small opening (8 × 8 cm) which can be closed by a guillotine door.

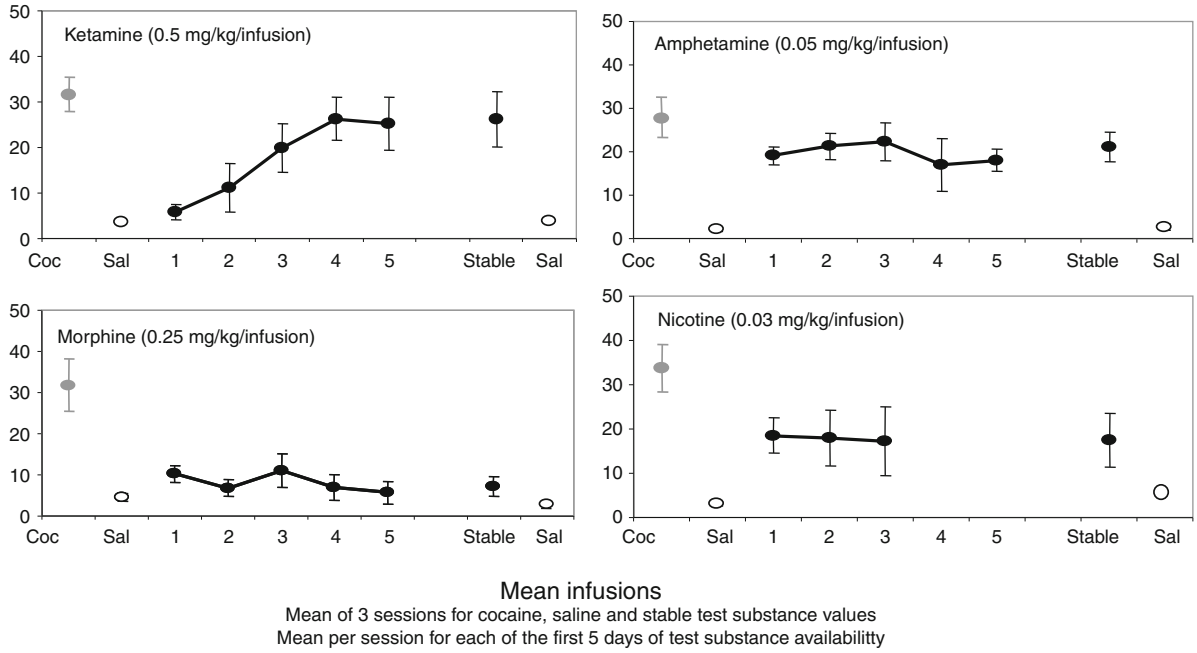

Fig. 3.17 Acquisition of four commonly abused substances (ketamine 0.5 mg/kg/infusion, amphetamine 0.05 mg/kg/infusion, morphine 0.25 mg/kg/infusion, and nicotine 0.03 mg/kg/infusion) in cocaine (0.5 mg/kg/infusion)-trained rats

For the first trial (T1), rats are placed individually into the lighted compartment. After 30 s, the door to the dark compartment is opened. When the rat has entered the dark compartment, the door is closed and the rat immediately receives a 0.8 mA shock (Coulbourn shock generator) for 2 s. The animal is removed immediately after the shock and is replaced in its home cage.

The animal is placed again 48 h later in the lighted compartment with the door closed for the second trial (T2). The door is opened after 30 s, and the animal's latency to cross to the dark compartment is recorded (cut-off time = 180 s).

Amnesia-inducing drugs cause a significant decrease in the step-through latency at T2.

Twenty rats are studied per group. The test is performed blind.

The test substance is usually evaluated at three doses, administered p.o. 60 min before T1, and compared with a vehicle control group.

Scopolamine (0.5 mg/kg s.c.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes five groups.

EVALUATION

The principal measure taken is the animal's latency to cross to the dark compartment at T2. This score provides an estimate of the animal's retention of the shock received at T1. Because the latencies measured at T2 have a 180 s cut-off, the scores in the control group are abnormally distributed because of the presence of numerous ceiling scores. It is therefore essential to apply nonparametric statistics, for example, the Mann-Whitney U test, to analyze the data.

A second measure taken is the animal's latency to cross to the dark compartment during T1. This measure is used to estimate the intrinsic effects of the test substance on exploration/locomotion. These scores are more normally distributed than at T2 but, for reasons of homogeneity, should also be evaluated using the Mann-Whitney U test.

A clear drug effect on T2 latencies in the absence of an effect on T1 latencies can be more clearly interpreted as an effect on memory.

CRITICAL ASSESSMENT OF THE METHOD

Passive avoidance procedures provide the most rapid and apparently simple index of a drug's impairing

effects on memory. They are therefore suitable as a first screen for potential cognition-impairing activity. The fact that the learning occurs within a single trial distinguishes this procedure from all other procedures described below. On the other hand, passive avoidance procedures suffer problems of interpretation in terms of the memory/learning processes involved. Is a decrease in step-through latency at T2 due to the fact that the animal did not learn the association between the dark compartment and the shock or because it could not retain this learning over the interest interval? A decrease in T2 step-through latency could also reflect an impairment of attention at T1 or even a decrease in pain sensitivity induced by the test substance during the learning trial.

A further problem with passive avoidance procedures is that, despite their apparent simplicity, the results obtained are notoriously variable on separate occasions and between different laboratories (Bammer 1982). Although part of the explanation for the interlaboratory variability lies with the multiplicity of passive avoidance procedures (see below), even using the same procedure from day to day provides varying results. To counteract the variability, it is therefore necessary to include more animals per group.

Passive avoidance procedures are essentially useful for detecting impairing effects of test substances. It is more difficult to demonstrate cognition enhancement because of ceiling effects. In terms of safety pharmacology, this is not a major drawback as impairment represents the major cognitive risk.

MODIFICATIONS TO THE METHOD

The passive avoidance procedure exists in multiple versions, but basically consists of two paradigms, step-down and step-through. The procedure described above is a step-through procedure involving two compartments, light and dark, whereas the step-down procedure involves placing the animal on a platform just above an electrifiable grid floor. Both oppose the animal's natural tendencies (aversion to bright light or remaining in a restricted space) with avoidance of an electric shock.

With the step-through procedure, the dimensions of the apparatus can vary, in particular the relative sizes of the two compartments. In general, it is the light compartment which is larger. The size of the light compartment can radically change the latencies with which the animal crosses to the dark compartment and, thereby, influence the amount the animal learns about this new environment before receiving electric shock. The manner of delivering shock can also vary. With some procedures, the animal receives shock for a brief but fixed period. With other procedures, the animal receives shock until it has returned to the light compartment. The same is also true for the step-down procedure. Indeed, the existence of so many variants no doubt explains much of the variability of data between different laboratories. As yet, no single "best" method has been recommended.

Most passive avoidance procedures used in CNS safety pharmacology employ shock levels of sufficient intensity to induce a high level of avoidance, thereby providing a behavioral baseline suitable for assessing cognitive impairment. Other variants either use lower levels of shock to induce less than maximal learning during a learning trial to permit demonstrations of improved performance or use multiple trials for establishing learning curves. Multiple trial procedures permit the progress of learning to be monitored and the effects of test substances on the learning process to be assessed, thereby providing more readily interpretable data. On the other hand, multiple trials are time-consuming, decreasing the major advantage of one-trial procedures. Using lower shock levels in a one-trial procedure has the disadvantage of decreasing the interpretability because there is no independent means of establishing whether learning was present or whether the shock level was simply insufficient to sustain a passive avoidance response.

Another variant is the manner of administering the test substance. One of the interpretational problems with passive avoidance is the occurrence of other drug effects, for example, on pain sensitivity or attention, which can confound the interpretation in terms of memory. Some of these effects can be controlled by administering the test substance immediately after T1, with the intention of acting on so-called memory "consolidation." Posttrial administration has other problems, however. Administration of the test substance may have positive reinforcing or aversive effects which can influence the scores at T2. Another problem is their onset of action. A test substance with slow onset could miss the "consolidation" period altogether and thereby appear to be devoid of amnesic potential when this is not the case. The best general screening procedure therefore remains that where the test substance is administered before the learning session, despite some difficulties in data interpretation.

Passive avoidance procedures generally use intertrial intervals of 24–48 h and, thereby, measure longer-term memory. It is possible to shorten the intertrial interval to, for example, 1 h to assess drug effects on shorter-term memory. On the other hand, passive avoidance procedures are not very frequently used for comparisons of short- and long-term memory, probably because other procedures (see below) offer better possibilities of interpretation.

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Example (See Fig. 3.18).

3.3.2.2 Morris Maze Test

PURPOSE/RATIONALE

Another fairly simple procedure, which allows a greater degree of interpretation, is the Morris water maze (Morris 1981). In this test, a mouse or a rat is placed into a circular tank containing water and has to find an escape platform in a fixed place just beneath the surface. The escape platform is not visible to the animal because the water has been rendered opaque. After swimming around for a certain time, the animal will eventually come across the hidden platform and climb up onto it to escape from the water. When placed again in the water on subsequent occasions, the animal will generally find the platform with increasing rapidity, indicating that it has learned the position of the platform. Although the maze is featureless, it is important that sufficient distinctive cues are present in the experimental room to allow the animal to orient itself.

PROCEDURE

The Morris maze we use consists of a circular water tank (150 cm in diameter) filled with water and maintained at 27°C with an escape platform (15 cm in diameter) 18 cm from the perimeter always in the same position 2 cm beneath the surface of the water. The water is made opaque by addition of milk powder rendering the platform invisible.

Rats are given a single training session on 1 day. A training session consists of four consecutive trials in the Morris maze separated by 60 s. For each trial, the animal is placed in the maze at one of two starting points equidistant from the escape platform and allowed to find the escape platform. The animal is left on the escape platform for 60 s before starting a new trial. If the animal does not find the platform within 120 s, the experimenter removes it from the water and places it on the platform for 60 s before beginning the next trial. During the four trials, the animals start the maze twice from each starting point in a randomly determined order per animal.

Twelve rats are studied per group. The test is performed blind.

The test substance is usually evaluated at three doses, administered p.o. 60 min before the session, and compared with a vehicle control group.

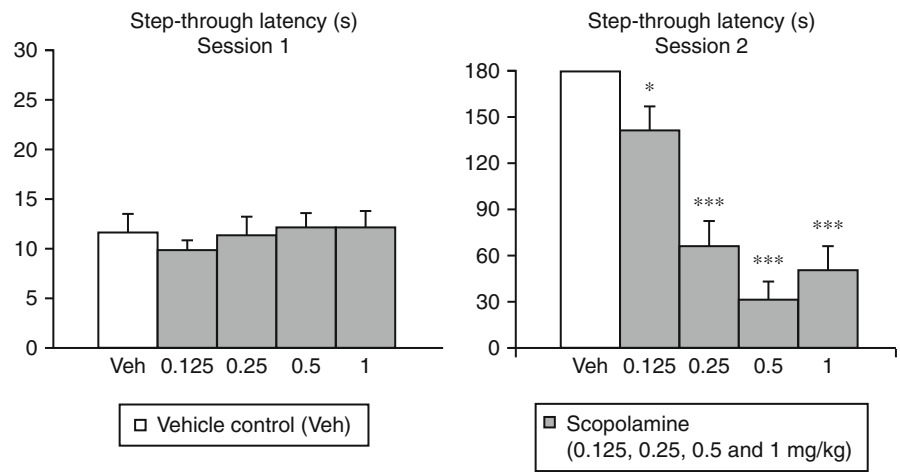

Fig. 3.18 Effects of scopolamine on passive avoidance behavior in the rat. Note the absence of effects of scopolamine on the step-through latency at trial 1 and the presence of a dose-dependent decrease in step-through latency at trial 2, indicating amnesia

Chlordiazepoxide (10 mg/kg p.o.) or scopolamine (0.5 mg/kg s.c.), administered under the same experimental conditions, is used as the reference substance.

The basic experiment therefore includes five groups.

EVALUATION

The principal measure taken is the escape latency at each trial. Decreases in the escape latency from trial to trial indicate learning. A drug-induced flattening of the learning curve therefore indicates impairment of learning. These effects can be analyzed statistically by comparing performance between the treated groups and vehicle control at each learning trial using Student's *t* tests. ANOVA with repeated measures (trials) provides a more sophisticated and sensitive assessment by including within the same analysis all the scores obtained per animal.

Another measure of interest is the animal's escape latency at the first trial. The absence of any treatment effect at the first trial, the animal's first experience of the test situation, can suggest that the test substance is devoid of intrinsic effects on swimming behavior which could confound interpretation of the subsequent data.

One other measure frequently taken, particularly when the animal's behavior is scored using computerized analysis of the video records, is the animal's swimming path during the trials. By this means, it is possible to quantify drug-induced changes in swimming patterns, for example, wall clinging (thigmotaxis), and to calculate the animal's swimming speed, thereby providing another assessment of the intrinsic effects of the test substance on swimming performance.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to the one-trial passive avoidance procedure, the Morris maze permits the progress of learning to be evaluated within the test. Furthermore, subsequent learning can be compared with initial swimming performance. Both factors allow a clearer interpretation of drug effects. Moreover, the Morris maze depends on the animal's use of extramaze visual cues. The behavior can therefore be more readily interpreted in terms of the animal's capacity to learn to orient itself in space (spatial learning) and the effects thereon of the test substance.

Forcing the animal to swim is a powerful motivator and thereby induces a stable behavioral baseline, once the animal has experienced the escape platform. On the other hand, the situation represents a major stress for the animal, particularly if the animal has difficulty finding the escape platform on the first trial. When this happens, drug effects on emotional factors, for example, "behavioral despair" (Porsolt et al. 2002), could confound interpretations in terms of learning/memory. The random nature of the first discovery of the platform is therefore a factor which increases variability, whereas subsequent learning varies little from one occasion to the next and is more clearly interpretable.

The capacity to conduct multiple variants of the procedure (see further details below) endows the Morris maze with a wide range of possibilities for interpreting drug-induced change in cognitive function. This together, with its functional simplicity, no doubt explains the popularity of the procedure.

MODIFICATIONS TO THE METHOD

The two major variants of the procedure are the grouping of the trials and the inclusion or not of probe trials.

In our own basic procedure, four trials are given on a single day with a 60 s timeout between trials. It is possible to repeat the same procedure over several consecutive days in the same test animals. Although more time-consuming, repeated sessions on different days enable drug effects on short-term memory and long-term memory to be distinguished. Short-term memory is best assessed by examining performance on repeated trials on the same day. The decreases in escape latency from trial to trial are a direct reflection of the animal's retention from the immediately preceding trial. In contrast, long-term memory can best be assessed by examining day-to-day performance. Indeed, the purest measure of long-term memory is the change in escape latency on the first trials of each day. This measure reflects the animal's retention from the previous day without confounding from the new learning occurring on subsequent trials on the same day. Indeed, another variant of the procedure is simply to give a single trial on each day. In this way, the influence of within-day learning is totally excluded.

In terms of economy of experimentation, we recommend giving multiple trial sessions on consecutive days and analyzing them as described above.

Another modification is to include probe trials, where no escape platform is present. The retention measure is the time spent in the maze quadrant associated with the escape platform. An animal which remembers the location of the platform will spend more time in that quadrant of the maze. An essential requirement is that the probe trial remains short, say 1 min, to avoid the animal learning that the platform is no longer present. If new learning can be thus avoided, probe trials provide the most sensitive index of the animal's retention of the position of the platform, unconfounded by random elements (the animal finding the platform by chance).

Further modifications are the size of the swimming tank and the position of the escape platform. Both parameters differ widely between different laboratories. In general, larger tanks increase the difficulty for the animal to find the escape platform with a consequently flatter learning curve. Indeed age- or drug-related changes in Morris maze performance depend critically on such factors.

References and Further Readings

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Examples (Figs. 3.19, 3.20).

3.3.2.3 Social Recognition Test

PURPOSE/RATIONALE

Social recognition refers to the fact that an adult rat, when exposed to the same juvenile rat on two occasions, demonstrates that it has recognized the juvenile by a decrease in the amount of investigatory behavior on the second occasion. Absence of a decrease in investigatory behavior at the second occasion suggests that the adult rat has forgotten the juvenile.

This simple model of memory differs from those described above in that it is based on a natural behavioral tendency rather than the situations imposed on the animal by exposure to aversive stimulation.

PROCEDURE

An unfamiliar juvenile rat (40–50 g, 3 weeks old) is introduced into the individual home cage (41 × 25 × 15 cm) of a mature adult rat (400–450 g, 3–4 months old) for 5 min. Following this first encounter (E1), the juvenile is returned to its isolation cage until a second encounter (E2) of 5 min with the same adult rat 30 min later.

Under such conditions, a mature adult rat recognizes the juvenile as familiar, as indicated by a reduction in the duration of social investigatory behavior at E2.

Twelve rats are studied per group. The test is performed blind.

The test substance is usually evaluated at three doses, administered p.o. 60 min before E1, and compared with a vehicle control group.

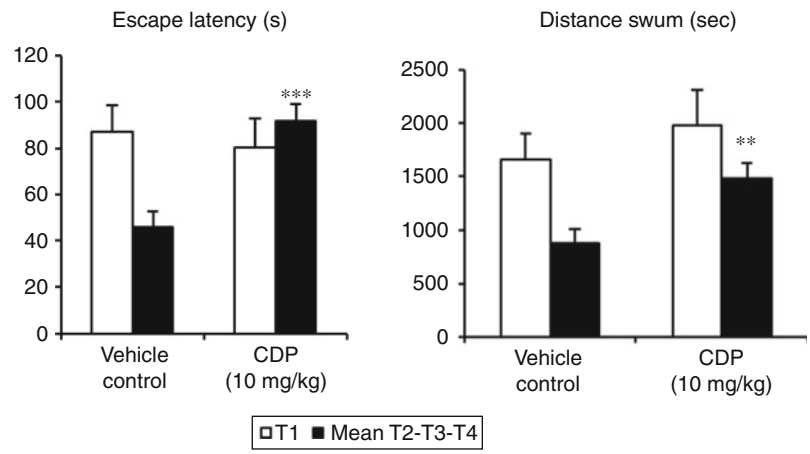

Fig. 3.19 Effects of chlordiazepoxide during the acquisition (single session) of a Morris maze task in the rat. Note the decrease in escape latencies and distance swum in the vehicle control group (short-term memory) and the absence of effect of CDP on performance at the first trial (absence of intrinsic effects on swimming performance). In contrast, it clearly attenuates this decrease (perturbing effect on short-term memory) during the last 3 trials

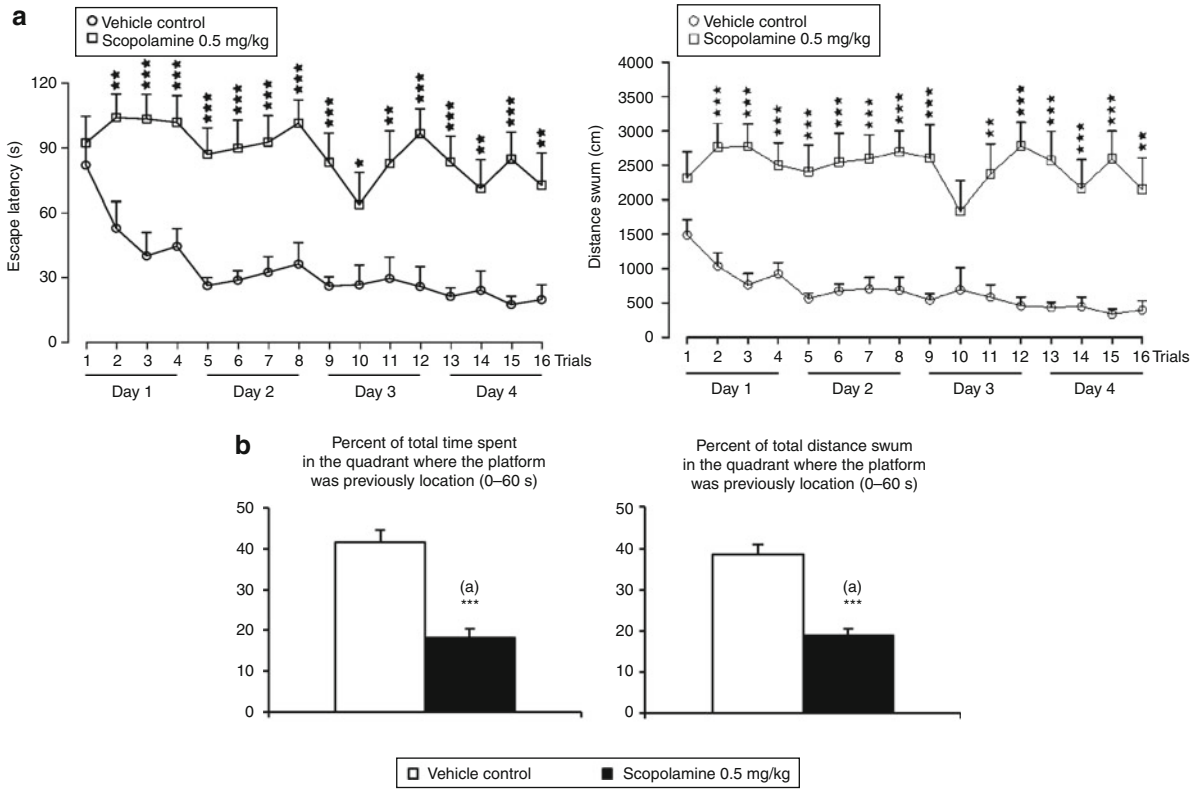

Fig. 3.20 (**a**, **b**) Effects of scopolamine on the acquisition and the retention of a Morris maze task in the rat. Note the clear decrease in escape latencies and distance swum in the vehicle control group from day to day (long-term memory) during the acquisition and the high percentage of time and distance swum in the target quadrant (clear retention) during the probe test. Scopolamine does not induce such a decrease in escape latencies and distance swum and clearly decreases the percentage of time and distance swum in the target quadrant (scopolamine-induced impairment)

Scopolamine (0.25 mg/kg s.c.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes five groups.

EVALUATION

The time the adult rat spends investigating (sniffing, grooming, closely following) the juvenile is recorded at each encounter.

These two measures permit the calculation of a recognition index which consists of the ratio between the durations of investigation at E1 and E2 (E2/E1). A recognition index of close to unity suggests that the mature adult has not remembered its previous encounter with the juvenile. A recognition index significantly less than unity suggests recognition of the juvenile by the mature adult.

These effects can be analyzed statistically by comparing treated groups and vehicle control at each test session using Student's *t* tests.

CRITICAL ASSESSMENT OF THE METHOD

Social exploration is a natural behavioral phenomenon which occurs when animals are confronted with one another. The behavior is not induced by the experimenter but occurs spontaneously. Such a method possesses therefore a certain face validity in that it appears to resemble memories for social encounters as they occur in man.

Caution has to be applied with such anthropomorphic interpretations, however. Whereas the basis of human social recognition is mainly visual, the major component in rodent social recognition paradigms is more likely to be olfactory (Sawyer et al. 1984). Thus, drugs which affect rodent social recognition may do so

by mechanisms not pertinent to social recognition in man, for example, by changing olfactory cues. Whatever the mechanism, it is clear that animals show memory in social recognition paradigms and that this memory is subject to disruption by drug treatment. The data obtained in a rodent social recognition paradigm may therefore usefully complement data obtained using the more classical approaches described above, where the learning/memory phenomena result from experimental manipulation of the animal's motivational state. With social memory procedures, no prior animal training or habituation is required to obtain the sought-after behavioral effects.

A problem with social recognition procedures is that, being based on spontaneous behavior, they are particularly susceptible to environmental changes. Thus, to obtain reproducible findings, it is essential that all environmental factors (temperature, lighting, ambient noise and temperature, cage size, bedding, age and weight of experimental animals, and time of day) be kept as constant as possible.

Moreover, to ensure that the drug effects observed are not due to intrinsic effects of the test substance on social exploration, it is desirable to precede the main social recognition experiment by a prior dose-response experiment to ensure that the selected doses do not affect baseline social investigation.

MODIFICATIONS TO THE METHOD

The basic paradigm described above ensures conditions under which a high level of social memory is obtained under control conditions. Other approaches aim to attenuate the level of social memory with the hope of the test being sensitive both to memory impairment and enhancement. A decrease in social recognition under the same experimental conditions can readily be obtained by increasing the time interval between E1 and E2, for example, to 120 min. Under these conditions, a vehicle-treated control group of normal mature adult rats will show less or no social recognition memory for the juvenile, whereas clear social recognition memory can be observed with a 30-min interval between E1 and E2 (Lemaire et al. 1994).

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Sawyer TF, Hengehold AK, Perez WA (1984) Chemosensory and hormonal mediation of social memory in male rats. Behav Neurosci 98:908–913

Example (See Fig. 3.21).

3.3.2.4 Delayed Alternation Test

PURPOSE/RATIONALE

One of the most powerful techniques for studying behavior under well-controlled experimental

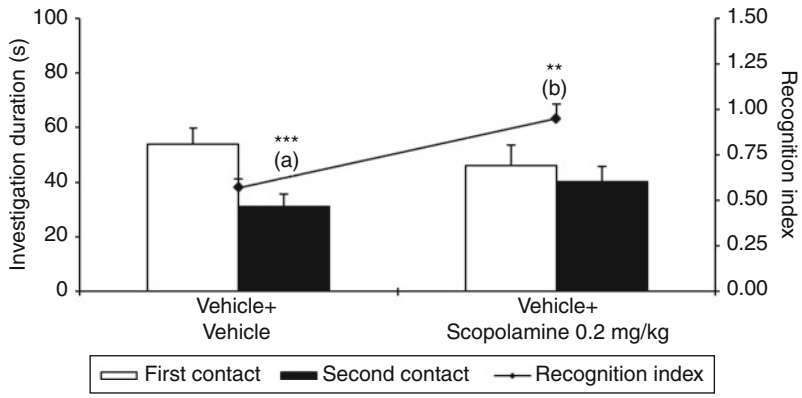

Fig. 3.21 Effects of scopolamine on social recognition memory in the rat. Note the clear decrease in social investigation in the vehicle control group between the first and the second exposure 30 min later (social memory) and the absence of effect of scopolamine on social investigation during the first exposure (absence of intrinsic effects of scopolamine on social investigation). In the scopolamine-treated animals, there is no decrease in social investigation at the second exposure, demonstrating impairment of social memory

conditions is operant behavior. The term operant behavior refers to a particular learned behavior whereby an animal obtains food reward or avoids punishment by pressing on a lever in a so-called Skinner box. Operant techniques permit a wide range of psychopharmacological effects (e.g., antipsychotic, antianxiety, antidepressant, analgesic) to be studied in a quantified fashion, using a standardized environment with a high degree of automation.

The technique also lends itself readily to the study of memory function, in particular by use of delayed responding procedures. In delayed responding, the animal is required to retain information over a short period (usually seconds) and then to show, by pressing an appropriate lever, whether it has correctly remembered the information (Dunnett et al. 1988). In the procedure described below, a rat in a Skinner box is presented with a lever, either on the left or on the right side of the food dispenser. The rat presses the lever, and the lever is withdrawn. Two levers are presented 2.5 s later, and the rat has to press the lever which was not presented previously to obtain a food reward (delayed alternation or delayed nonmatching to sample). If the rat presses the same lever as that previously presented, the lever is withdrawn, but no food is given. In this fashion, the animal can be trained to retain a piece of information (position of a lever) and, thereby, demonstrate its short-term memory capacity.

PROCEDURE

We use standard Skinner boxes (MED Associates, St Albans, VT 05478, USA) equipped with two retractable levers and a food distributor (Roux et al. 1994). The experiments are controlled and the data collected automatically using specialized software (MED Associates, St Albans, VT 05478, USA).

During the 7 days immediately preceding the experiment, the animals are placed on a restricted food regime (15-g standard diet per day) with free access to water. Before being given the standard diet each day, they are also given several 45 mg food pellets (those used as reward in the delayed alternation procedure) to habituate them to this novel food.

The delayed alternation experiment consists of two separate phases:

- Acquisition of lever pressing (single lever)
- Acquisition of delayed alternation (two levers)

Acquisition of Lever Pressing

The aim of this phase is to train animals, on the presentation of a single retractable lever, to press on it to receive a food pellet reward.

Animals are given daily 30-min sessions for about 5 days.

At the end of this phase, between 80% and 100% of the animals acquire the lever-press response. Animals which fail to learn are discarded from the experiments. If some animals are close to establishing steady lever-pressing behavior, they are given extra training with the aim of attaining at least 10 animals per group.

Acquisition of Delayed Alternation (Drug Test)

Subsequent to lever-press acquisition, all animals are submitted to delayed alternation sessions over 10 days. During this phase, the Skinner boxes are fitted with two retractable levers one on each side of the food distributor.

A training session consists of 36 successive trials separated by 10 s. Each trial starts by presenting the animal with one lever (left or right). When the animal presses on the lever, it is given a food pellet, the lever is retracted, and 2.5 s later, two levers are presented. The animal has to learn to press on the lever not previously presented in order to gain a food reward (delayed alternation). If the animal does not lever press within 20 s of a one- or two-lever presentation, the lever(s) is withdrawn, and the next trial commences 10 s later.

The test substance is usually evaluated at three doses, administered p.o. 60 min before each session, and compared with a vehicle control group.

Scopolamine (0.25 mg/kg s.c.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes five groups.

Drug administrations commence at the beginning of delayed alternation training and continue over the weekend between the two test weeks. The experiment therefore includes a total of 12 administrations.

EVALUATION

Three principal measures are taken:

- Percent correct responses (the number of times the animal correctly alternates expressed as a percentage of the total number of trials)
- Simple reaction time (mean time taken to respond to a single-lever presentation)

– Choice reaction time (mean time taken to respond to a two-lever presentation)

Response accuracy reflects the animal's capacity to remember the lever previously presented and therefore represents a measure of short-term memory. Simple reaction times reflect the animal's rapidity to respond to an unpredictable spatial stimulus and therefore represent a measure of attention. Choice reaction times reflect the animal's rapidity to choose between two levers and therefore represent a measure of decision taking or information processing speed. Performance on all parameters improves over the acquisition period and therefore indicates the animal's capacity to learn a new task.

These effects can be analyzed statistically by comparing performance between the treated groups and vehicle control at each learning session using Student's *t* tests. ANOVA with repeated measures (trials) provides a more sophisticated and sensitive assessment by including within the same analysis all the scores obtained per animal.

CRITICAL ASSESSMENT OF THE METHOD

The delayed alternation task provides a clear index of the animal's short-term memory capacity. Because the animal can be given numerous trials (lever presentations) within a single test session of about 20 min, the procedure provides a very dense behavioral sampling from which to estimate the effects of a drug. Furthermore, measuring the animal's simple and choice reaction times provides additional information about drug effects on other aspects of cognitive performance more related to attention and information processing speed. The operant delayed alternation paradigm thereby provides a useful multiparameter measure of possible impairing effects of a test substance on cognitive function. Indeed, results obtained in our laboratory suggest that different classes of test substance can differentially affect the three parameters measured, demonstrating the usefulness of the procedure for characterizing drug effects.

One of the principal qualities of operant behavior is the stability of the behavioral baselines between different executions of the procedure. Even with the present learning task, where the behavior is by definition labile, the possibility of obtaining comparable results on different occasions represents a major advantage of the procedure. The stable behavioral baselines undoubtedly result from the highly standardized experimental environment, where the behavior is measured automatically and there is a minimum of experimenter influence. Automation also permits numerous animals to be tested simultaneously by the same technician, thereby offsetting the cost of the apparatus and the time-consuming nature of the training procedures themselves.

Despite the stability of the behaviors observed on different occasions, the use of a learning task where the behavior evolves markedly during the course of the experiment constitutes a sensitive background against which to evaluate drug effects.

In view of the above, we recommend the delayed alternation procedure as being the most useful single procedure for evaluating possible deleterious effects of test substances on cognitive processes.

MODIFICATIONS TO THE METHOD

Delayed alternation behavior can be studied at two phases, during acquisition (as above) or when performance has been stabilized after continued training.

Delayed alternation acquisition is probably the most sensitive procedure for detecting potential adverse effects of different kinds, but lends itself less readily to unequivocal interpretation. A flattening of the learning curve could reflect either a drug effect on learning itself or result from drug-induced impairment of the short-term memory necessary for correct performance of the task. If the animal cannot remember which lever was presented previously, it will have difficulty learning to alternate between the two levers.

This interpretational problem can be diminished by training the animals up to stabilized performance. Once stabilized, the behavior will more clearly reflect short-term memory because the learning component is no longer present. Indeed, once alternation performance is stabilized, it is possible to introduce delays of different lengths and thereby assess decreases in response accuracy as the retention delay is increased (delay-dependent forgetting). Thus, once animals have been trained up to stabilized performance, they can be used repeatedly to evaluate different drug treatments.

Another term for describing delayed alternation is "delayed nonmatching to sample." This terminology refers to the first lever presentation (left or right) as a sample and a subsequent response on the opposite lever (alternation) as a "nonmatching to sample." Another variant of the procedure therefore is "delayed matching to sample" whereby the animal must always

press on the same lever as that previously presented to obtain a food reward. Both procedures have been used in experimental studies of adverse drug action with essentially similar results. On the other hand, our own experience in aged rats suggests that age-related deficits in response accuracy during acquisition and stabilized performance are more readily demonstrated using a "nonmatching to sample" procedure.

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Example (See Fig. 3.22).

3.3.3 EEG Studies

The Japanese guidelines suggested that electroencephalogram (EEG) studies should be conducted when necessary (Category B), whereas ICH S7A merely mentions electrophysiology examinations as part of the recommended follow-up studies. Electrophysiology covers a wide range of procedures from in vitro intracellular and extracellular studies to whole animal in vivo studies such as EEG. EEG itself is a very broad concept and refers to all aspects of the electrical activity of the brain, from superficial recordings outside the skull to depth electrodes recording from within the intracerebral structures at extracellular levels. As far as CNS follow-up safety pharmacology is concerned, two major EEG applications, the quantified EEG (QEEG) and the sleep/wake cycle, appear to be of particular relevance because they involve the conscious freely moving animal.

3.3.3.1 QEEG

PURPOSE/RATIONALE

QEEG makes use of the Fourier theorem to analyze the electrical power in the different frequency bands of the EEG (Itil 1981). Animals, with electrodes fixed to the skull (cortical leads) and sometimes also implanted stereotaxically in selected structures (e.g., hippocampus, striatum), are exposed to brief measurement periods during which they are either left free to move spontaneously or activated by means of a treadmill to ensure a stable heightened level of vigilance.

The EEG signals are continuously fed into a computer which divides them into short fragments which are decomposed by fast Fourier formation (FFT) into a series of sine waves. The spectral power, a measure proportional to the wave amplitudes, is then estimated and integrated over predefined frequency ranges. The frequency ranges studied in the rat closely match those which are used to characterize the human EEG: 1.5–4 Hz (comparable with the delta-band in the human EEG), 5–8 Hz (theta-band), 8.5–12.5 Hz (alpha-band), 13–35 Hz (beta-band), and 36–64 Hz (gamma-band). The total power 1.5–64 Hz is also calculated.

The protocol described below is for QEEG evaluation in the rat. Despite the considerable differences in function and structure between human and rat brain, psychoactive drugs showing effects in humans affect the rat brain similarly. Thus, the rat EEG is generally a reliable predictor for human CNS drug effects (Easter et al. 2009).

PROCEDURE

Animal Preparation

Rats are anesthetized (sodium pentobarbital 55 mg/kg i.p., plus supplementary doses of 5–10 mg/kg if necessary) and are implanted with two surface electrodes, consisting of miniature titanium screws, placed over the frontoparietal cortex, and two depth electrodes, consisting of twisted platinum-iridium wires, placed stereotaxically into the hippocampus CA1 area (Paxinos and Watson coordinates interaural, AP + 5.0 mm, L ± 2.5 mm, V + 7.0 mm). The electrodes are connected to small plugs, and the whole assembly is secured on the skull with acrylic dental cement. Following surgery, implanted animals are kept in individual Makrolon cages (30 × 18 × 19 cm) and are allowed at least 10 days to recover.

In order to ensure a minimum of eight animals completing the experimental treatments, a total of 14 rats are implanted.

Testing Procedure

Animals are placed individually on a stationary treadmill (30 × 10 cm) and are connected to a signal conditioning system (Coulbourn Model V75-01) via

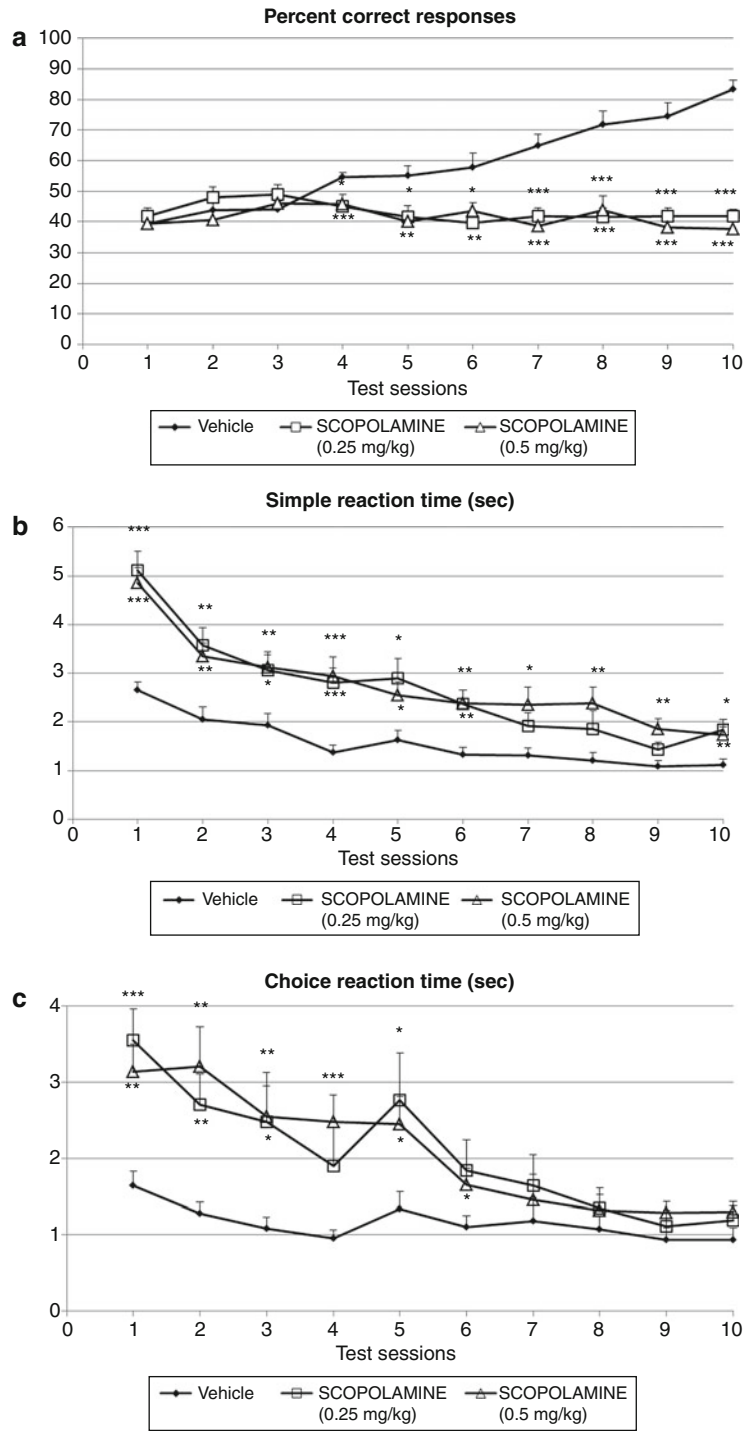

Fig. 3.22 (**a**–**c**) Effects of scopolamine on the three parameters measured during the acquisition of a delayed alternation task in the rat. Note that scopolamine significantly decreases the number of correct responses (impairment of learning/memory) and significantly increases simple reaction times (impairment of attention) and choice reaction times (impairment of information processing speed)

a shielded multicore cable, connected at one end to the plug on the animal's head and at the other end to a swivel mounted 30 cm above the center of the treadmill. Animals are then given a 60-min session with alternating periods of 10 min with the treadmill turned on (speed 1.5 m/min) and off.

Recording sessions take place between 10 a.m. and 5 p.m. with several animals recorded in parallel.

Before beginning drug testing, all animals are submitted to at least one habituation session (without administration) to ensure correct locomotion in the apparatus. The quality of electrical signals for each animal is also checked during this session.

Each animal receives all the treatments in separate 60-min sessions over periods of weeks. The testing procedure during each test week is as follows:

- Day 1: Control session (vehicle administration)
- Day 2: Drug test session (test substance administration)
- Day 3 to day 7: Washout period

Each animal is tested at the same time of day in the same treadmill during different test sessions.

Eight rats are studied per test substance and receive all the different treatments in an order balanced between the animals. The test is performed blind.

EVALUATION

EEG signals recorded by the signal conditioning system are filtered between 1 and 64 Hz and with a notch filter of 50 Hz. Amplification levels are adjusted to avoid saturation of the signal input to the computer. The differential output between the two cortical electrodes is digitized online at a sampling rate of 256 Hz/channel and the data stored in raw data files.

EEG is quantitatively analyzed by spectral analysis using a fast Fourier transform algorithm. In our laboratory, this analysis is performed using the specialized software (ecgAUTO). The mean total spectral power between 1.5 and 64 Hz and the power in five subfrequency bands (1.5–4, 5–8, 8.5–12.5, 13–35, and 36–64 Hz) are calculated for the periods during which the treadmill is on.

For each hour, the mean relative power (% of total spectral power) in the subfrequency bands delta, theta, alpha, beta, and gamma is estimated during treadmill-on phases.

To take into account interindividual variability in the EEG amplitudes, results are expressed as percentage change in the absolute power of corresponding spectra between vehicle and test substance.

The Wilcoxon signed-rank test (two-tailed) is used to compare baseline recordings and recordings after substance administration.

CRITICAL ASSESSMENT OF THE METHOD

Evaluation of drug effects on the EEG was already being performed in the early 1930s, by the discoverer of the human EEG himself, the psychiatrist Hans Berger. Visual analysis of EEG traces is nonetheless a time-consuming, painstaking procedure and, therefore, impractical on a routine basis for safety pharmacology. Apart from the subjective nature of visual trace analysis, subtle changes in EEG activity can often escape visual detection. With the advances in digital data processing, automatic EEG analysis (QEEG) has become possible and the potential usefulness of QEEG for drug screening has been recognized (Van Rinsen and Glatt 1993). Drug-dependent QEEG effects have been shown in various species (Krijzer and van der Molen 1987). Although QEEG has frequently been characterized as an efficacy pharmacology procedure for identifying specific drug activity (Krijzer et al. 1993), QEEG also has undoubted usefulness in safety pharmacology. The same techniques are used for both, but the interpretation is different. Whereas the efficacy approach emphasizes the pattern of power changes in various brain structures, the main question for the safety approach is the presence or absence of such changes.

While there is debate as to whether QEEG, by virtue of the different profiles observed, is capable of identifying specific classes of psychotropic agent, there is general consensus that the QEEG can detect basic stimulant, sedative, or even convulsant activity (Van Rinsen and Glatt 1993). This could have clear relevance for drug safety by corroborating in terms of brain activity, data obtained from behavioral observation. Furthermore, the QEEG can serve as a direct index of cerebral bioavailability, to determine up to which dose a new drug, intended for a non-CNS application, is devoid of effects on the brain (Danhof and Visser 2002).

Analysis of the EEG traces can permit early detection of pathological changes in brain activity, frequently in the absence of overt effects on behavior. This appears to be particularly true for recordings from subcortical structures such as the hippocampus. Like other parts of the limbic system, the hippocampus has a lower threshold for convulsions than the cortex. Early signs of seizures, whether intentionally induced with a convulsant or as a side effect of drug treatment, mostly appear first in the hippocampus. In man, the relevance of the hippocampus is demonstrated by its involvement in temporal lobe epilepsy (Quesney 1986). Moreover, the hippocampus is a key structure not only for pathological epileptic processes but also for memory processing (Squire et al. 2004). Thus, for safety pharmacology purposes, the hippocampus

constitutes a key structure for QEEG analysis. The results from such studies can be critical for deciding whether the development of a substance should be discontinued or shifted into another direction.

Even if EEG activity is usually assessed in the rat, larger species such as the dog are however widely used not only in drug development, to evaluate cardiovascular risk, but also in toxicology. We have data suggesting that the dog might be a particularly suitable species for evaluating human proconvulsant risk (Dürmüller et al. 2007).

MODIFICATIONS TO THE METHOD

Since EEG activity varies with the level of vigilance, the maintenance of a constant vigilance level is essential for QEEG studies. Free-floating vigilance levels can lead to unwanted variability in the EEG signal.

Several procedures have been described to keep animals awake. One is to stimulate the animals' somatosensory system, using tactile or auditory stimuli (Sala et al. 1995). This method is effective for short recording periods, but is neither practical nor reliable for longer-term recording because the animals habituate rapidly to sensory stimuli.

Another approach has been to invert the light/dark cycle and test the animals during their active period (Dimpfel et al. 1992). A persisting difficulty with an inverted light/dark cycle is that even nocturnal animals, such as the rat or the cat, will still show considerable sleep during the dark phase. Inversing the light/dark cycle is also time-consuming in that the animals require several days to habituate to the inversion, thereby increasing costs. It is also virtually impossible to eliminate all sources of disturbance, such as cage cleaning or ambient noise levels, without very sophisticated installations. A final disadvantage is that such inversions are not applied for other CNS safety pharmacology procedures, reducing the comparability of the data obtained.

For the above reasons, forcing the animals to remain active would appear to constitute the optimal procedure for maintaining a constant level of vigilance during QEEG studies. An equivalent approach to the treadmill is the use of a treadwheel (Glatt et al. 1983; Krijzer and van der Molen 1987). With both methods, animals can be kept awake for periods sufficiently long to permit adequate EEG sampling. We favor the treadmill approach because rats do not need any particular training, in contrast to the treadwheel approach where up to 30 days of habituation are necessary. Data obtained in our laboratory using a treadmill show clearly that spectral power is more variable during the periods of rest (data not shown).

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Van Rinsen H, Glatt AF (1993) Introduction and history of the use of electroencephalography in animal drug studies. Neuropsychobiology 28:118–121

Example (See. Fig. 3.23).

3.3.3.2 Sleep/Wake Cycle

PURPOSE/RATIONALE

Studies of the sleep/wake cycle are to be distinguished from QEEG by the fact that the animals are studied over

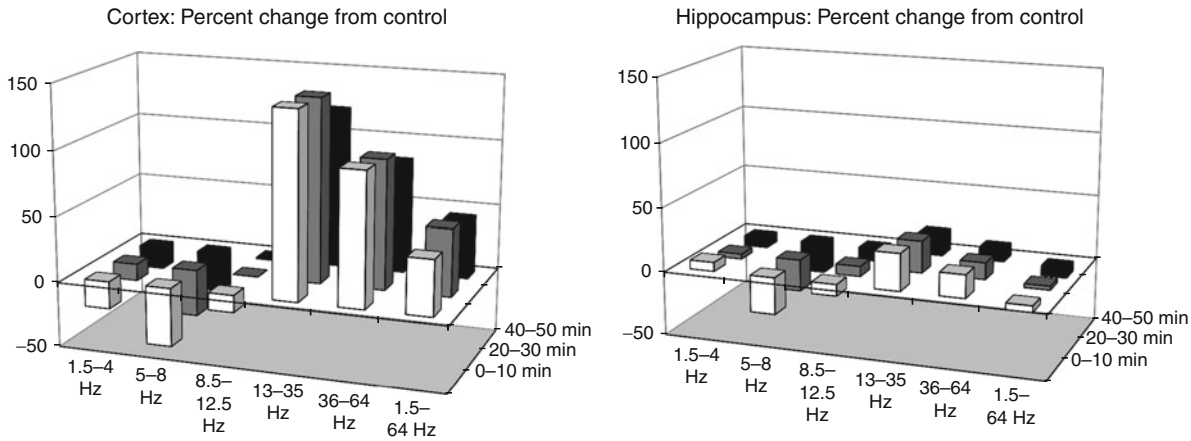

Fig. 3.23 Effects of diazepam on the power spectrum of the quantified EEG. Diazepam (8 mg/kg p.o.) decreases power in the lower frequency range, with an inversion of this effect at high frequencies in the cortex. The effects are weaker in the hippocampus. Similar profiles are observed with other benzodiazepines and benzodiazepine-like substances such as chlordiazepoxide or zolpidem (data not shown)

much longer periods of time (up to several days) (Jouvet 1969). In contrast to QEEG, the aim is to determine the effects of the test substance on spontaneous changes in sleep/waking activity in freely moving subjects and, in particular, whether the test substance induces alterations in the architecture of natural sleep. Although a power spectrum analysis can also be undertaken, the primary analysis is usually confined to changes occurring in the relative durations of the different phases of sleep, the hypnogram (Ruigt et al. 1989).

Although the rat is a nocturnally active animal with polyphasic sleep, experimental data show that sleep is similarly affected by drugs in both humans and rats (Lancel 1999). Thus, studies on the sleep-wake cycle in the rat offer the possibility to test drugs for their capacity to enhance sleep (efficacy pharmacology) or adversely affect the architecture of sleep (safety pharmacology). Unwanted sedative effects for nonhypnotics can be disclosed as well as sleep disturbance induced by both hypnotic and nonhypnotic drugs. From a safety pharmacology standpoint, the aim is to determine up to which dose the test substance is devoid of effects on sleep function.

PROCEDURE

Animal Preparation

Rats are anesthetized (sodium pentobarbital 55 mg/kg i.p., plus supplementary doses of 5–10 mg/kg if necessary) and are implanted with two surface electrodes, consisting of miniature titanium screws, placed over the frontoparietal cortex, and two depth electrodes, consisting of twisted platinum-iridium wires, placed stereotaxically into the hippocampus CA1 area (Paxinos and Watson coordinates interaural, AP −5.0 mm L ± 2.5 mm, V + 7.0 mm). A further two straight platinum-iridium electrodes are implanted into the neck muscle for recording electromyographic activity. The electrodes are connected to small plugs, and the whole assembly is secured on the skull with dental cement. Following surgery, implanted animals are kept in individual Makrolon cages (30 × 18 × 19 cm) and are allowed at least 10 days to recover. One day after surgery, the rats are moved with their cages for adaptation into the recording environment, which is a soundproof, ventilated, and temperature-controlled Faraday room (1.8 × 2.4 × 2.3 m). This room is kept under a 12/12-h light/dark cycle with lights on at 10.00 a.m. Following recovery, rats are subjected to a 2–3-day habituation period in the recording cages (30 × 40 × 48 cm) made out of Plexiglas with sawdust-covered floors, during which they get used to being connected via recording cables to the signal conditioning system (Grass Polygraph Model 7, installed with preamplifiers/amplifiers 7P5/DA and P511). The cables are connected at one end to the plug on the animal's head and at the other end to a turning commutator mounted on the top of the recording cage.

To ensure a minimum of eight rats completing all the experimental treatments, a total of 14 rats are generally implanted.

Testing Procedure

After the habituation period, the test substance is generally evaluated at two doses administered p.o. or i.p.

Recording sessions are performed as follows with nine rats recorded in parallel:

Day 1: Baseline session 1 (treatment 1); without treatment
Day 2: Baseline session 2 (treatment 1); vehicle administration
Day 3: Drug session 1; treatment 1 administration
Day 4 to day 7: No testing
Day 8: Baseline session 1 (treatment 2); without treatment
Day 9: Baseline session 2 (treatment 2); vehicle administration
Day 10: Drug session 2; treatment 2 administration

Administrations are performed at 10.00 a.m. (immediately after switching on the lights), and recording starts 15 min later and lasts 23 h. Half of the animals receive the high dose first and the other half the low dose first (balanced crossover). Each animal is tested in the same recording cage, with free access to food and water during the whole experimental period.

EVALUATION

EEG signals read into the signal conditioning system are filtered between 1 and 75 Hz for the cortex, 1 and 35 Hz for the hippocampus, and 10 and 3,000 Hz for the neck muscle, and all signals are notch filtered at 50 Hz. Amplification levels are adjusted to avoid saturation of the signal input to the computer. The differential output between the two cortical sites, the two hippocampal sites, and the muscle leads is digitized online at a sampling rate of 256 Hz/channel and the data stored in raw data files.

Sleep-wake cycle analysis is performed off-line using the specialized software (ecgAUTO) and the results presentation and statistical analysis with MS Excel.

Sleep-wake cycle analysis is based on power spectral analysis. A fast Fourier transform algorithm calculates from the raw EEG signal total power and the power of subfrequency bands for cortex and hippocampus from 8,192 data points which corresponds to a spectral point covering 16.4 s of real time EEG. For muscle activity, the total energy is calculated, i.e., the sum of the square amplitudes of the EMG over the same time periods/1,000. Under visual inspection of the spectral time-point curve, thresholds are placed manually between minima and maxima of the spectral amplitudes for the different frequency bands. The energy-time curve of the EMG is analyzed the same way. The program then automatically assigns individual spectral points to vigilance stages to form a hypnogram. These hypnograms together with the spectral power values are then transferred into Excel.

The latencies to the first occurrence of SWS and REM are calculated. Further, the absolute time spent in SWS, REM sleep, and wakefulness is calculated for the following time intervals: the total recording sessions (23 h), the half cycles (0–12 h and 12–23 h), and the five time segments (0–4, 4–8, 8–12, 12–18, and 18–23 h).

In addition, the ratio of alpha to delta spectral power, a measure which defines the depth of SWS and the proportion between total and active wake, is calculated for each interval. Differences between vehicle control and test substance are calculated for all variables and for each animal and averaged over the animals per treatment group. A paired *t* test is used for the statistical analysis of each comparison.

CRITICAL ASSESSMENT OF THE METHOD

Human sleep is generally subdivided in five phases, phase 1–4 sleep and REM sleep. Non-REM sleep in the rat has two phases, light sleep and deep sleep, corresponding to the human phases 1 and 2 and 3 and 4, respectively. Since it does not appear to be technically easy to implement an algorithm to differentiate between the two phases, rat sleep is rarely subdivided by computer programs. From a pharmacological point of view, such a differentiation may be of importance to show drug effects which deepen or lighten sleep without affecting other sleep parameters. To date, however, such drugs have not been described.

As with many other animal experimental models, most sleep-wake cycle studies are performed in normal rats. This is less of a problem for studies in safety than in efficacy pharmacology. In efficacy studies, a drug which may be useful to normalize sleep disturbances may not show effects when tested on the normal sleep-wake cycle. There are, on the other hand, sleep-wake cycle models which match human sleep disturbance.

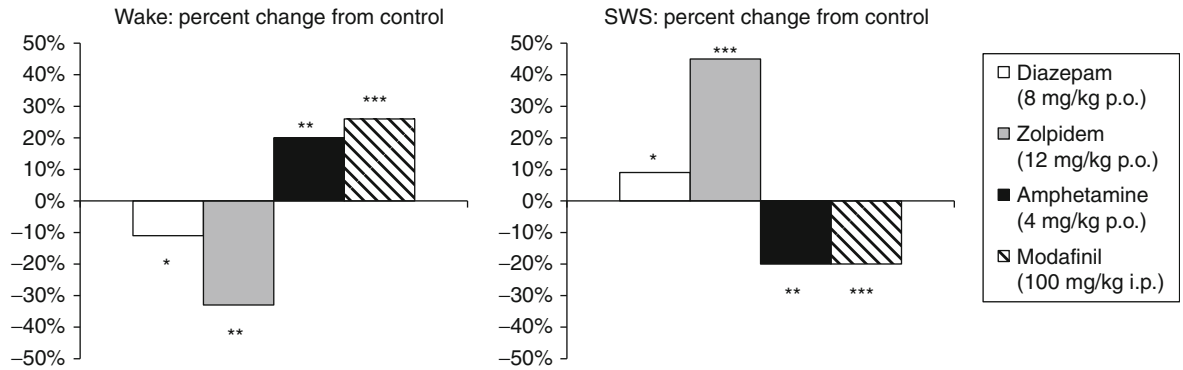

Fig. 3.24 Effects of diazepam, zolpidem, amphetamine, and modafinil on the sleep/wake cycle in the rat during the first 12 h after drug administration. Both diazepam and zolpidem decrease the amount of wakefulness and increase the amount of slow-wave sleep, whereas opposite effects are observed with amphetamine and modafinil

One example is sleep deprivation, another is selective CNS dopamine-depletion, to model sleep disturbance related to Parkinson's disease.

Computerized analysis of the sleep-wake cycle by means of spectral analysis requires that each phase of the cycle expresses its unique activity pattern. Under physiological conditions, this is the case, but may be less evident after certain drug administrations. In exceptional cases, computerized analysis may become impossible because of drug-induced abnormalities in the EEG. In these cases, however, visual analysis would also be impossible because the spectral analysis approach uses the same information as visual analysis. In these cases, measurement of more physiological parameters (see below) may be more helpful.

MODIFICATIONS TO THE METHOD

Telemetry is becoming increasingly used and is slowly replacing the cable technique even if it is relatively expensive. Indeed, telemetry permits simultaneous recording of physiological parameters such as heart and respiratory rate and body or brain temperature in addition to the electrical brain and muscle activity used to differentiate between the sleep-wake cycles phases. This may provide supplementary information about sleep dynamics and approach techniques closer to those used in human polysomnography. Video recording is also useful for capturing behavioral patterns in parallel with cerebral activity (Moscardo and Rostello 2010).

The drug administration time point is an important variable for sleep EEG experiments. Some drug effects are seen only when the drugs are administered before the dark or before the light phase of the cycle. Independent of pharmacokinetic considerations, most laboratories find that the "best" time for evaluating drug effects is several hours from the beginning of the light phase.

In recent years, a new mathematical approach, based on the theory of nonlinear dynamics has been used to analyze EEG signals through the sleep-wake cycle. This kind of analysis has not been shown superior to the commonly used spectral analysis for pharmacological studies. This may rapidly change with the appearance of more powerful computers. In contrast to spectral analysis, which is basically not more than an automation of visual analysis, nonlinear analysis could go well beyond the visual analysis of the EEG trace.

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Examples (See Fig. 3.24).

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Methods in Cardiovascular Safety Pharmacology

4

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4.1 Background

The inclusion of pharmacological studies (also known as *general*, *secondary*, *or ancillary pharmacology*) in the safety evaluation of new drugs is a well-established practice (Zbinden 1966; Alder and Zbinden 1973). These studies contribute to the safety profile of potential new drugs and provide pharmacological data that can be used for optimization of further compounds and the ultimate selection of compounds suitable for clinical development. The emergence of safety pharmacology as a specialty area distinct from toxicology was facilitated by the appearance of the ICH S7A guideline in which the rationale for safety pharmacology studies was laid out, and study types were defined (The European Agency for the Evaluation of Medicinal Products. Human Medicine Evaluation Unit 2000). However, one topic in particular was instrumental in focusing attention on safety pharmacology studies, namely the concern about drugs causing severe ventricular arrhythmias, including torsades de pointes and, in some cases, sudden death. One must not forget, however, that the purpose of conducting cardiovascular safety pharmacology studies is not just to define a specific proarrhythmic risk but to examine potential effects on the peripheral vasculature, the heart, or any other effect that may secondarily lead to an activation or depression of cardiovascular performance (Sarazan et al. 2011).

Studies designed to detect potential drug-induced effects on the cardiovascular system need to include direct effects on myocardial contractile function as well as peripheral effects on the vasculature that can lead to reflex changes altering the autonomic tone of the heart. Therefore, the key physiological parameters

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that need to be assessed include the heart rate and arterial blood pressure, best reported as both diastolic and systolic pressures since the evaluation of only mean arterial pressure can potentially miss important, drug-induced effects. An electrocardiogram is typically included in such a study to detect potential effects on myocardial depolarization and intramyocardial conductance and repolarization, either using limb leads or in some cases using an electrode attached directly to the heart. Overall cardiovascular performance is perhaps best assessed by measuring cardiac output, but this parameter is easiest to measure in anesthetized animal models with invasive techniques including flow probes or dye/thermal dilution approaches. Cardiac output is, however, the product of heart rate and stroke volume. Heart rate is easily measured, and stroke volume can be measured using various imaging techniques; echocardiography being the most widespread. Measurement of left ventricular pressure is also possible and allows one to assess changes in myocardial contractility using the maximal rate of pressure development in the left ventricle (LVdP/dtmax). Since drug-induced effects on myocardial contractility are not uncommon, it is highly recommended to include this type of assessment in cardiovascular safety pharmacology studies (Sarazan et al. 2011).

Numerous strategies for the conduct of cardiovascular safety studies have been suggested (Pourrias et al. 1999; Lacroix and Provost 2000; Champeroux et al. 2000; Gralinski 2000; Kinter and Valentin 2002; Guth et al. 2004) based on the regulatory requirements as well as the needs for efficient drug development. This chapter will present the methodologies included in most of these strategies.

4.1.1 General Considerations

The ICH S7A guidance on safety pharmacological studies for human pharmaceuticals (The European Agency for the Evaluation of Medicinal Products. Human Medicine Evaluation Unit 2000) states a preference for in vivo studies using experimental models with unanesthetized animals. This is a major deviation from previous general practice, based on the recommendations of the Japanese Guidelines for Nonclinical Studies of Drugs Manual (Japanese Ministry of Health and Welfare 1995), in which it was stated that anesthetized animal preparations are typically used for cardiovascular safety evaluations. The new regulatory preference for the use of conscious animals is based on the possible complicating effects that anesthetic agents can have on the cardiovascular system. For example, pentobarbital anesthesia is known to have direct effects on ventricular repolarization (Bachmann et al. 2002). Furthermore, nonoptimized anesthesia can lead to nonphysiological conditions that also may mask drug-induced effects. An example of this is the tachycardia often seen in animals overdosed with pentobarbital anesthesia. Thus, experimental models using conscious animals for measuring cardiovascular parameters will be highlighted. However, due to their great utility when carefully performed, cardiovascular models using anesthetized animals should still be included in the cardiovascular safety pharmacology portfolio and will therefore be addressed.

4.1.1.1 Conscious Versus Anesthetized Animal Use

Despite the theoretical advantages, the use of conscious animals brings with it new challenges for an effective study design. For example, the cardiovascular system of a conscious animal, as compared to an anesthetized animal is much more variable, as the animal reacts to its environment. Subtle, drug-induced effects may not be readily apparent in animals that are reacting to external visual or acoustic stimuli in the laboratory environment. Furthermore, drug-induced central nervous system effects, or any other drug-induced effect that impacts the animals' well-being (e.g., anxiety, gastrointestinal disturbances, etc.) may result in secondary effects on the cardiovascular system. These aspects can introduce a substantial amount of variability in the cardiovascular parameters measured, such as heart rate. Given the reliance of the QT interval duration on heart rate, a variable heart rate can also lead to challenges for the analysis and the assessment of proarrhythmic potential due to delayed ventricular repolarization. With the use of conscious animals, the amount of training needed to achieve the desired basal physiological conditions is associated with increased time, effort and, consequently, expense. Therefore, despite the preference for conscious animal studies for the safety pharmacological evaluation of new drugs, including the cardiovascular system, these models are complex and need a considerable amount of time and effort to conduct them optimally. Models using anesthetized animals are usually simpler

and highly stable models, in which a variety of invasive measurements (e.g., cardiac output, ventricular function, regional blood flow, etc.) can be made, that are not easily accessible in the conscious animal. Thus, there is still an important role for the use of anesthetized animals, particularly in the study of cardiovascular effects of drugs such that this type of model is still included in this text.

4.1.1.2 Animal Reuse and Dose Selection

The use of conscious animals for conducting cardiovascular safety pharmacology studies introduces the possibility of reusing animals. Techniques are available for obtaining the basic cardiovascular data (heart rate, arterial blood pressure, ECG, and even left ventricular pressure and LVdP/dt) without affecting the health status of the animal and without the need to euthanize the animal at the end of the study. The possibility of reusing animals will be affected by the doses of test article selected for use in the safety pharmacology studies. The ICH S7A guideline states that doses should include and exceed the therapeutic range with the goal of defining the dose–response relationship of any adverse effect observed. In practice, studies are typically designed to include therapeutically relevant doses of the test article and predefined multiples based on the expected side effect profile and needed therapeutic window for a given clinical indication. The dose applied should always be related to the actual plasma drug concentration achieved. Since drug-induced effects are most likely to coincide with the peak plasma drug concentration reached, the Cmax is usually the most relevant pharmacokinetic parameter for safety pharmacological assessments. The duration of the exposure is of interest to correlate to the reversibility of a given effect. As an example, a maximal dose could be selected to achieve a 30-fold higher plasma drug level than anticipated for therapeutic use for a non-life-threatening clinical indication. Alternatively, one may target a lesser maximal plasma drug level for compounds used to treat a life-threatening disease, where side effects may be acceptable when the overall benefit-risk assessment is positive. Additionally, for cytotoxic agents, one should seriously consider the use of an anesthetized animal model in which higher doses can be given without the fear of causing the animals distress and they are euthanized while still anesthetized. The study design in conscious animals should avoid discomfort or pain for the animals used, as emphasized in the ICH S7A guideline. Furthermore, safety pharmacology studies are usually single-administration studies in which effects, when seen, are usually fully reversible such that after an appropriate washout of the test article, the animals are suitable for use in further studies. This results in a reduction in the number of animals needed for conducting safety pharmacology studies but does introduce the need to monitor the health status of the animals to qualify them for further use.

4.1.1.3 Species Selection

The ICH S7A guideline proposes the use of relevant animal models without explicitly stating which animal species are most appropriate for use. For the cardiovascular evaluation of new drugs, the dog has been the preferred species in the past, and there is a wealth of comparative data available from studies performed using the dog (Gralinski 2003). As such, it is likely that the dog will remain the preferred species for such studies despite ethical pressure to limit the use of companion animals for drug testing. Use of the dog may be of particular advantage when the dog is also the species used for toxicological studies. Nevertheless, other animal species including the minipig, and nonhuman primates are considered to be appropriate models for cardiovascular safety pharmacology studies.

The rat is also a useful species for conducting cardiovascular safety pharmacology studies. However, since the evaluation of the electrocardiogram is an essential element of a safety pharmacology cardiovascular assessment, the rat is in this regard not ideal. The rat is not a suitable species for testing the effects of a drug on ventricular repolarization since its repolarization is not dependent upon IKr and IKs, as in larger mammalian species, but rather on I_{to}. Despite this limitation, the rat still is an attractive cardiovascular model for detecting effects on arterial blood pressure and heart rate, on the autonomic nervous system, or even other types of arrhythmia not related to altered ventricular repolarization. Technology is available for studying rats in the conscious state, and they may also be reused, as mentioned above in conjunction with dogs or other larger animals. The use of the rat as a cardiovascular model is probably best suited for early pharmacological testing of drug candidates and may provide useful data for lead optimization at a time when amounts of a given test article are not adequate to perform studies in the larger species used as

cardiovascular models. Finally, since the rat is still a standard species for toxicological studies, there is interest in knowing the cardiovascular effects of a test article in that species. Therefore, the conscious rat model is included in this text as providing a useful cardiovascular safety pharmacology model. Alternately, the guinea pig can be used for specific studies of effects on ventricular repolarization and offers interesting and relevant models for early detection QT prolongation and proarrhythmic properties (Yao et al. 2008) which may be particularly useful in early stages of development.

4.1.1.4 In Vitro Studies for Electrophysiological Effects

There is a clear association between drug-induced QT prolongation and arrhythmia and the blockade of myocardial IKr. This suggests the measurement of drug effects on IKr or a model mimicking IKr in studies in vitro. The minimal requirement for addressing the risk of QT-based arrhythmia appears to be two study types: (1) effects on IKr, the specific myocardial membrane current thought to be mechanistically responsible for most drug-induced QT prolongation and (2) effects in vivo on the QT interval of the electrocardiogram from an appropriate (i.e., nonrodent) animal model, the currently used biomarker for proarrhythmic risk.

The CPMP "points to consider" paper of 1997 (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products 1997) pointed out that a thorough electrophysiological characterization of a drug requires evaluation on multiple levels of physiological complexity. In particular, the assessment of the effects of a drug on the myocardial action potential is an important link between the possible effects on a single ion channel type (such as hERG) and the consequent effects seen in the electrocardiogram (such as QT prolongation). Models using isolated Purkinje fibers, papillary muscles, myocardial wedge preparations, or even isolated hearts were proposed to allow one to assess the effects of a drug on the integrated electrophysiology of the heart. These types of models have been shown to detect the effects of many of the known proarrhythmic agents that cause their action through action potential prolongation. However, comparative evaluations, most notably that organized by ILSI (International Life Sciences Institute), provided data suggesting that not all of the know proarrhythmic agents produced effects that could be easily detected in the dog Purkinje fiber model. The observation of such false-negative results leads to dropping this model as a mandatory study type. Nevertheless, the usefulness of this type of model is still acknowledged, and these studies may play an important role for early drug discovery in providing a more thorough risk assessment at a time when in vivo studies are still not feasible due the lack of adequate amounts of a test article. Thus, both in vitro study types (IKr and similar models, and myocardial action potential models) are included in this text.

4.1.1.5 Models of Repolarization-Dependent Arrhythmia

Perhaps the most convincing evidence that a drug is proarrhythmic would be to show a dose-dependent increase in the incidence of a torsades de pointes–like arrhythmia in an animal model. Indeed, the ICH S7B guidance also acknowledges the theoretical interest in the use of this type of model. However, due to the complexity of both these experimental models and their unclear relationship to a proarrhythmic activity clinically, there is no requirement to provide such data. Rather, there is simply the call for a further development of these types of models to determine their predictive value for risk in humans. There are a few models that appear to be useful, should one choose to evaluate proarrhythmic action of a drug directly, and these will be presented in this text. Whereas these models are not considered to be part of the basic cardiovascular safety pharmacology evaluation of a drug, they may be useful as follow-up studies.

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4.2 In Vivo Experimental Models for Cardiovascular Safety Pharmacology

4.2.1 Cardiovascular Safety Studies in Conscious Dogs and Other Species

PURPOSE AND RATIONALE

The preferred model for performing safety pharmacology studies on the cardiovascular system according to the ICH S7A guideline is the conscious animal under unstressed, physiological conditions (The European Agency for the Evaluation of Medicinal Products. Human Medicine Evaluation Unit 2000). This includes the evaluation of drug-induced effects on systemic arterial hemodynamics and the electrocardiogram. Important cardiovascular parameters are, however, only accessible through invasive measurement techniques that can interfere with the physiological status of the animal subject. Telemetry-based systems allow one to monitor hemodynamic parameters including heart rate, arterial blood pressure, and the ECG (and, with some systems, left ventricular blood pressure and left ventricular dP/dt) with the animals in their home cage thereby serving to reduce the stress associated with the collection of data. It should be noted that the use of telemetry is not essential for the performance of this type of study. For example, animals can be trained to the laboratory environment such that they can be examined using light restraint (e.g., sling or chair) under physiological conditions.

PROCEDURE

There are two commonly used full-implant telemetry systems employed for this type of study (Data Science International (DSI), USA, and Integrated Telemetry Services (ITS), USA). They typically measure arterial blood pressure and a single electrocardiogram, but they can also be configured for the measurement of left ventricular pressure or multiple ECG leads. A single ECG lead with clearly definable wave forms is usually sufficient for detecting drug-induced effects, however. Beagles are frequently used for these studies, but larger dogs have the advantage of a simplified implantation procedure and, in general, lower heart rates at the time of the subsequent studies. There is some evidence that drug-induced effects on the QT interval duration are more pronounced in females. For the dog, this difference, if present, appears to be very small and can be disregarded such that animals of both sexes can be included in these studies. All animals should be adequately trained for adaptation to their home cage and laboratory environment prior to their being instrumented.

4.2.1.1 Instrumentation

The chronic implantation of the telemetry systems is a complex procedure requiring experience for the best results. For the best results, it is highly recommended to observe and learn the procedure at an experienced laboratory prior to embarking on one's own. The detailed information is meant only to give the reader a sense for the procedure and is not intended to be prescriptive.

The transducers of the telemetry implant are calibrated prior to implantation, and the unit is sterilized using a low-pressure ethylene oxide process.

Dogs are anesthetized with a combination of Rompun (xylazine hydrochloride, 1 ml/10 kg, i.v.) and Ketavet (ketamine hydrochloride, 0.7 ml/10 kg, i.v.) after premedication with Temgesic (buprenorphine, 0.2 mg/kg i.m.) and ventilated with 66% N_2O and 33% O_2 and 1% isoflurane. All procedures are performed under aseptic conditions using sterilized equipment.

For this example, the implantation of a T27 unit from ITS is described. The dog is placed in a lateral recumbency with the left side facing the surgeon. An incision is made in the fifth intercostal space, beginning from the dorsal tip of the scapula for approximately 20 cm. The latissimus dorsi, serratus dorsalis, and iliocostalis thoracis muscles are divided using a scalpel. The incisions are then covered with saline-soaked gauze. The battery and transmitter of the implant are positioned by making an 8-cm incision right-angled on an imaginary line between the sternum and the end of the costa fluctuans. A small pocket is opened between the external and internal oblique abdominal muscle layers, just large enough to place the battery (dorsal) and the transmitter (ventral) inside this "pocket." The pocket is closed using sutures of 2-0 vicryl (V517H) stitches. A pocket is opened for the switch-on antenna that is placed under the skin. The cables with both pressure transducers and ECG leads extending from the ventrally implanted transmitter are guided subcutaneously to the lateral incision. The antenna used to send the telemetry signals is guided subcutaneously from the ventral transmitter location dorsally toward the spine and then runs parallel to the spine for ~25 cm. The distal end is fixed in place with a suture. The small incisions required to place the antenna are then closed, and the initial ventral incisions required for battery and transmitter placement are closed.

The aortic pressure transducer is implanted next. The intercostal muscles are incised. A rib retractor is then inserted and gently and gradually opened to give the required access. Lung lobes are packed away with saline-soaked gauze to expose the aorta. The 5-cm segment of the descending aorta is then isolated to allow implantation of the aortic pressure transducer. The isolated segment is partly clamped proximally and distally to maintain blood flow underneath the clamp. A microscalpel is used to make a hole in the aortic wall, and the pressure transducer is introduced via this hole into the aorta using a Russian forceps. The transducer is sutured into place, and blood flow is restored.

The left ventricular pressure transducer is then implanted. The pericardium is opened so that the apex of the heart is exposed. The heart is gently held in one hand, and two stitches in the ventricular wall for fixation are made. A purse-string suture is made around the apex. A sharp spike is then placed into the apex of the left ventricle, and the transducer is placed into the ventricle and sutured firmly in place.

The lung is then inflated, and the intercostal muscles are sutured closed. The ECG lead is then fixed close to the sternum in the sixth intercostal space. The muscle layers are then closed, and the lungs are inflated once more to displace all residual air. The skin is then closed.

The gas anesthesia is then turned off, all incisions are treated with topical antibiotics and dressed, and dogs are allowed to wake up. Animals are extubated when their swallow reflex has returned. Analgesics and antibiotics (Temgesic and Tardomycel, benzylpenicillin-benzathine, and benzylpenicillin-procaine) are administered for 7 days following the procedure. Dogs are allowed to recover for 14–21 days before experiments using test substances are initiated.

Studies are conducted with the animals in their home cages that have been equipped with antennae to pick up the transmitted physiological signals. Drugs can be treated orally, intravenously, subcutaneously, or inhalatively (Markert et al. 2004). After treatment, dogs are returned to their home cages for the duration of the study that can last up to 24 h if needed. This is usually based on the duration of the expected drug exposure or possible pharmacological activity of the test article. Longer studies should allow for feeding of the animal and water should be available ad libitum.

4.2.1.2 Experimental Design

Studies can be conducted as group comparisons if sufficient numbers of animals are available. Alternatively, the use of a Latin square crossover experimental design allows for studies with fewer animals (e.g., $N = 4$).

The experiment starts after an equilibrium period of 60–120 min to allow the dogs to acclimate to the measurement pens. The administration of the test compound is started after a 30–45 min control period. Experiments involving intravenous infusions or frequent blood sampling are performed in smaller cages to allow close monitoring of indwelling catheters. Continuous measurements should not, however,

exceed 6–8 h without allowing for a short pause for exercise. Continuous measurements for up to 24 h are acceptable if the animals can be kept in larger cages.

EVALUATION

The hemodynamic and ECG parameters include systolic, diastolic, and mean aortic pressure; aortic pressure peak; systolic and end-diastolic left ventricular pressure; left ventricular pressure LV dP/dt max and dP/dt min; heart rate; and PQ, QRS, and QT intervals. NOTOCORD-software (or equivalent) is used for acquisition of data, whereas Excel (or equivalent) is used for data analysis. Data are summarized at predefined time points by calculating median values + SD. Of particular interest are time points corresponding to the time of Cmax.

4.2.1.3 Correction of the QT Interval for Heart Rate Changes

The QT interval duration is heart rate dependent. Correction of QT values for changes in heart rate is the most common procedure used to discriminate drug-induced changes in QT interval from physiologic changes of heart rate (nycthemeral cycle, emotional reactions) or direct or indirect drug-induced effects on heart rate. The use of correction formula derived from clinical data (Bazett, Fridericia, etc.) is not appropriate for use with dog ECGs. Individual correction of QT interval is now the preferred way to derive any QT heart rate correction. The principle of individual correction involves the establishment of individual QT/RR relationships for each animal during a treatment-free period. A 24-h period is usually sufficient to characterize QT values achieved at low heart rate levels, usually during the night period or at high heart rate levels during the daylight periods of activity. The slope of the QT/RR relationship is then derived individually for each animal and used in a correction formula to achieve a null slope when applied to the treatment free QT/RR relationship. The latest refinement of individual correction methods is the probabilistic method (Holzgrefe et al. 2007) and an individual correction method (Markert et al. 2011). In addition to individual correction, this method involves calculation of mean QT and RR values from a large number of successive beats, 250 beats at least in dogs for each time points. Its main advantage is to markedly reduce QT interval variability related to the autonomic nervous system rhythms and to the hysteresis phenomena (delayed adaptation of QT interval due to rapid heart rate changes). The probabilistic method achieves a sensitivity threshold less than 10 ms for the QT interval in conscious dog studies, i.e., similar to that obtained in clinical trials.

The QT interval is also dependent on body temperature. In conscious dogs, mean changes in the QT interval of 14 ms per degree of changes in body temperature have been reported (Van der Linde et al. 2008). Consequently, body temperature is a parameter that should always be recorded in parallel to QT interval measurements in conscious dogs for an accurate and reliable interpretation of cardiovascular safety studies.

4.2.1.4 Drug Exposure

It is essential to relate any drug-induced effect to the plasma drug levels achieved. It is possible to take blood samples during this type of study, but it must be accepted that this disrupts the hemodynamic status of the animals for up to 30 min. An alternative approach is to conduct pharmacokinetic studies in the same animals (or other similar animals) on another day.

4.2.1.5 Animal Reuse

Animals instrumented for this type of study may be reused. Since the instrumentation is fully implanted without externalization of wires or catheters, there is little risk of developing sepsis after successful implantation. The batteries are designed to provide long life well in excess of a year. After the completion of a given study and an appropriate washout time, further studies can be conducted. This is facilitated by the fact that studies are typically single-administration studies using doses that are not intended to cause irreversible effects. The qualification of animals for subsequent use should be based on the health status of the animals. Given the ease of obtaining hemodynamic data from these animals, one can maintain historical data on heart rate and blood pressure, parameters that are sensitive to the overall well-being of the animal. Additionally, clinical chemistry parameters can be monitored to detect possible effects on kidney and liver function.

MODIFICATIONS OF THE METHOD

4.2.1.6 Species

Similar telemetry-based cardiovascular safety pharmacology studies can be conducted in minipigs

(Stubhan et al. 2008) and monkeys (normally *Macaca mulatta* (rhesus) or *Macaca fascicularis* (cynomolgus), Authier et al. 2007). These two alternative species are of interest due to their use in toxicology studies where the dog is deemed inappropriate. The main technical disadvantage when using freely moving cynomolgus monkeys is the poor quality of ECGs when using subcutaneous electrodes which does not allow automated calculation of cardiac conduction times. For this species, epicardial placement of ECG leads or the ITS telemetry system is highly recommended to achieve an optimal quality of ECGs, enabling automation of determination of the QT interval (Holzgrefe et al. 2007). Pharmacological sensitivity to drugs causing QT prolongation in the cynomolgus monkey is close to that of beagle dogs (Champeroux et al. 2011). In cases of autonomic nervous system–mediated tachycardia, drug-induced QT prolongation might be masked or markedly reduced in conscious dogs (Champeroux et al. 2010). In such a situation, cynomolgus monkeys offer an interesting alternative animal model since this species seems to be less sensitive than dogs to effects of the autonomic nervous system on the QT interval duration.

Whereas rats are also used in toxicological studies, they are not appropriate for use in cardiovascular safety pharmacology studies since they are not sensitive to drug-induced effects on ventricular repolarization dependent upon a blockade of the myocardial rectifying current IKr. Rats may nevertheless prove useful for detecting hemodynamic effects of drugs, particularly early in drug research when amounts of test article may be limited (see below).

The guinea pig has been shown to demonstrate drug-induced effects on the QT interval duration (Hamlin et al. 2003) and is suitable for use with telemetry-based systems. However, their intolerance of arterial catheters limits their use for simultaneously measuring arterial blood pressure.

4.2.1.7 Critique of the Method

The telemetric assessment of cardiovascular effects of drugs is considered the gold standard model for safety pharmacology studies. Animals can be studied in optimized, physiological conditions with neither anesthesia-dependent effects nor with the possible interference from other external influences. This is thought to best mimic a clinical setting and thereby best demonstrate possible drug-induced effects. The reuse of animals instrumented for the telemetric assessment of cardiovascular parameters also allows for a reduction in the numbers of animals needed for such studies. The disadvantages of this experimental approach relate primarily to the costs for establishing and maintaining such a system, as well as the dedicated laboratory space required. Furthermore, a high level of training is required, particularly for the successful implantation of the telemetric equipment. Due to the fact that data can be collected continuously for a long period of time, attention has to be paid to the manner in which the collected data is collected, reduced, managed, and statistically evaluated.

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4.2.2 Cardiovascular Safety Pharmacology Studies in Anesthetized Dogs and Other Species

PURPOSE AND RATIONALE

The basic parameters needed for a safety pharmacology evaluation of possible cardiovascular effects of drugs include heart rate, arterial blood pressure, and the electrocardiogram. These parameters can also be measured in anesthetized dogs or other appropriate animal species. One must recognize the potential influence of the anesthesia on the parameters measured, but this type of experimental model offers certain advantages. The use of an anesthetized animal allows for more invasive techniques to be applied, thereby giving a more in-depth evaluation of possible drug effects on heart and vascular function in a variety of perfusion beds. A well-performed study using an anesthetized animal model is characterized by a highly stable hemodynamic state with very low variability of the measured parameters. This translates into a high sensitivity for detecting possible drug-induced effects. The oral route is, however, not accessible in the anesthetized animals although intraduodenal administration may provide a reasonable alternative administration route if intravenous administration is not feasible or desirable.

Measurements possible in this model include end-diastolic and systolic pressure of the left ventricle, contractility of the heart (usually using peak positive LVdP/dt or LVdP/dt at a developed pressure of 40 mmHg), heart rate, cardiac output, and arterial blood flow in a given local perfusion bed. Test compounds can be classified in terms of a variety of pharmacological actions:

- Positive and negative inotropic effects
- Arrhythmogenic effects
- Hyper- or hypotensive effects
- Tachycardic or bradycardic effects

In summary, despite the preference for studies in conscious animals stated in the ICH S7A guideline, the use of anesthetized animals for assessing cardiovascular effects of drugs is a valid and useful approach with a variety of practical advantages in comparison to conscious animals.

PROCEDURE

Male or female dogs weighing between 15 and 25 kg are used. Various anesthesia regimens are appropriate for conducting this type of study including injectable anesthetics (e.g., pentobarbital, alpha-chloralose) as well as inhalative anesthetics (e.g., isoflurane). Animals are ventilated mechanically, and blood gas parameters should be monitored to confirm appropriate ventilation rate and depth. Venous and arterial catheters are placed either in the neck (A. carotis, V. jugularis) or groin (A. femoralis, V. femoralis) for vascular access and the measurement of arterial blood pressure (using external pressure transducer), blood sampling, and intravenous administration of test compounds.

Left ventricular pressure can be measured by use of a catheter-tip micromanometer placed in the left ventricle through the carotid artery. Alternatively, left ventricular pressure can be measured with a pressure transducer placed directly into the ventricle through the apex and secured with a purse-string suture; however, this approach requires a thoracotomy. The use of a solid-state manometer, as opposed to a fluid-filled catheter, is essential for the measurement of left ventricular pressure when one also wants to assess myocardial contractility using LV dP/dt, the derivative of the left ventricular pressure signal. Fluid-filled catheter systems do not have a frequency response capable of capturing subtle changes in LV dP/dt and give damped measurements.

Cardiac output can be measured using dye- or thermal-dilution techniques or by placement of an electromagnetic flow probe around the pulmonary

artery. Insertion of a pressure transducer or fluid-filled catheter into the pulmonary artery and pulmonary vein allows for the calculation of pulmonary vascular resistance. The measurement of flow through the proximal aorta may also be useful but does not include coronary blood flow and as such is not equivalent to total cardiac output. Selective arterial blood flow can be measured using either electromagnetic or Doppler flow probes around the vessel of interest. Commonly measured regional flows include coronary (circumflex or anterior descending arteries), femoral artery, or renal artery blood flow.

The electrocardiogram can be obtained using standard limb leads and/or precordial leads. A lead should be selected that is stable over time and that has a sharp demarcation at the end of the T wave to facilitate the measurement of the QT interval duration. One can also position a monophasic action potential electrode catheter through the femoral or carotid artery to obtain endocardial monophasic action potentials (see below Modification of the Method).

4.2.2.1 Experimental Course

It is imperative to allow sufficient time after the instrumentation is complete to achieve a stable, steady-state hemodynamic condition prior to administering a test compound. Continuous monitoring of arterial blood pressure and heart rate should demonstrate no changes in the parameters measured for at least 20 min. A test article can then be applied by the chosen route (intravenous, intraduodenal, inhalative) in doses appropriate for the compound. Sufficient time is then allowed for the development of any hemodynamic effects, if any (usually at least an additional 20–30 min), and further higher doses of the test article can then be administered. It is advisable to take blood samples at the end of each dosing interval for the determination of drug levels. The overall duration of the study can last for hours if care is taken to keep the animal's body temperature in a normal range and to replace fluids through the intravenous administration of warmed saline or other suitable volume replacement. Whereas possible drug-induced effects can be assessed by comparing data at each dosing interval to the pretreatment measurements, it is advisable to include a vehicle-treatment arm in which animals are treated identically but with an equal volume of the vehicle used. This is particularly important when excipients are needed to keep the test article in solution.

4.2.2.2 Parameters Measured

The following parameters should be measured in all studies:

1. Heart rate (HR) (beats/min)
2. Arterial blood pressure (BP, diastolic and systolic, mean) (mmHg)
3. Electrocardiogram (PR interval, QRS duration, QT interval) (ms)

If used, the following additional measurements can yield additional insight into possible drug actions:

1. Left ventricular end-diastolic (LVEDP) and peak systolic pressure (mmHg)
2. LV dP/dt or LV dP/dt_{40} (mmHg/s)
3. Cardiac output (CO, ml/min)
4. Regional arterial blood flow (ml/min)

Other parameters can be calculated from those measured including:

1. Stroke volume (SV = CO/HR) (ml)
2. Total peripheral resistance (TPR = BPm/CO × 79.9) (dyn s/cm^5)
3. Left ventricular stroke work (LVSW = (BPm–LVEDP) × SV × 0.333 × 10^{-3})
4. Left ventricular minute work (LVMW = LVSW × HR)

Studies are typically conducted with at least four animals per treatment group (active vs. vehicle). Statistical analysis can be done using an analysis of variance for repeated measures.

4.2.2.3 Critique of the Method

Until the appearance of the ICH S7A guideline, this experimental approach was the most common for studying drug-induced effects on the cardiovascular system and was specifically mentioned in the Japanese guidelines for general pharmacology as the standard test. As such, there is a vast amount of experience with this type of study and a large amount of comparable data available. The primary reason why anesthetized animal models lost their primary role in pharmacological studies was due to the recognition of the possible effects of, particularly pentobarbital, anesthesia on ventricular repolarization and therefore on drug-induced effects on the measured QT interval duration (Bachmann et al. 2002; Weissenburger et al. 2000). Nevertheless, a well-performed study using anesthetized animals, as supported recently by the Japanese PRODACT investigators (Tashibu, et al. 2005), can provide a useful and sensitive model of detecting drug-induced effects on the QT interval duration.

Furthermore, some drug-induced cardiovascular effects, in particular on blood pressure, might be masked because of baroreflex-mediated compensatory mechanisms. Interestingly, most anesthetics reduce baroreflex sensitivity and might offer favorable conditions for detection of such important properties. The use of anesthetized animals becomes particularly interesting when heart rate is affected in conscious animals, because of possible interferences of direct or indirect neurobehavioral effects, direct cardiac effects, or baroreflex-mediated compensatory mechanisms.

MODIFICATIONS OF THE METHOD

Pigs (juvenile farm pigs or minipigs) may be used for this type of study and is an attractive alternative if the dog is not suitable for any reason. Adult domestic pigs are difficult to use due to their size, such that if adult animals are preferred, one of several breeds of mini- or micropigs may be used. The induction of anesthesia is different than with the dog, and typically, an intramuscular sedative is administered first (e.g., ketamine) followed by the anesthesia used for the remainder of the study. Halothane anesthesia should not be used in pigs due to a high incidence of hyperthermic reactions.

Hey et al. (1996) analyzed the ECG wave in anesthetized guinea pigs to determine QT interval, QTc interval, PR interval, QRS interval, and heart rate after the administration of the second-generation antihistamines ebastine and terfenadine. In separate studies in conscious guinea pigs, the effect of oral ketoconazole on the ECG parameters after oral ebastine and terfenadine was studied. Fossa et al. (2007) have assessed electrophysiological and hemodynamic effects of antidepressants in anesthetized guinea pigs.

Measurement of the monophasic action potential (MAP) in anesthetized dogs for evaluation of arrhythmogenic activity of drugs was recommended by Usui et al. (1998) and Weissenburger et al. (2000).

A quad-polar electrode catheter is inserted through the left femoral artery and positioned at the noncoronary cup of the aortic valve to record a His bundle electrogram. A bidirectional steerable monophasic action potential (MAP) recording/pacing combination catheter is inserted through the left femoral vein and positioned at the endocardium to obtain MAP signals. The signals are amplified with a DC amplifier.

The amplitude of MAP is measured as the distance from the diastolic baseline to the crest of the MAP plateau phase. The duration of the MAP signal is measured as an interval from the MAP upstroke to the desired repolarization level along a line horizontal to the diastolic baseline. The interval (ms) at 90% repolarization is defined as MAP_{90}. The heart is driven electrically through the pacing electrodes of the combination catheter. Stimulation pulses are rectangular in shape, about twice the threshold voltage (1.5–2.2 V) and of 1-ms duration. The MAP_{90} is measured during sinus rhythm (MAP_{90}(sinus)) and at a pacing cycle length of 400 ms (MAP_{90}(400)) and 300 ms (MAP_{90}(300)).

The effective refractory period (ERP) of the right ventricle is assessed with a programmed electrical stimulator. The pacing protocol consists of eight beats of basal stimuli in a cycle length of 400 ms followed by an extra stimulus of various coupling intervals. Starting in late diastole, the coupling interval is shortened by 5–10 ms steps until refractoriness occurs. The difference ERP–MAP_{90}(400) is calculated to predict the vulnerability of the myocardium.

The amplified MAP signals together with systemic blood pressure, left ventricular pressure, heart rate, and ECG are continuously monitored using a high-fidelity recording system or electronically.

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4.2.3 Cardiovascular General Pharmacology Studies in Conscious Rats

PURPOSE AND RATIONALE

There is a clear preference for performing safety pharmacology studies for drug effects on the cardiovascular system in a nonrodent species in order to capture possible effects on ventricular repolarization. Nevertheless, it must be acknowledged that there may be great value in conducting studies to examine drug effects on the cardiovascular system in smaller animals such as rats. One reason is that toxicity studies are conducted in rats, and drug effects on cardiovascular parameters in rats is of relevance to such studies. Furthermore, cardiovascular studies in nonrodents (usually the dog) require substantially more compounds than studies in rats. Therefore, as part of the lead optimization, selection, and development process, cardiovascular studies in rats can be invaluable.

4.2.3.1 Telemetry System

Cardiovascular parameters such as arterial blood pressure and heart rate can be measured in conscious, free-moving rats using commercially available radiotelemetry systems (Deveney et al. 1998). Such systems include (1) an implantable transmitter with battery capable of sending the pressure signal from a fluid-filled catheter implanted into the abdominal aorta, (2) a receiver unit that detects the transmitted signal and converts it into a digital format, (3) a pressure reference module that adjusts the measured aortic pressure for atmospheric pressure, and (4) data acquisition software for data storage and computation.

The rats are anesthetized and undergo a one-time aseptic procedure to implant the aortic catheter distal to the renal arteries. The catheter is secured using cyanoacrylate glue. The transmitter and battery unit are placed in the abdomen and attached to the abdominal musculature using a suture. After closure of the surgical wound, the animals are given sufficient time to recover from the procedure before being used in a study, usually at least 1 week.

4.2.3.2 Experimental Procedure

Rats can be studied while still in their home cage. However, since the transmitter frequency used is the same for all animals, they must be held alone during the study. This means that in most cases, the rats are moved from multiple housing cages to single cages for the duration of a study. The cages are placed directly on the receiver units to provide a close contact between the receiver and the animal. The transmitters are turned on before starting the study; the transmitter units can be turned on and off using a magnet thereby lengthening battery life. A control period of up to an hour allows the animals to acclimate to the environment and to provide a steady state prior to administration of a test article. The oral administration of compounds is most convenient in rats, using gavage, but intravenous and intraperitoneal administration is also feasible. Inhalative administration of compounds is also possible by nebulizing the compound into a small exposure box or through the use of an application system bringing the nebulized compound to the nose of the animal. The initial 15–30 min following the administration of a compound is affected by the excitement inherent with the administration process. Usually within a half hour, the animals have returned to a relaxed hemodynamic steady state.

4.2.3.3 Study Design

Studies can be conducted with a group comparison or with crossover design. Individual parameters can be averaged over predefined time periods, but it may not always be possible to predict the time of drug-induced effects. Also, in freely moving animals, drug-induced changes may not be continuous, but there may be overall changes that may be missed if one assesses only short time periods. An alternative approach is to calculate areas under the parameter-time curve and test for drug-induced effects. In this way, the analysis is not restricted to a given time point. With crossover design, an appropriately long washout phase is needed between treatments. A treatment arm with vehicle is also useful to demonstrate the stability of the system. Measurements can be made continuously or at regular intervals as long as desired. Water is usually provided ad libitum, and food can be given should the measurements be needed over an extended time period.

Instrumented animals may be reused after appropriate washout periods on the assumption that the compounds in the doses tested cause no reversible damage. Since the cardiovascular parameters measured are sensitive to the overall well-being of the animals, changes in baseline conditions should be examined for detecting possible compound-related toxicities. Assuming such toxicities are not observed, animals can be maintained for over 1 year with appropriate care.

MODIFICATIONS OF THE METHOD

Due to the fact that whole body plethysmographs for rats are constructed out of plexiglass, they can be used in conjunction with telemetry systems without interfering with the transmitted signals. Therefore, this offers the opportunity to combine studies for determining cardiovascular function using telemetry, with whole body plethysmograph studies for simultaneously measuring respiratory function (Schierok et al. 2000).

4.2.3.4 Critique of the Method

The most important limitation of the rat as cardiovascular model for safety pharmacology studies is its lack of utility for detecting hERG-related drug effects. Whereas this is a substantial limitation, there are nevertheless other drug-induced effects that can be observed in the rat model including arterial blood pressure, heart rate, and contractile ventricular function. The rat, due to its rather small size, requires substantially less compound to perform a study in comparison to that what is needed for larger animals. Thus, this model can be used at an earlier time during drug optimization and selection when amounts of a given test compound are limited. One should keep in mind, however, that a rat instrumented for telemetric collection of cardiovascular data, if kept for longer periods of time, grows considerably in comparison to the "typical" laboratory rat and can grow to over 800 g.

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4.2.4 Assessment of Effect on the Autonomic Nervous System in Conscious Rats

PURPOSE AND RATIONALE

Serious side effects can be expected from drugs able to impair the autonomic nervous system. The main risk is orthostatic hypotension for drugs which alter the baroreflex sensitivity, usually sympatholytic agents. Sympatholytic drugs can also be responsible for bradycardia as well as parasympathomimetic drugs. Conversely, parasympatholytics and sympathomimetics can be expected to induce tachycardia. Mechanisms involved in these interferences can be related to effects on the central nervous system as well as to peripheral effects on ganglionic systems and peripheral cardiovascular system. The conscious rat is an interesting model to assess possible effects on the integrity of the autonomic nervous system function because this species exhibits a good equilibrium between the sympathetic and parasympathetic components of the autonomic nervous system, allowing detection of both parasympatholytic/parasympathomimetic and sympatholytic/sympathomimetic properties.

4.2.4.1 Assessment of the Baroreflex Function

The integrity of the baroreflex function is assessed under dynamic conditions by causing very short decreases or increases in blood pressure (Rocchiccioli et al. 1989). This procedure leads to the activation or deactivation of the baroreflex system. In response to such short hypotensive and hypertensive events, one of the consequences of the activation/deactivation of the baroreflex is to slow or accelerate the cardiac rate, respectively. These short bradycardic or tachycardic events are very well correlated to blood pressure changes in terms of duration and amplitude. With this technique, it is possible to activate or deactivate partially or totally baroreflex function.

4.2.4.2 Experimental Procedure

Animals are instrumented with polyethylene catheters in the femoral artery for blood pressure measurement and the femoral vein for administration of vasoactive agents. Surgery is performed under short anesthesia with sodium pentobarbital (45 mg/kg, i.p.) 24–48 h before measurements. Arterial and venous catheters are exteriorized at the neck, placed in the protective sheath of a chest vest, and connected to a leak-proof rotating joint system placed above the cage, leaving the animals to move about freely. The baroreflex curve is built by causing short-lasting hypotension and hypertension alternately by bolus administration of nitroprusside (1–50 μg/kg) and phenylephrine (1–25 μg/kg) respectively. A total of 15–20 points over a period of 60–90 min is necessary to draw the

baroreflex curve. The baroreflex curve is then fitted to a sigmoid Emax model. The slope of the curve is an index of the baroreflex sensitivity. The lower plateau of the curve (blood pressure vs. heart rate) provides the maximum slowing capacity of the heart in response to hypertension. The upper plateau provides maximum acceleration capacity of the heart in response to hypotension. In rats, the lower and upper plateaus depend on parasympathetic and sympathetic systems, respectively. Consequently, changes in maximum slowing or acceleration capacity allow a direct assessment of drug effects on the autonomic nervous system.

4.2.4.3 Critique of the Method

Since 60–90 min is needed to construct the entire baroreflex curve, this method is not useful for assessing short-lasting effects (<1 h) on the autonomic nervous system. Usually, drugs are given by the oral route, and the baroreflex curve is constructed during the period corresponding to Cmax. Alternatively, drug infusion can be also used.

Although this method does not directly show signs of orthostatic hypotension, it provides direct evidence of the mechanism by which orthostatic hypotension might occur. Most anesthetics impair baroreflex function. Since this method is conducted in conscious animals, it provides direct assessment of drug effects on the autonomic nervous system and baroreflex sensitivity without the risk of interferences caused by anesthetics.

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4.3 In Vitro Cardiac Safety Pharmacology Models

In vitro studies are particularly useful in the context of cardiac electrophysiology as most of the (off-) targets are distinct and clearly defined molecular entities with known biophysical properties that are easily accessible to modern molecular biology and single-cell electrophysiology. For example, recent work has highlighted the human ether-à-go-go-related gene (hERG) K^+ channel as the molecular target for a wide range of drugs whose administration is associated with an increased risk of an unusual life-threatening form of arrhythmia known as torsades de pointes. hERG is a potassium channel alpha subunit (coded by the KCNH2 gene) that, as a tetramer, makes up the ion-conducting pore of the channel responsible for IKr, an important contributor to the delayed outward potassium current. This suggests the evaluation of drug effects on IKr or a model mimicking IKr in vitro as part of a cardiovascular risk assessment. However, the delayed outward potassium current in heart muscle cells of several species including humans is comprised of a rapidly (IKr) and a slowly (IKs) activating component. Therefore, one should not forget that for special structural classes or special primary targets, screening for effects on different cardiac ion channels (e.g., the substrate for IKs, KV7.1/minK, or a variety of other channels) might be necessary.

The CPMP "points to consider" paper of 1997 (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products 1997) pointed out that a thorough electrophysiological characterization of a drug requires evaluation on multiple levels of physiological complexity. In particular, the assessment of the effects of a drug on the myocardial action potential is an important link between the possible effects on a single ion channel type (such as hERG) and the consequent effects seen in the electrocardiogram (such as QT prolongation). Models using isolated Purkinje fibers, papillary muscles, myocardial wedge preparations, or even isolated hearts were proposed to assess the effects of a drug on the integrated electrophysiology of the heart. These types of models have been shown to detect the effects of many of the known proarrhythmic agents having a QT prolongation as mechanism of action. However, comparative evaluations, most notably one organized by ILSI (International Life Sciences Institute), provided data suggesting that not all of the known proarrhythmic agents produced effects that could be easily detected in the dog Purkinje fiber model (Hanson et al. 2006). It was then suggested that this lack of sensitivity should lead to dropping this model as a recommended study type. Nevertheless, the usefulness of this type of model is still acknowledged, and these studies may play an important role for early drug discovery in providing a more thorough risk assessment at a time when in vivo studies are still not

feasible due the lack of adequate amounts of a test article, in vivo PK data, or test article formulations. Thus, both in vitro study types (isolated ion channel studies, and the integrated action potential models) are included in this text, although the action potential models are not an absolute regulatory requirement.

In vitro models for cardiac safety are available at several levels of complexity. The lowest level is represented by ion channel assays that aim at studying the interaction of a given test article with one isolated ion channel type in a heterologous expression system. Due to the strict focus on a single channel type, these assays usually provide unequivocal answers. However, it may be challenging to derive potential net effects in more integrated systems from such data. Therefore, follow-up in vitro assays may also be performed, for example, using isolated primary cardiomyocytes, acute primary tissue preparations, or whole acutely isolated organs. The use of heterologous expression systems provides the opportunity to study human channels, whereas primary cells, tissues, and organs need to be taken from a suitable animal species with very few exceptions.

References and Further Reading

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4.3.1 "High-Throughput" hERG Assays

PURPOSE AND RATIONALE

The high number of drug-like small molecules inhibiting the hERG channel suggests the need to screen for this activity at an early stage of drug development. Indeed, this could be even addressed by screening drug libraries to identify compounds having this potential. Alternatively, one might consider using hERG activity as an early secondary screen of hits emerging from a first high-throughput assay. In either case, experimental approaches are needed that allow the testing of large numbers of compounds thereby necessitating a high degree of automation. Several test systems have emerged as potential high-throughput approaches for detecting effects on the hERG-mediated current IKr. It must be recognized, however, that these relatively new technologies are still in a development stage and have not gained acceptance by regulatory authorities. Nevertheless, they are mentioned in this text, at least briefly, since they may still provide useful approaches for early drug discovery. Such techniques have been reviewed and critically assessed previously (Netzer et al. 2001, 2003).

An important consideration when doing off-target screening using an increased throughput format is how this approach would drive decision-making. When screening for primary targets, false negatives are usually less critical than false positives as these would imply the waste of considerable follow-up work. When screening for an off-target, false negatives would lead to the very same situation and are very likely to occur due to limited solubility when screening at high concentrations or due to stickiness of lipophilic compounds due to an unfavorable surface to volume ratio of the liquid handling system.

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4.3.1.1 Binding Competition Assays

Assay Principle

The principle of this type of assay is to determine the competitive binding of a test compound in comparison to a radio-labeled compound known to be a high-affinity ligand of the hERG channel. Radio-labeled, potent blockers of hERG such as [^{3}H]-dofetilide (Finlayson et al. 2001a, 2001b) or [^{35}S]MK-499 (Wang et al. 2003) can be used in conjunction with myocardial cells, stably transfected cell lines, or membrane preparations. With this approach, the hERG channels are fully blocked with excess, for example, [^{3}H]-labeled dofetilide. The test compound is then incubated with the hERG-dofetilide system and can compete for the binding sites initially occupied by

dofetilide as a function of its potency for binding the same site. The amount of displaced radioligand is determined in the supernatant by scintillation counting.

Critique of the Method

The test system has relatively low costs and can provide a high throughput. However, compounds that block hERG-mediated currents but do not compete for the radioligand's binding site will not be detected. Due to the heterogeneity of chemical structures known to block the hERG channel, it is assumed that many binding sites may exist. Moreover, this test system detects binding but does not demonstrate any change in the electrophysiological function of the channel proteins used. Indeed, there are differences with respect to results of electrophysiological assessments of drug activity. Using a [^{35}S]MK-499-based test system, astemizole and terfenadine produce IC_{50} results that compare favorably with electrophysiological recordings (Wang et al. 2003). In contrast, the measured value for cisapride using this assay is threefold higher than in electrophysiological studies, and MK-499 is 30-fold lower as compared to electrophysiological data (Wang et al. 2003). Such discrepancies also appear using a [^{3}H]-dofetilide-based system and may even be more pronounced. Thus, the specificity of the binding site for the radio-labeled compound, versus the test compound, may limit the usefulness of this approach.

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4.3.1.2 Two-Electrode Voltage Clamp Systems Using Xenopus Oocytes

Assay Principle

In order to heterologously express ion channels, oocytes are injected individually with cRNA encoding for the channel protein of interest. Currents are studied using the two-microelectrode voltage clamp method: Two sharp electrodes are inserted into the oocyte, the first of which serves as a current injection electrode and the second as a probe for membrane potential. Voltage clamp is achieved by current injection controlled by a feedback amplifier which compares actual and command membrane potential. Ion channel inhibition is determined by reduction of injected current in relation to test item concentration. Voltage clamp experiments on Xenopus oocytes can be performed manually on single oocytes; however, systems have been developed that provide automated cRNA injection and voltage clamp measurements in a 96-well format (Leisgen et al. 2007). Although the 96 wells still need to be addressed sequentially, a higher throughput is possible as compared to manual systems.

Critique of the Method

Voltage clamping as a method to directly determine channel activity is currently the best method to quantitate the pharmacology of test articles with respect to ion channels. Heterologous expression in Xenopus oocytes is particularly superior over mammalian expression systems when a large variety of different gene products are intended to be screened, as transient expression is achieved by cRNA injection into individual oocytes. This saves the work of transient transfection or even establishment of a stable cell line for each gene product as it would be necessary with mammalian expression systems. Moreover, in most cases, a much higher expression level can be achieved than with a mammalian expression system. In the context of delayed repolarization, the advantage of individual injection of each oocyte with a suitable amount of engineered cRNA could become relevant when a test article's differential action on various human genotypes is to be determined or the analysis of the binding mode of a particular test article is desired using point mutations of putative binding sites.

However, it is known that in Xenopus oocytes, pharmacology is usually right-shifted (i.e., higher concentrations of test article are needed for a given effect) with respect to results from mammalian expression systems, most likely due to the large amount of yolk contained in the oocytes acting as a sink for the test article, especially in case wherein it is highly lipophilic (Witchell et al. 2002). Therefore, it is recommended to cross-check results with a mammalian system and, in case of a large shift, to use results not in absolute

numbers but rather for rank ordering. In some cases, measurements in Xenopus oocytes might even be impossible because the right shift drives the needed test article concentrations above their solubility limit.

Compared to the patch clamp technique (see Sect. 3.1.4), voltage clamping with two electrodes avoids the difficulties associated with seal resistance to access resistance ratio and hence is better amenable to automation.

References and Further Reading

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4.3.1.3 Automated Patch Clamp Systems

Assay Principle

New systems are currently in development that attempt to incorporate the patch clamp technique (see Sect. 3.2) into an automated and, thereby, higher-throughput format. Whereas these systems cannot be described as being truly high throughput, their goal is to speed the study process through automation while preserving the manner in which a study in a mammalian expression system is usually conducted. Thus, cells heterologously expressing hERG are used, and a patch clamp methodology is applied. Patching of the cells, addition of the test article, and most of the data analysis is, however, done on an automated basis. There are systems that run in the "whole-cell" mode (see Sect. 3.2), whereas others are designed to gain electrical access to the cells in the "perforated patch" mode. In the "perforated patch" mode, the cell membrane is not ruptured as in the "whole-cell" mode but rather permeabilized using a pore-forming agent such as amphotericin. In order to boost success rates and current amplitude, systems have been developed which patch clamp several cells in parallel ("population patch"). For this method a vast excess of cells is needed in order to make sure that all holes in the planar substrate are occupied by a cell to prevent an empty whole from acting as a short circuit.

Generally, all automated patch clamp systems require cells in suspension.

Critique of the Method

A critical step to the performance of a patch clamp experiment is the formation of a tight, high-resistance seal between the cell membrane and the glass micropipette or hole in a planar substrate. The formation of this seal is one limiting factor with this experimental approach, with success rates in the order of 50–80%. The subsequent rupture of the patch to gain access to the cell interior is another critical step which often reduces the success rate of automated systems down to less than 50%. Alternatively, the use of the "perforated patch" method helps increasing success rates; however, the resultant recordings may not be optimal due to a high and instable access resistance and loss of control over the electrolyte composition in the cytosol.

In most automated systems, the options for fine-tuning the recording with respect to compensation of offset, series resistance, whole-cell capacitance, and leak currents are limited as compared to manual patch clamp.

The assay principle is not limited to hERG studies and, indeed, automated electrophysiology has been used frequently for primary target screening. However, on a given automated electrophysiology platform, each cell line, gene product, and assay protocol needs to be validated as both the cell line itself is critical for success rates, and the biophysical properties of the gene product are crucial for the question whether the limitations in fine-tuning options are acceptable.

As in all screening platforms, liquid handling is critical for data quality, as test article precipitation or adsorption to surfaces may compromise exposure of the target. In systems requiring a vast excess of cells in order to enhance the success rate for sealing, the high cell density might also act as a sink for the test article.

4.3.2 Manual Patch Clamp Studies

PURPOSE AND RATIONALE

The introduction of the patch clamp technique revolutionized the study of cellular physiology as it provided the first high-resolution and high-fidelity method of observing the function of individual ion channels in a variety of cell types. The goal of patch clamp electrophysiology for in vitro pharmacology is to analyze the effect of a given test article on ion channel conductance under strict control of the membrane potential and the driving forces for every ionic species.

In the so-called whole-cell mode of the patch clamp technique, the net current through all open ion channels contained in the membrane of a single cell is measured. The whole-cell mode is obtained by rupturing the membrane patch under the orifice of a patch pipette sealed to the cell membrane (Hamill et al. 1981). This is achieved by applying suction to the interior of the patch pipette. In the whole-cell mode, the cell interior is dialyzed by the electrolyte solution contained in the patch pipette. The resulting low-resistance electrical access to the cell interior combined with the dialysis of the cytoplasm with a defined electrolyte solution provides the basis for a successful recording.

The patch clamp technology is considered the gold standard methodology for the assessment of a test article's effects on ion channels as it provides functional data under the most precisely defined conditions. In its standard application, the patch clamp technique is a time-consuming sequential process which requires the permanent attention of highly skilled technical staff who is not only experienced in the delicate manipulation of single cells but also understands the complex biophysical processes behind ionic currents transmitted by channel proteins. Therefore, only a limited number of compounds can be tested using this approach.

According to ICH S7B, a manual patch clamp study so far is the only acceptable way to address potential hERG channel interactions. Other ion channel liabilities are not mentioned as mandatory studies in the ICH S7B guideline, but it is recommended to employ the same technique when performing supplementary studies addressing other ion channels.

References and Further Reading

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4.3.2.1 Patch Clamp Studies in Heterologous Systems

Assay Principle

Heterologous expression in mammalian cell lines has become the most convenient assay system for patch clamp studies on a specific ion channel type in isolation. The whole-cell current for each individual cell is determined (base line recording, vehicle control), and then an arbitrary number of escalating concentrations of the test article are applied to the cells while the resulting changes in current are observed. In many cases, four concentrations escalating in a semi-log fashion and three replicates (cells) are sufficient to construct a valid concentration-response curve.

PROCEDURE

Cells are placed at the bottom of an incubation chamber superfused with a buffer solution which needs to be of appropriate electrolyte content for the particular ion channel to be analyzed. For hERG, a buffer of the following composition is suitable (mM): NaCl (137), KCl (4.0), $MgCl_2$ (1.0), $CaCl_2$ (1.8), sucrose (10), and HEPES (10) at pH 7.4 with NaOH. Patch pipettes are filled with an electrolyte solution which again needs to be adapted to the properties of the particular ion channel to be studied. For hERG measurements, the following composition is useful (mM): K-Aspartate (130), $MgCl_2$ (5.0), EGTA (5.0), ATP-K_2 (4.0), and HEPES (10.0), pH 7.2 with KOH. After reaching the whole-cell configuration, the net membrane current is recorded using a patch clamp amplifier in the voltage clamp mode. To elicit hERG-mediated potassium currents, cells need to be stimulated with a pulse pattern similar to the following: hold: −80 mV, activation/inactivation: +20 mV for 2,000 ms, deactivating tail current: −50 mV for 1,000 ms repeated at 10–20 s intervals. The peak tail current is used for analysis as current of interest. Other more sophisticated stimulation protocols are possible and results may depend on the specific stimulation protocol used. Drug effects on current amplitude are typically expressed as fraction of baseline current. Concentration-response data may then be fitted to a sigmoidal concentration-response curve model. It is generally recommended to check the concentration of the test article in the superfusate using a suitable analytical method. The superfusion buffers used are typically protein free, meaning that in comparison to the in vivo situation, one needs to consider potential protein binding of a compound. In case of very high protein binding, it is advisable to experimentally verify the expected protein shift.

Critique of the Method

HEK293 and CHO cells are most commonly used for this purpose. HEK293 cells may have an endogenous

transient outward potassium current (Snyders and Chaudhary 1996), whereas CHO cells do not have such a current (Teschemacher et al. 1999). The amount of background current in HEK293 cells may be variable between batches of cells and could interfere with hERG measurements, making CHO cells in this regard superior. HEK293 cells may, however, be more consistent in their level of heterologous expression in comparison to CHO cells (Witchell et al. 2002). Issues relating to the use of stably versus transient transfection have been reviewed (Witchell et al. 2002). The quality of data obtained by this assay is only optimal if the assay is performed in a best practice fashion. Poor quality results are likely to be obtained in case numerous technical details are not properly accounted for. The most common pitfalls leading to poor data include inadequate exposure to test article due to solubility or adsorption problems; incomplete equilibration with the test article due to slow on rate (some test articles may take up to 15 min to equilibrate with the target!), leaky cells, or poorly tuned recordings; and small assay window due to poor expression level, resulting in a low signal-to-noise ratio.

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4.3.2.2 Patch Clamp Studies in a Native Environment

Assay Principle

Patch clamp techniques using isolated ventricular myocytes may be used to analyze a test article's effect on the complex interplay of the various ion channels contributing to the cardiac action potential. For this purpose, acutely isolated cardiomyocytes from several species may be used, but also cardiomyocytes differentiated from stem cells have recently emerged as attractive assay systems (Kattman et al. 2011). Patch clamping is performed in the same way as with heterologous systems. Depending on the desired study endpoints, cells may be kept in the current clamp mode (observation of action potential configuration, determination of refractory period) or in the voltage clamp mode (analysis of a net current).

Critique of the Method

Ion channel pharmacology is best studied in a heterologous, more or less, artificial environment; however, when the concerted action of all relevant ion channels in their native environment is to be studied, experimental conditions should be as close to the natural environment as possible. This is particularly challenging for the solution loaded into the patch pipette, as the dilution of intracellular protein kinases and phosphatases together with their substrates may considerably alter channel properties. Hence, the "perforated patch" technique may be appropriate in this case.

Patch clamping cardiomyocytes provides the advantage of analyzing net effects of multiple target drugs in an environment which contains these targets in their natural relative frequency. In contrast, it is advantageous only in rare cases to attempt to isolate these targets in their native environment. Pharmacological isolation is problematic as only few drugs exhibit the necessary selectivity. Biophysical means may help to separate, for example, fast inactivating channels from slowly inactivating channels; however, isolation of all molecular entities is impossible in most cases. Moreover, native expression levels of most ion channels are lower than in heterologous systems which results in small assay windows.

References and Further Reading

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4.3.3 Myocardial Action Potential Configuration

PURPOSE AND RATIONALE

Studies of myocardial action potential configuration to assess the potential of a drug to affect ventricular

repolarization, and therefore a prolongation of the QT interval duration, focus on the pivotal role of hERG-mediated potassium current in mediating this effect. Nevertheless, it is well established that even a potent blockade of the hERG channel does not necessarily lead to a QT prolongation. For example, the calcium channel blocker verapamil does not lead to delayed repolarization at concentrations even above its IC_{50} for hERG. The reason for this apparent discrepancy is simply that the myocardial action potential configuration is the net result of the concerted activity of numerous ion channels, and effects on a given channel may be masked by the activities of other channels; further examples are given in Yuill KH et al. (2004). It is this recognition that suggests that drug profiling should examine the effects of a compound not only on hERG channels but on the overall myocardial action potential. This allows one to assess the physiological relevance of any activity on the hERG channel that may be present and, moreover, opens the possibility to recognize liabilities independent of hERG (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products 1997; Haverkamp et al. 2000). Whereas in vivo data will also add significantly to this preclinical risk assessment, performance of hERG channel assays together with action potential assays may provide an early risk assessment with a better correlation to the in vivo and clinical setting than with just one of these assays alone (Guth et al. 2004).

Drug effects on the myocardial action potential can be measured in myocardial tissue in vitro typically using Purkinje fibers, papillary muscles, ventricular wedge preparations (albeit less commonly), or the entire isolated heart (Franz 1991). The focus of all these approaches is to assess the action potential duration, measured usually in terms of the time to a reach a certain percentage of repolarization after the initial upstroke, for example, the time to 90% repolarization (APD_{90}). Additionally, some of these models may respond to delayed repolarization with early afterdepolarizations (EADs), thought to be a source for arrhythmia. The use of the myocardial wedge preparation also addresses the important issue of potential transmural differences in drug-induced effects on repolarization (Antzelevitch et al. 1999).

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4.3.3.1 Studies in Isolated Purkinje Fibers

PURPOSE AND RATIONALE

Studies of Purkinje fibers in isolated Purkinje fibers can detect compound-induced effects on the action potential configuration and possible early afterdepolarizations. This type of study is a logical adjunct to studies examining effect on hERG-mediated current since it examines the potential relevance of any potential hERG-blocking activity on the overall myocardial action potential. Purkinje fibers from the dog or the rabbit have been used typically, while other species, including the pig (Gintant et al. 2001) may also be appropriate models.

CRITICAL ASSESSMENT OF THE METHOD

The Purkinje fiber is a noncontractile tissue, which facilitates electrode positioning and stability. In comparison to the papillary muscle or the monophasic action potential in the intact heart, drug-induced effects on the action potential duration are considerably larger in the Purkinje fiber model, particularly from the rabbit. Whereas this is not necessarily a disadvantage, the rabbit Purkinje fiber model has been viewed as possibly being too sensitive for drug-induced effects on repolarization. Furthermore, a low repolarization reserve has been reported in

rabbit Purkinje cells (Dumaine and Cordeiro 2007). Conversely, in the canine Purkinje model, not all hERG-blocking compounds produce the expected prolongation of the action potential. One notable example is terfenadine (Gintant et al. 2001), that even in supratherapeutic concentrations failed to prolong the action potential duration in this model. This has lead to action potential studies receiving secondary status from regulatory authorities due to the perception of a risk for false-negative results. Interestingly, 80% of torsadogenic agents exhibit mixed effects in the canine Purkinje model, corresponding to combined INa/ICaL and hERG blocking properties, while drugs causing QT prolongation show a more "pure" hERG blocking profile (Champeroux et al. 2005). Since INa and ICaL currents inhibition produces shortening in action potential duration and QT interval, these concomitant ancillary electrophysiological properties were proposed to be an aggravating factor which could enhance the transmural heterogeneity of the action potential duration and consequently the risk for torsades de pointes (Champeroux et al. 2011).

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4.3.3.2 Studies in Isolated Guinea Pig Papillary Muscles

Assay Principle

Studies in isolated guinea pig papillary muscles can detect compound-induced effects on the action potential configuration as well as inotropic effects. This type of study is a logical adjunct to studies examining effect on hERG-mediated current since it examines the potential relevance of any potential hERG-blocking activity on the overall myocardial action potential. The most commonly used model is the guinea pig papillary muscle (Kii et al. 2005) due to its appropriate size; use of papillary muscles from larger species may be compromised by ischemia in the core of the muscle during the experiment, as oxygen supply for this type of preparation is diffusion-limited.

Critique of the Method

In contrast to the Purkinje fiber, the papillary muscle is a contractile tissue, lending it also for the measurement of contractile force, together with the action potential configuration. Since effects on the inotropic state of the myocardium can also affect the action potential, this is a useful secondary measurement to have to interpret possible drug-induced effects. However, the contraction of the muscle makes the placement of the electrode more difficult, and changes in the contractile function can lead to loss of the electrode placement in the course of a study.

As with the Purkinje fiber model, some compounds known to block hERG channels potently and to cause QT prolongation in the clinic do not demonstrate the expected action potential prolongation; this includes astemizole and terfenadine. In the case of the guinea pig papillary muscle, it has been suggested in conjunction with the PRODACT evaluation in Japan that the way in which the data are evaluated may be key for detecting the drug-induced effect on the action potential configuration (Kii et al. 2005). In conjunction with these studies, the investigators employed APD90-30, which is the interval between 30% and 90% repolarization, as a proarrhythmic marker. With this approach, these investigators were able to demonstrate effects of astemizole in the isolated guinea pig papillary muscle. However, terfenadine did not impact APD90-30; it was therefore suggested that other limitations must prevent terfenadine from demonstrating its expected APD prolongation in this in vitro setting.

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4.3.3.3 Arterially Perfused Wedge of Canine Left Ventricle

Assay Principle

The M cells in the arterially perfused wedge of canine left ventricle are a unique myocardial cell type found in the deeper layers of the ventricular wall (Antzelevitch et al. 1999). These cells respond more sensitively to agents that block hERG channels and, as such, contribute to possible drug-induced transmural heterogeneity of ventricular repolarization and thereby the proarrhythmic potential. The perfused myocardial wedge preparation (Antzelevitch et al. 1996) is designed to allow the study of differences in drug action on transmural repolarization and may therefore provide a better assessment of a possible proarrhythmic potential of a test article.

Critique of the Method

The measurement of a transmural ECG together with local action potentials from across the ventricular wall of the dog provides one of the most sophisticated in vitro approaches for determining drug-induced effects on repolarization as well as having implications for proarrhythmic potential. This is why M cells of the midmycardial wall are most sensitive to drug-induced effects on repolarization and this contributes to a transmural heterogeneity of repolarization that provides a proarrhythmic substrate. Thus, objectively assessed, this model is perhaps the best in vitro model to examine drug-induced effects on repolarization of the heart. Its main disadvantage is the experimental complexity which makes it accessible only to the specialty laboratory and may require extensive training to master the technical aspects. A further complicating factor is the use of dogs for studies in which only small portions of the excised heart are utilized, which has both ethical and financial aspects that need to be considered.

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4.4 Models for Proarrhythmic Potential

The goal of pharmacological profiling of drugs for effects on hERG channels, the action potential, or the ECG is ultimately to predict the potential for proarrhythmic activity. None of these approaches is fully predictive since even potent hERG-blocking drugs that produce a prolongation of the action potential duration in vitro and QT interval in vivo do not necessarily lead to an increased rate of arrhythmia in man based on clinical data; however, the number of unreported cases is rather elusive. Still, it would therefore appear useful to assess the proarrhythmic potential of a drug in a model designed to detect arrhythmias. Nevertheless, the available proarrhythmia models failed to win the confidence of regulatory agencies, and the use of them has neither been mandated nor widely recommended. Such models could, however, prove useful in cases where the results from other types of tests (hERG, action potential, in vivo) are not conclusive.

4.4.1 Studies of Arrhythmogenic Effects in Isolated Heart Preparations

PURPOSE AND RATIONALE

The evaluation of isolated heart preparations of arrhythmogenic, antiarrhythmic, and antifibrillatory effects in the Langendorff heart preparation is described in Sect. 3.1.2 of the book.

Generally, proarrhythmia isolated heart models are designed in two ways: either it is attempted to lower the arrhythmia threshold as much as possible in order to drive the probability of overt arrhythmia up to a degree which makes detection possible even in a study comprising as few as only a handful of preparations, or a sophisticated combination of several surrogate markers is sought which may yield an abstract proarrhythmic index; the validity of which may be shown using tool compounds for which clinical evidence exists.

As an example for the first option, Eckardt et al. (1998) and Johna et al. (1998) proposed the isolated perfused rabbit heart as a model to study proarrhythmia induced by class III antiarrhythmic drugs.

The other path is followed in a model developed by Hondeghem 1994. The assessment of proarrhythmic

potential is based on a more in-depth analysis of drug-induced effects on the action potential including action potential duration, instability of the action potential prolonging effects, reverse use dependency, and triangulation of the action potential. (Hondeghem 1994; Valentin et al. 2004). This information is synthesized into a proarrhythmic assessment that is claimed to discriminate between compounds that are proarrhythmic and those that are not, independently from their effects on the action potential duration per se. In fact, it is claimed that compounds that prolong the action potential but without being instable, reverse-use-dependent, nor causing triangulation are actually antiarrhythmic (Hondeghem et al. 2001).

4.4.1.1 Critique of the Method

Direct observation of overt arrhythmia is certainly the most convincing readout of an arrhythmia model. However, the measures used to drive an isolated heart to the threshold of arrhythmia (i.e., simulated hypokalemia) leave room for interpretation as it is not predictable how a 100% incidence of arrhythmia in a hypokalemia-driven model would translate into the incidence under normokalemic conditions.

In the search for a proarrhythmic index derived from several markers, the relative importance of instability, triangulation, and reverse use dependency contribute is unclear. The contribution of each of these markers may differ from drug to drug, and synergistic actions are likely. Nevertheless, drugs that lead to instability are considered to be the most problematic. Triangulation is, in general, a better predictor of proarrhythmic activity than reverse use dependency, making reverse use dependency the least predictive parameter of the three. The use of the rabbit is based on its having IKr as the main repolarizing current in the heart, but it has little IKs activity (Nattel 1999) and is therefore not sensitive for drugs that potentially affect this current. Furthermore, as with all in vitro test systems, effects due to metabolites of drugs are not detected. Nevertheless, the performance of this test system for differentiating between proarrhythmic drugs and nonproarrhythmic drugs has been impressive (Valentin et al. 2004). The model has not been reproduced outside of the laboratory that developed it, thereby limiting its availability and its acceptance.

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Renal System in Safety Pharmacology

5

Susan G. Emeigh Hart

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5.1 General Functional Assessments

5.1.1 In Vivo Assessments

5.1.1.1 Assessment of Glomerular Filtration Rate (GFR) and Renal Blood Flow (RBF)

Assessment of GFR by Plasma Chemistry

PURPOSE AND RATIONALE

If a metabolic end product or protein is small enough to be filtered at the glomerulus and is neither extensively reabsorbed or secreted, the excretion rate is primarily determined by the glomerular filtration rate (GFR) (Price and Finney 2000), which is considered a sensitive index of functional nephron mass (Newman and Price 1999). Thus, assessment of the plasma level of such a constituent can serve as a biomarker of renal function, and several have been used extensively for this purpose.

The ideal endogenous marker of GFR, in addition to being primarily excreted through the kidney, freely filtered at the glomerulus and neither secreted nor reabsorbed by the tubule, should also be delivered in a steady fashion to the plasma, and be readily detectable using available technology. This requires that production of the substrate and its delivery to the plasma occurs at a stable rate and is not influenced by other disease or physiologic processes, and that the substrate is either non- or minimally protein bound (Price and Finney 2000). Several endogenous small molecules (urea, creatinine, 2-(a-mannopyranosyl)-L-tryptophan) or small (less than 66 kDa) proteins (cystatin C (γ-trace), prostaglandin D synthase (β-trace protein), α_1-microglobulin, β_2-microglobulin, and

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retinol-binding protein) meet these criteria and have been used to assess GFR in many species.

PROCEDURE

Serum or plasma samples are collected from the test animals (if plasma is used, blood should be collected in heparinized tubes). Assay methods vary, and are outlined below:

Urea is most commonly assayed by combined urease methods, in which the urea is first converted to two ammonium ions. The ammonium generated is then measured by either enzymatic or chemical methods. Urea nitrogen values determined by this method (mg/ml) are converted to urea values by the use of appropriate factors (2.14 for urea in mg/ml, 0.357 for urea in mmol/l) (Newman and Price 1999; Emeigh Hart and Kinter 2005).

Creatinine is most commonly measured by the Jaffe reaction of creatinine with picrate to generate an orange chromogen. Several enzymatic assays (based on reactions with creatinase or creatinine deaminase) have also been developed. These are equal in sensitivity to the Jaffe method but are less likely to be subject to interference by endogenous or exogenous chromogens (this is of particular concern in the dog and mouse compared to other species) (Meyer et al. 1985; Finco et al. 1995; Finco 1997; Newman and Price 1999; Schwendenwein and Gabler 2001; Dunn et al. 2004). A recently developed HPLC assay has been shown to be much more sensitive than the Jaffe method for mouse plasma (Dunn et al. 2004).

2-(a-mannopyranosyl)-L-tryptophan (MPT) is measured by HPLC (Takahira et al. 2001). Cystatin C assays are all antibody based (nephelometric, agglutination or sandwich ELISA) (Pergande and Jung 1993; Finney et al. 1997; Jensen et al. 2001), and have been used successfully in dogs, rats, mice and cats (Hakansson et al. 1996; Bökenkamp et al. 2001; Braun et al. 2002; Martin et al. 2002).

Cystatin C assays are all antibody-based (nephelometric or sandwich ELISA), and there are several commercially available assays (Pergande and Jung 1993; Finney et al. 1997; Jensen et al. 2001). Although the antibodies used in these tests are specifically directed against human cystatin C there is significant homology across species (Poulik et al. 1981; Esnard et al. 1988) and these tests have been used successfully to detect cystatin C from dogs, rats, mice, and cats (Hakansson et al. 1996; Bökenkamp et al. 2001; Braun et al. 2002; Martin et al. 2002).

The majority of the additional small protein analytes used to assess GFR are detected by immunoassays with reagents specific for the human proteins, with variable cross-reactivity with analogous proteins from other species (Loeb 1998).

EVALUATION

Analyte levels are compared either to those of a concomitant control group (using appropriate statistical methods of the group size is large enough) or to laboratory-specific reference intervals for the species, strain, age, and sex in question. Elevation of analyte levels outside of the reference range indicate a decrease in GFR, with the magnitude of the elevation being roughly proportional to the degree of the decrement.

Despite these potential shortcomings, serum creatinine assessment has been considered the "gold standard" for assessment of GFR in humans, and is also widely accepted as an index of GFR in most animal species (Finco 1997; Starr et al. 2002). In humans, GFR can be reliably estimated from single point in time creatinine measurements, using one of several algorithms that correct for the effects of age and gender on muscle mass (Cockcroft and Gault 1976; Porter and Finn 1998; Newman and Price 1999). The most commonly used algorithm is that of Cockroft and Gault (1976), which corrects for the effects of age and gender on muscle mass and is reasonably accurate in adults between 30 and 100 years of age:

$$\text{GFR} = \frac{(140 - \text{age}) \times \text{weight(kg)} \times K}{72 \times \text{Serum creatinine(mg/dl)}}$$

where $K = 0.85$ for women, 1.00 for men

A similar algorithm has been calculated for dogs. The study from which this was derived demonstrated no influence of diet, gender, or age on the predictive value of point-in-time plasma creatinine levels for GFR in this species (Finco et al. 1995):

$$\text{GFR} = 2.6/\text{Serum creatinine(mg/dl)}$$

A number of algorithms for calculation of GFR based on serum cystatin C measurements have been generated for adult humans; these also have been

shown to be applicable to children (Le Bricon et al. 2000; Filler and Lepage 2003; Hoek et al. 2003; Larsson et al. 2004; Grubb et al. 2005; Sjöström et al. 2005). All of them are based on the following relationship (with appropriate correction factors applied):

$$\text{GFR} = 1/\text{Serum cystatin C}$$

The accuracy of these methods is dependent upon the method used for serum cystatin C determination. Calculation of GFR using both methods simultaneously in the same patient will improve the accuracy of the final result compared to the use of either analyte alone (Tidman et al. 2008).

Measurement of both urea and creatinine and calculation of the urea:creatinine ratio can provide some useful information about the etiology of the underlying process. Since both creatinine and urea nitrogen are most commonly expressed in mg/dL, this ratio will be unitless if the raw data values for the analytes are used. However, in order for results to be compared between laboratories and across studies it is considered more accurate to convert the values for both analytes to their Standard International (SI) unit values (mmol/l for urea and μmol/l for creatinine, see "Fractional Excretion Methods" for conversion factors for common analytes) before calculating the ratio; alternatively, in order to keep the ratio unitless both analytes can be converted to their μmol/l equivalents by multiplying the creatinine value by 88.4 and the urea value by 357.

If the ratio is elevated as the consequence of elevation of both analytes the cause may be primary renal disease; however, extremely high urea:creatinine when both are elevated (where urea is elevated markedly out of proportion to creatinine) more likely indicates decreased renal blood flow (because decreased tubular flow enhances creatinine excretion while increasing urea reabsorption), urinary tract obstruction (for the same reason), or extravasation of urine into the peritoneal cavity (because urea is more readily reabsorbed than creatinine from the peritoneum). Elevation in the urea:creatinine ratio as a consequence of pure urea elevation can be seen with gastrointestinal hemorrhage, high protein diet (as a transient effect), increased protein catabolism, or loss of muscle mass (in this circumstance, plasma creatinine may be reduced). A decreased ratio can indicate early acute tubular necrosis but more likely pinpoints primary liver dysfunction (due to decreased urea synthesis), decreased protein intake, ingestion of high quality protein diets used in the management of renal failure, extremely muscular individuals, or circumstances of tissue anabolism (Baum et al. 1975; Newman and Price 1999).

LIMITATIONS OF THE METHOD

Effects on prerenal (dehydration, blood loss, altered vasomotor tone, age-related decreases in renal blood flow in rats) and/or postrenal factors (obstruction or extravasation of urine to the peritoneal cavity) may cause elevations of the commonly measured analytes that do not reflect effects on the GFR or loss of functional nephron mass. When these parameters are elevated as a consequence of primary renal injury, they cannot be used to determine the location of that injury (glomerulus versus tubule or tubule segment affected) (Baum et al. 1975; Corman and Michel 1987; Finco 1997; Newman and Price 1999).

The delivery of urea to the peripheral circulation is variable because the rate of synthesis is not constant, depending on both dietary protein intake and the functional integrity of the liver. Baseline plasma urea levels will be elevated in circumstances of increased protein catabolism, including increased dietary protein intake (particularly pronounced in dogs), gastrointestinal hemorrhage, fever, severe burns, corticosteroid administration, sustained exercise, or muscle wasting, whereas urea synthesis and baseline plasma levels decrease with low protein diets, modest food restriction in rodents, hepatic insufficiency, hyperglycemia, and decreased circulating plasma amino acid levels (Pickering and Pickering 1984; Finco 1997; Hamberg 1997; Tauson and Wamberg 1998; Newman and Price 1999). In extreme malnutrition, however, the effect of diminished protein intake is offset by increased urea synthesis resulting from muscle catabolism as well as concomitant decreases in renal plasma flow and dehydration, with the net result that baseline plasma urea levels are usually elevated (Levin et al. 1993; Benabe and Martinez-Maldonado 1998).

In contrast, creatinine is synthesized and delivered to the plasma at a fairly consistent rate and the rate of synthesis decreases as plasma levels increase, presumably due to a feedback control over synthesis (Watson et al. 2002a). Thus, the day-to-day plasma level in

a given individual does not fluctuate widely, which has the disadvantage of limiting the sensitivity of creatinine as an index of GFR. Consumption of high meat diets may elevate plasma creatinine and baseline levels will be elevated in individuals with higher muscle mass or following sustained exercise or acute muscle damage. Conversely, serum creatinine will be lower in individuals who have undergone loss of muscle mass (Finco 1997; Newman and Price 1999). Diurnal variations in serum creatinine levels have also been documented for humans (Finco 1997) and dogs (Loeb and Quimby 1999), with plasma levels in general being slightly higher in the afternoon in both species.

Because of the confounding influences of fluctuating plasma baseline levels, urea and creatinine elevations in plasma are in general not sensitive enough to detect low-level alterations (less than 75% loss) of GFR. Additional contributions to the lack of sensitivity of these analytes comes from the contributions of renal secretion and/or reabsorption to their overall excretion, (which can compensate for their decreased filtration) and to inherent imprecision in the assays used (Finn and Porter 1998; Price 2002; Starr et al. 2002; Shemesh et al. 1985). In general, urea will underestimate GFR due to extensive tubular reabsorption with decreased GFR (Baum et al. 1975; Kaplan and Kohn 1992; Newman and Price 1999), and creatinine tends to overestimate GFR because it is secreted by the tubule in many species and secretion increases both with reduced GFR and increase in some disease states that do not affect GFR or renal blood flow directly (Shemesh et al. 1985; Andreev et al. 1999; Newman and Price 1999a; Rocco et al. 2002; Sandsoe et al. 2002; Starr et al. 2002).

The Jaffe reaction for creatinine is subject to interference by numerous endogenous substrates and drugs or compounds (Schwendenwein and Gabler 2001; Sonntag and Scholer 2001; Dunn et al. 2004). This effect can be minimized by using appropriate substrate extraction or by the use of kinetic assessments. The urease assay is specific for urea, but increased circulating ammonia (such as occurs in aged plasma samples, metabolic disorders, and portocaval shunting) will react with the subsequent reaction and result in falsely elevated plasma levels (Newman and Price 1999).

Plasma 2-(a-mannopyranosyl)-L-tryptophan (MPT) may be a superior indicator of GFR. Unlike creatinine, its baseline plasma levels are not influenced by muscle mass. The point-in-time measurement of this tryptophan glycoconjugate correlated extremely well with the inulin clearance; furthermore, MPT clearance correlated better with inulin clearance (the "gold standard" for assessment of GFR) than creatinine clearance does, suggesting it may be a useful endogenous marker for GFR in many species. However, the renal handling of MPT has not been examined to determine if plasma levels may be influenced by either reabsorption or secretion (Horiuchi et al. 1994; Gutsche et al. 1999; Takahira et al. 2001). The analyte requires detection by HPLC methods, which will limit its utility in clinical or laboratory settings.

Plasma cystatin C has been extensively compared to serum creatinine measurements in human medicine, where it has been shown to be either identical or superior in sensitivity to serum creatinine as an index to GFR in all age groups and renal disease states. It may be superior to creatinine in prediction of alteration of GFR in circumstances of low-level impairment of renal function. It is also not affected by nonrenal diseases that augment creatinine secretion (Newman et al. 1994; Price and Finney 2000; Baigent et al. 2001; Buehrig et al. 2001; Donadio et al. 2001a, 2001b, 2001c; Woitas et al. 2001; Kazama et al. 2002; Laztera et al. 2002; Oliveri et al. 2002; Rocco et al. 2002). Cystatin C has been shown to be an equally sensitive marker of renal injury in the dog (Almy et al. 2002; Braun et al. 2002) and the rat (Bökenkamp et al. 2001), but not the cat (Martin et al. 2002), and its utility has not been explored in other species.

Other small molecular weight proteins in the serum that have been used as indices of GFR in humans are generally better predictors of low-level decrements of GFR than serum creatinine, but none of them are superior to serum cystatin C in this respect. Additionally, their serum levels in humans have been shown to be significantly influenced by age, inflammation or febrile illness, liver disease, and/or corticosteroid administration (Jung et al. 1987; Donaldson et al. 1990; Melegos et al. 1999; Donadio et al. 2001a, 2001b; Priem et al. 1999, 2001; Woitas et al. 2001; Bökenkamp et al. 2002; Filler et al. 2002). Furthermore, the cross-reactivity of the antibody detection reagents with other species and the usefulness of these markers in animal models have not been well established (Loeb 1998).

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Assessment of GFR and RBF by Clearance Methods

PURPOSE AND RATIONALE

The renal clearance of a compound is the volume of plasma from which that compound is completely removed by the kidneys per unit time (Pitts 1968). The material removed from the plasma is generally excreted in the urine.

Clearance methods can be used for a variety of purposes:

1. Determination of effects on glomerular filtration rate (GFR) and renal blood flow (RBF)
2. Determination of the mechanism(s) involved in the renal excretion of a substrate (more in 1.E.1.1.2.2.3)

PROCEDURE

Clearance procedures can be conducted in all laboratory animal species; anesthetized or conscious animal models may be used. Measurement of arterial pressure

is advisable, especially in anesthetized preparations, to insure that renal perfusion pressure remains within the autoregulatory range (usually 80–120 mmHg). Vascular access ports can be helpful to provide continuous arterial access for pressure measurements (Mann et al. 1987).

Carefully timed and complete collection of urine is critical to clearance studies. Point-in-time samples are more easily collected from larger species, either by free-catch, urethral catheterization, manual compression of the bladder, or cystocentesis (withdrawal of urine from directly from the bladder with a needle and syringe). Catheterization may require sedation or anesthesia, especially in females of all species and in male pigs whose urethral recess makes this process difficult (Van Metre and Angelos 1999). Point-in-time samples from laboratory rodents may be obtained by taking advantage of the fact that these animals frequently urinate when they are handled or shortly after being removed from their home cage. A skilled, quick, and prepared operator with a ready small container or plain microcapillary tube may be able to obtain a small sample in the first instance (Loeb and Quimby 1999); in the second, the animal can be placed in a small confined space on plastic food wrap and observed carefully for urination, as it has been shown that most rodents will urinate within 20 min after removal from their home cages. The sample thus generated can be collected by micropipette (Kurien and Scofield 1999). Manual compression of the bladder can also be performed on rodents, and cystocentesis may also be performed by a skilled operator using a small (25 gauge) needle and syringe (Loeb and Quimby 1999). For repeated point-in-time urine samples from rats over short periods of time (1–2 weeks after the surgery), the urethra or the ureter can be cannulated. Cannulated rats can also be used to collect accurate and complete timed urine samples (Mandavilli et al. 1991; Horst et al. 1988). Care must be taken to ensure complete urine collection in catheterized/cannulated models by flushing the bladder with saline (adding the wash to the urine volume) and instilling air at the end of the collection to completely empty the bladder.

Timed urine collections in all species (including large animals) can also be obtained by the use of specially designed metabolism cages. The use of metabolic cages for timed urine collection is outlined in detail in "Urinalysis."

In all cases, samples must be collected in clean containers and must be kept free of contamination from food, drinking water, feces, blood, and bacteria. For best results, samples should be analyzed promptly (ideally within 1 h) after collection, but where analysis must be delayed, the sample must be protected from chemical degradation and evaporation, best accomplished by using collection containers that are appropriately sized for the species in question, tightly sealing the containers when possible, and keeping the specimen cold (4°C) until analysis. If chilled or frozen, samples must be allowed to equilibrate slowly to room temperature before analysis.

Either endogenous substances or exogenously administered tracers may be used in clearance studies. Creatinine and cystatin C have both been used as endogenous tracers for the determination of GFR. Either endogenous or exogenous creatinine clearance can be used to estimate GFR. For endogenous creatinine clearance, a single timed urine collection and matched plasma sample are used, with clearance determined as described below. Detection of creatinine by either enzymatic or HPLC methods are recommended if endogenous creatinine clearance is used, due to the limitations of the Jaffe method (outlined in "Assessment of GFR by Plasma Chemistry.") Exogenous creatinine clearance is performed by bolus administration of creatinine and determination of plasma clearance; the Jaffe reaction may be used for creatinine determination in this circumstance. This method compensates for the insensitivity of the Jaffe detection method at low creatinine concentrations in plasma by artificially increasing plasma creatinine levels and, additionally, removes the underestimation of GFR that results from the fact that endogenous chromagens are present in plasma but not urine (Brown 1994; Finco 1997, 2005; Watson et al. 2002b). In rats, exogenously administered cystatin C has been used to determine GFR. For this tracer, plasma clearance must be used to estimate GFR due to the fact that cystatin C is reabsorbed and degraded by the proximal tubule and does not normally appear in the urine (Roald et al. 2004; Tenstad et al. 1996).

Exogenous tracers are administered intravenously to achieve near steady state concentrations, generally by using a priming dose to load the plasma and extracellular compartments, and subsequent infusion to replace renal losses. Once steady state plasma tracer levels are approached, a series of timed urine

collections (clearance periods) are performed, with blood samples collected at the either the midpoint or beginning and end of the clearance periods. The urine and blood (plasma or serum) samples are analyzed for tracer(s) and the test compound.

Tracers for the determination of GFR must be freely filtered and then neither secreted nor reabsorbed from the tubular filtrate. This allows the assumption that the amount of plasma cleared of the tracer per unit time represents that which has been filtered through the glomeruli (i.e., GFR). A number of exogenous tracers meet these criteria. The fructose polysaccharide, inulin (mw ~ 5,200) is the most commonly used tracer in all species and serves as the "gold standard" to which others are compared (Finco 2005; Brown et al. 1996; Ragan and Weller 1999). Other indicators include isotopes of vitamin B_{12}, sodium iodothalamate, iohexol, and radiolabeled metal chelates of ethylenediaminetetraacetate (EDTA) and diethylenetriaminepentaacetic acid (DPTA) (Sarkar et al. 1988, 1991; Gaspari et al. 1997; Ragan and Weller 1999; Finco 2005). Other sugars such as the polyfructosan sinistrin (available commercially as Inutest®) and a commercially available artificial sweetener derived from sucrose (LC Sugar®) have also been used as tracers in both humans and animals (Perez-Rojas et al. 2005; Pill et al. 2005). As indicated above, clearance of the endogenous substances creatinine and cystsin C have also been used to evaluate GFR.

Tracers for the assessment of renal plasma flow must be completely cleared (combination of filtration and nearly 100% first pass tubular secretion to the urine) on first pass through the kidney. This allows the assumption that the volume of plasma cleared per unit time represents that which was either filtered by the glomeruli or bypassed the glomeruli and perfused the tubules. *p*-aminohippurate (PAH) is used for the assessment of RPF, because it is both freely filtered by the glomeruli and actively secreted by the organic acid transport pathway of the proximal tubule. First-pass PAH extraction by the kidneys varies from about 70–90% in rats, dogs, and humans (Brenner et al. 1976), but for the purpose of estimating RBF it is assumed to be 100%. Using this assumption, RPF is always slightly underestimated. Tetraethylammonium bromide (TEA), a substrate for the renal cation transporter, may also be used and is subject to the same limitations (Ragan and Weller 1999).

Inulin is measured colorimetrically, either by acid hydrolysis to generate a green product, or by a series of enzymatic reactions based on inulinase or one of several fructosidases. The resulting glucose is either oxidized using glucose oxidase and H_2O_2 and reacted with anthrone reagent to generate a colored product or is converted to glucose-6-phosphate or sorbitol with subsequent reduction of NADH, which is detected spectrophotometrically (Davidson and Sackner 1963; Day and Workman 1984; Sugita et al. 1995; Kuehnle et al. 1992). More recently, additional colorimetric electron acceptors have been developed which are equally or more sensitive and allow for adaptation of these methods to automated analyzers (Kimata et al. 2009). Similar enzymatic methods are used to detect the polyfructosan tracer reagents (Pérez-Rojas et al. 2005; Pill et al. 2005). A fluorescein-isothiocyanate (FITC) labeled version of sinistrin has been synthesized that can be measured fluorometrically, with comparable accuracy to the enzymatic methods (Pill et al. 2005). HPLC methods are used for the remaining exogenous GFR tracers. PAH and TEA are measured colorimetrically (Newman and Price 1999).

EVALUATION

Renal clearance (Cl) of any compound (X) can be determined by comparing the urinary excretion rate of compound X to the plasma concentration of compound X.

The urinary excretion rate is calculated as:

$$\text{Urinary excretion rate}(\text{mg/min}) = U_X(\text{mg/ml}) \times V(\text{ml/min})$$

Where U_x represents the concentration of substance X in urine (in mg/ml) and V represents the volume of urine collected per unit time (in ml/min). Thus, the clearance equation may be constructed:

$$Cl_x(\text{ml/minute}) = U_X(\text{mg/ml}) \times V(\text{ml/min})/P_X(\text{mg/ml})$$

where Px is the concentration of compound X in plasma (in mg/ml)

GFR is estimated by calculating the clearance of the tracer or endogenous substance. RPF estimated using PAH clearance is often designated effective renal

plasma flow (ERPF). Renal plasma flow is converted to renal blood flow (RBF) by dividing ERPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct):

$$\mathrm{RBF} = \mathrm{ERPF}/(1 - \mathrm{Hct})$$

The clearances of other compounds can be compared with inulin clearances to determine how the kidney functions in the elimination of the test compound. A clearance ratio is constructed by dividing the renal clearance of the test compound (X) by the renal clearance of inulin:

$$\text{Clearance Ratio} = \mathrm{CL_X}, (\mathrm{ml/min})/\mathrm{Cl_{inulin}}, (\mathrm{ml/min})$$

A clearance ratio <1.0 indicates reabsorption of the test substance following filtration, whereas active secretion will result in a clearance ratio of > 1.0.

MODIFICATIONS OF THE METHOD

For any of the tracers that meet the criteria specified above, plasma clearance may be used to estimate GFR or RPF as an alternative to renal clearance. This is the only method that can be used for cystatin C due to the fact that cystatin C is reabsorbed and degraded by the proximal tubule and does not normally appear in the urine (Tenstad et al. 1996). This eliminates the need for accurate timed urine collections, reduces inaccuracies resulting from matrix effects in the analyte assays, and allows the use of tracers that are not excreted into the urine.

Plasma clearance may be determined either after bolus administration of the tracer or by cessation of infusion following the establishment of a steady state plasma level as described above. Multiple timed plasma samples are then collected for the evaluation of the plasma tracer levels and calculation of clearance based on determination of area under the plasma concentration curve (AUC), as follows (Bailey et al. 1970; Frennby et al. 1996; Rönnhedh et al. 1996):

$$\mathrm{Cl} = \frac{\mathrm{Q_{tot}}}{\mathrm{AUC}}$$

If volume of distribution is known or can be determined for a given tracer in any species, plasma clearance can be estimated from the plasma concentration of the tracer collected at a single point in time following the establishment of steady state plasma concentration after IV bolus administration, as follows (Jacobsson 1983):

$$\mathrm{Cl} = [1/(\mathrm{t/V} + 0.0016)] \times \ln[\mathrm{Q_{tot}}/(\mathrm{V} \times \mathrm{C_t})]$$

where $\mathrm{Q_{tot}}$ = total quantity of tracer injected, V = volume of distribution, Ct = plasma concentration at time t. The formula corrects for non-immediate mixing and nonuniform distribution of the tracer in plasma. Although the corrections were derived initially for inulin clearance in humans, they have been shown to be accurate for a number of tracers in humans and rats (Sterner et al. 1996; Orlando et al. 1998; Katayama et al. 2010).

Alternatively, tracers may be administered by continuous intravenous infusion until steady state is reached; at this point, plasma levels are determined at 3–5 time points from samples collected from the arterial system. The median arterial concentration is determined and clearance is calculated from the following formula:

$$\mathrm{Cl_X}(\mathrm{ml/min}) = \frac{\mathrm{Ix}(\mathrm{mg/ml}) \times \mathrm{Iv}(\mathrm{ml/min})}{\mathrm{P_X}(\mathrm{mg/ml})}$$

where Ix = concentration of the tracer in the infusion fluid, and Iv = infusion rate, and Px = median arterial concentration of the tracer at steady state.

Simultaneous determination of GFR and RPF (using either inulin or iothalamate combined with PAH in the infusate) has been performed in rats using this method (Rönnhedh et al. 1996; Fischer et al. 2000).

LIMITATIONS OF THE METHOD

First-pass extraction of PAH is highly variable both between species and between individuals within a species, which adds to the inherent inaccuracy of the estimate of RBF by this method. Furthermore, the test compound may interfere with the extraction of either PAH or TEA by competing for transport by the organic anion or cation transporters (Newman and Price 1999; Ragan and Weller 1999).

The limitations of the Jaffe method for creatinine determination have been discussed in Sect. 1.1.1. Exogenous creatinine clearance compensates for the insensitivity of the method as well as the interference by endogenous chromagens by artificially increasing the plasma creatinine concentration (Finco 1997).

Sinistrin (and consequently, FITC-sinistrin) are available in Europe but availability may be limited in other geographic areas. Similarly, LC sugar is only available in South America and is not currently available as a sterile injectable formulation suitable for repeated administration or clinical use. (Pérez-Rojas et al. 2005).

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Assessment of RBF by Intravascular Doppler Flow Probes

PURPOSE AND RATIONALE

Probes utilizing electromagnetic or Doppler technology may be positioned around or within a renal artery to allow direct measurement of renal blood flow (Yagil 1990; Haywood et al. 1981). Detection of blood flow using Doppler systems is based on changes in the emitted ultrasonic frequency, a Doppler shift, caused by reflection of the signal of moving blood cells. The Doppler shift is proportional to the velocity of blood flow, as indicated by the following equation:

$$\Delta f = \frac{2 f_0 v \cos\theta}{c}$$

where Δf = Doppler peak frequency shift, f_0 = transmission frequency, v = instantaneous peak velocity, θ = angle of incidence of the beam to the bloodstream (assumed to be 0° for linear probes) and c = speed of sound in blood (1,570 m/s) (Chilian et al. 1982).

This technique is particularly useful when rapid or continuous assessment of effects on renal blood flow or assessment of renal vasoreactivity need to be assessed.

PROCEDURE

Doppler probes have been applied to rats, rabbits, cats, dogs, pigs, monkeys, and humans; these must be appropriately calibrated prior to experimental use. Animals are anesthetized and the probes are placed within the renal artery via the abdominal aorta, which may be accessed via the femoral or carotid arteries. The diameter of the renal artery at the placement site of the Doppler probe must be determined simultaneously to correct velocity measurements for determination of renal blood flow (see "Evaluation").

EVALUATION

A number of parameters can be derived from the Doppler measurements, most commonly including average peak velocity (APV), pulsatility index (PI), and resistive index (RI). Renal blood flow (in ml/min) can be calculated as follows:

$$\mathrm{RBF} = \frac{\mathrm{APV} \times \pi \times \mathrm{D}^2 \times 60}{4}$$

where D = renal arterial diameter (Doucette et al. 1992).

Resistive index (RI) has been shown in several species to be positively correlated with tubular dysfunction or postrenal obstruction and its return to normal may serve as a prognostic indicator of resolution of tubular disease (Rivers et al. 1997; Shokeir et al. 1996; Tsuji and Taira 2001).

LIMITATIONS OF THE METHOD

Dopper probe assessment of velocity is accurate primarily in straight blood vessels of small diameter (<4.76 mm) and at relatively low flow rates (<200 ml/min), both of which may be exceeded in normal renal arteries in many species (Lerman and Rodriguez-Porcel 2001).

In dogs, acute, severe normovolemic anemia significantly altered renal artery Doppler parameters without having an influence on Doppler assessment of splanchnic blood flow; the technique may thus be inaccurate in anemic animals (Koma et al. 2006). Furthermore, it is invasive and the size of the test species to which it can be applied depends on the availability of appropriately sized and calibrated probes (Lerman and Rodriguez-Porcel 2001).

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Assessment of GFR and RBF by Scintigraphic Imaging

PURPOSE AND RATIONALE

Radioactive indicators (^{99c} Tc-DPTA, ^{113m}In-DPTA, ^{99c}Tc-mercaptoacetyltriglycine) may be used to measure renal blood or plasma flows or glomerular filtration rate (Reba et al. 1968). These techniques require intra-arterial or intravenous administration of the tracer followed by monitoring of the amount of tracer in the kidneys with an external gamma camera (Fommei and Volterrani 1995).

In species where extrarenal clearance of tracers used in the determination of GFR or RPF is high or variable, external detection methods may actually be more accurate than plasma clearance methods because the extrarenal component of plasma removal is eliminated (Drost et al. 2003).

PROCEDURE

Tracer compounds with the same characteristics described earlier under clearance methods are utilized for the determination of GFR or RPF, but these are labeled with radionuclides suitable for scintigraphic imaging such as ^{99m}Tc, ^{131}I, ^{125}I, ^{67} Ga, ^{68} Ga, or ^{51}Cr. Tracers are administered either by intra-arterial or intravenous administration and are followed by monitoring the amount of tracer in the kidneys with an external gamma camera (Fommei and Volterrani 1995). External measurements of radioactivity retained in the kidney are made either at timed intervals (rate of accumulation, or slope method) or at a selected interval, usually corresponding to either first pass or peak level of the tracer (percent accumulation, or integral method) (Kampa et al. 2002).

$$\text{Predicted GFR } 1/4\ 0.44 \cdot \%\text{dose uptake} + 0.87$$

EVALUATION

With scintigraphic imaging, renal blood flow and and glomerular filtration rate are estimated using the Fick Principle:

$$Flux = \text{DAk} * \frac{\text{C1} - \text{C2}}{\text{X}}$$

(where A = area, x = distance, C = concentration and D = diffusion coefficient)

LIMITATIONS OF THE METHOD

These techniques do not yield absolute flow, but rather flow per unit volume or tissue mass. However, algorithms can be developed in the species of interest to allow conversion of tissue uptake to GFR or RBF expressed in standard units by simultaneous determination of plasma clearance of the tracer in preliminary experiments (Kelleher et al. 1991; Kampa et al. 2003), as the correlations between methods for any given tracer are in general high (Delpassand et al. 2000).

In general, assessment of GFR or RBF by external detection methods is less accurate than assessment by clearance methods, although the correction algorithm used for the external detection method can influence the degree of accuracy achievable (De Santo et al. 1999; Itoh et al. 2000; Itoh 2003). Integral methods (calculated based on the percent of the dose accumulated) are in general more accurate than rate of accumulation methods because they eliminate variability resulting from variable durations of the uptake phase (Kampa et al. 2003). Manual selection of the region of interest (ROI) for the collection of the scintigraphy data also improves the accuracy (Kampa et al. 2002).

MODIFICATIONS OF THE METHOD

Depending on the tracer used, either GFR or RBF can be estimated from the results. The table below lists

different tracers that can be used for the assessment of either GFR or RBF (Emeigh Hart and Kinter 2005):

Radiopharmaceuticals used for estimating:	
Glomerular filtration rate	Renal blood or plasma flow
^{3}H-inulin	^{3}H-para-aminohippuric acid (PAH)
^{14}C-inulin	^{14}C-PAH
^{14}C-carboxy inulin	^{99m}Tc-hippuran analogs
^{14}C-hydroxy-methyl inulin	^{99m}Tc-iminodiacetic PAH (PAHIDA)
^{131}I-chloroiodopropyl inulin	^{99m}Tc-mercaptoacetyltriglycine (^{99m}Tc-Mag3)
^{131}I-propargyl inulin	^{99m}Tc-mercaptosuccinyltriglycine (^{99m}Tc-MSG3)
^{125}I-diatrizoate	^{99m}Tc-N,N'bis(mercaptoacetyl)-2,3-diaminopropanoate (CO2-DADS-A)
^{125,131}I-iothalamate (Conray – 60)	^{125,131}I-iodopyracet (Diodrast)
^{131}I-diatrizoate (Hypaque, Renografin)	^{131}I-orthoiodohippurate (Hippuran, OIH)
^{51}Cr-, ^{99m}Tc-, ^{111,113m}In-, ^{140}La-, ^{169}Yb-EDTA	^{67,68} Ga-N-succinyl desferioxamine
^{51}Cr-, ^{99m}Tc-, ^{111,113m}In-, ^{140}La-, ^{169}Yb-DTPA	^{97}Ru-ruthenocenyl-glycine (Ruppuran)
^{57,58}Co-hydroxycobalamin	^{99m}Tc-thiodiglycolic acid
^{57,58}Co-cyanocobalamin	

In addition to their use in estimating glomerular filtration rate and renal blood flow, imaging techniques are extremely useful for viewing renal tissue in smaller animals.

Radiopharmaceuticals have been used for this purpose, but more recently, fluorescent probes and multiphoton microscopy techniques have been used to allow evaluation of blood flows in living animals in real time (Peti-Peterdi 2005; Kang et al. 2006). Regardless of the label or method, probes used for imaging should be rapidly and specifically extracted from blood by the kidney and retained for a sufficient period in the renal tissue to permit imaging. The ideal probe for imaging the renal parenchyma should be inert, 100% extracted in a single pass, irreversibly bound to the parenchyma, not accumulated by other tissues and not excreted in the urine.

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Assessment of RBF by Microspheres

PURPOSE AND RATIONALE

Microsphere techniques can be used to assess blood flow in a number of tissues and organs (Rudolph and Heymann 1967) and have been extensively used to assess renal blood flow (Katz et al. 1971). Microspheres of a diameter that allows them to be contained in the microcirculation without separating from the bloodstream during circulation (usually 15 μm) are injected into the arterial circulation (either systemic or specific to the organ of interest), and the tissue is

subsequently harvested and the number or concentration of microspheres is evaluated. Distribution of the microspheres to the organ or tissue of interest is in proportion to blood flow and thus the numbers of microspheres lodging in organ pieces estimates the blood flow distribution.

PROCEDURE

Accurate determination of renal blood flow by microsphere methods requires that the microspheres are completely mixed in the central circulation, do not separate from the blood during circulation, are completely (100%) extracted in the first pass, are retained within the tissue of interest once deposited, and can be accurately measured (e.g., the label remains attached to the microsphere during tissue processing). Administration of the microparticle suspension (including the suspension vehicle used) must not disturb the circulation, either generally or to the organ of interest, and adequate numbers of microparticles must be administered to ensure that samples contain at least 200–400 microspheres each, which requires the administration of very large numbers of microspheres, especially in larger animals (Buckberg et al. 1971; Prinzen and Bassingthwaighte 2000).

Radiolabeled microspheres were the first ones developed and are most commonly used (many of the isotopes outlined in "Assessment of GFR and RBF by Scintigraphic Imaging" have been used for this purpose). Microspheres are administered by slow bolus injection into the left ventricle, at a dose sufficient to ensure delivery of adequate number of microspheres to the tissue of interest, as described above. During delivery of the microspheres, a reference sample of arterial blood is withdrawn at a measured rate to provide an estimate of the cardiac output. Following the injection, the kidneys are harvested and weighed and the microsphere count in representative weighed tissue samples is determined by liquid scintillation methods (McDevitt and Nies 1976; Prinzen and Bassingthwaighte 2000).

EVALUATION

The reference sample is used to provide an estimate of cardiac output (CO), using the following formula (McDevitt and Nies 1976):

$$CO = \frac{Q_i \times \text{Reference sample withdrawal rate}}{Q_{rs}}$$

where Q_i = total quantity of microspheres injected and Q_{rs} = total quantity of microspheres in the reference sample.The renal blood flow (RBF) is then calculated based on the following relationship (Rudolph and Heymann 1967):

$$RBF = \frac{Q_{kid} \times CO}{Q_i}$$

Or, more simply (Glenny et al. 1983):

$$RBF = \frac{Q_{kid} \times \text{Reference sample withdrawal rate}}{Q_{rs}}$$

where Q_{kid} = quantity of microspheres in the kidney.

MODIFICATIONS OF THE METHOD

More recently, microspheres have been developed that are labeled with colored or fluorescent dyes. Colored and fluorescent microspheres are extracted from tissues and blood by digestion (either with KOH or protease solutions) with potassium hydroxide and subsequent microfiltration; the dyes are then recovered from the filtered microspheres within a defined volume of an appropriate solvent, and their concentrations are determined spectrophotometrically, fluorometrically, or by HPLC methodology. (Kowallik et al. 1991; Glenny et al. 1993; Hakkinen et al. 1995; Mazoit et al. 1998).

Fluorescent microspheres may also be quantified using flow cytometry (FACS) following tissue extraction or in histologic sections, using manual counting of particles or automated image analysis methods. Both FACS and histologic techniques have shown good correlation to extraction methods and radionuclide techniques, and histologic methods have been used to evaluate regional differences in blood flow within a tissue with reasonable accuracy (Austin et al. 1993; Luchtel et al. 1998; Bernard et al. 2000; Kelly et al. 2000).

Microparticles may also be administered by intravenous injection in smaller animals such as rodents (Bernard et al. 2000; Schimmel et al. 2001), with acceptable results, provided adequate reference samples are collected.

LIMITATIONS OF THE METHOD

Although microsphere methods do provide accurate estimates of total renal blood flow when compared to

other methods, in general they cannot be used to evaluate regional blood flow in the kidney accurately because they are retained to a disproportionately greater extent in the cortex compared to the medulla, due both to trapping of microspheres in the glomeruli and "skimming" of microspheres at the arcuate arteries, resulting in disproportionately decreased delivery to the medulla (Clausen et al. 1979; Knox et al. 1984). However, changes in regional blood flows can be evaluated by determining differences in regional deposition of differentially labeled microspheres that are administered to the same animal before and after an experimental manipulation. If fluorescent microspheres are used for this purpose, the fluorochromes must be selected carefully to ensure adequate separation of the excitation and emission wavelengths to prevent "spillover" (Katz et al. 1971; Glenny et al. 1993). Thirteen such fluorochromes have been identified which do not result in spillover when administered simultaneously (Schimmel et al. 2001).

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5.1.1.2 Assessment of Renal Tubule Functions

Urinalysis

PURPOSE AND RATIONALE

More than 99% of the reabsorption of glucose, protein, and electrolytes occurs in the proximal tubule. Electrolyte concentrations can be affected by other tubule segments, but even low-level changes in proximal tubular function will be reflected by increased urinary excretion of protein and glucose (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price 1999; Aleo et al. 2002). Standard and modified urinalysis techniques can be successfully used to assess the effects of compounds on proximal tubule uptake processes (Katsuno et al. 2007).

PROCEDURE

Collection of a good quality sample from the test species is paramount to obtaining high quality data from the urinalysis (see "Assessment of GFR by Plasma Chemistry"). Timed urine collections in all species (including large animals) can also be obtained by the use of specially designed metabolism cages. A metabolism cage consists of an animal chamber mounted above an excrement collection system. The animal chamber must be equipped with feeder and waterer units if an animal is to be housed in the metabolism cage for more than a few hours. These need to be sized appropriately to the test species of interest (especially regarding the collection container, which must minimize surface area available for evaporation) and designed to eliminate contamination of the urine by food, drinking water, or feces. In selecting the size of the collection container, the anticipated urine volume should be considered: a mouse will produce 0.25–1 ml

in 24 h of urine; a rat, 10 ml; a hamster, 5–8 ml; a rabbit, 600 ml; and a dog or minipig, 500 ml or more (Loeb 1998; McClure 1999; Van Metre and Angelos 1999). Whatever type of metabolism cage is used, the following general precautions are offered:

1. For studies of >24 h duration, test animals should be acclimated to the metabolism cage for several days prior to study initiation. During this period, animals should be monitored frequently to insure that they learn to use the feeder and waterer systems properly. Test animals should be maintaining or gaining weight prior to study initiation.
2. Feeder and waterer systems should provide ample food and water to meet the animal's needs for the duration of urine collection and all separator systems should function properly. For chronic studies (>5–7 days duration), it is useful to have a complete exchange of feeder, waterer, and urine/feces collector and separator systems so that soiled units may be rapidly exchanged with clean, dry/filled units at regular intervals.
3. Cages should be decontaminated, cleaned, rinsed with distilled/deionized water, and thoroughly dried prior to use. Surfaces used to collect urine may be siliconized or sprayed with a suitable hydrophobic material (PAM®, General Foods) to facilitate urine collection.
4. All surfaces contacting urine should be rinsed with distilled/deionized water or appropriate solvents to collect any residuals at appropriate intervals.
5. To preserve the quality of the specimens during prolonged collection times, the opening of the collection vial needs to be small to prevent evaporation and the vial should be surrounded either with wet ice or frozen cold packs to chill the sample promptly once it is deposited (Loeb 1998; Loeb and Quimby 1999). Urine may also be collected under mineral oil to prevent evaporative losses. For very small or antidiuretic animals (e.g., hamsters, gerbils) placing the cage over a shallow pan of oil and skim feces from the surface may be necessary while collecting urine with a pipette from under the oil. To maximize the volume of the sample collected in rodents in metabolism cages, food should be withheld (this also reduces the risk of contamination of the sample) and water provided (Lee et al. 1998).

Routine urinalysis consists of visual assessment (color, clarity), volume, specific gravity or osmolality, pH, and quantitative or semiquantitative determination of total protein and glucose and microscopic evaluation of urine sediment (Weingand et al. 1996). Urine constituents can be measured semiquantitatively using commercially available "dipstick" test strips (Chemstrips®, Roche Diagnostics or Multistix®, Bayer Heath Care Diagnostics), which contain reagents for the determination of specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, hemoglobin, nitrite, and leukocyte esterases (Newman and Price 1999). These strips may be read manually or using automated analyzers specific to each product.

If quantitative assessment of urine analytes is required (see under "Evaluation"), either urine volume or urine creatinine concentration must be measured and used to "normalize" the concentration of the measured analyte. This will negate the effects of differences in urine concentration between animals and allow for accurate comparison of the results. The daily excretion of creatinine is fairly consistent in all species and therefore its quantity in a spot urine sample serves as an accurate index of the 24 h urine output. This adjustment has been shown to accurately correct for incomplete timed urine sample collection in rodents (Haas et al. 1997a), and works well for other species. Urinary creatinine can be measured by the same methods used for plasma (see "Assessment of GFR by Plasma Chemistry").

EVALUATION

Urine volume, combined with assessment of urine concentration (specific gravity or osmolality) can serve as an index to renal function. With severe acute loss of functional nephron mass or renal perfusion, urine output is decreased (oliguria) or absent (anuria), while loss of the ability of the kidney to adequately concentrate urine results in the excretion of large volumes of dilute urine.

The commercially available dipstick reagents do not detect the low levels of glucose found in normal urine and thus positive results indicate significant glucosuria in most species (the exception is the gerbil, where dipstick positive glucosuria is normal due to the high urine concentration in this species) (McClure 1999). If glucosuria is detected, time-matched plasma glucose levels are needed to rule out that this result has not resulted from an increase in the filtered glucose load. If plasma glucose is normal, the appearance of glucose in the urine may indicate a functional deficit in the proximal tubule (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price 1999; Aleo et al. 2002).

Because of inherent inaccuracies in the dipsticks when applied to animal urine (see below under "Limitations of the Method"), positive results by dipstick for protein (or glucose in some species and under some conditions) must be followed by detailed qualitative and quantitative assessment.

Quantitative assessment of urinary protein excretion is necessary to rule out a contribution from glomerular malfunction. In general, excretion of markedly elevated levels of protein is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/low-grade glomerular injury (Peterson et al. 1969; Finco 1997).

A number of different methods have been developed for quantitative assessment, including turbidometric, colorimetric (biuret and Lowry assays as examples) and dye-binding assays. All have their advantages and limitations; in general, biuret assays tend to detect all types of proteins with equal sensitivity but require fairly large sample volumes, turbidometric assays can suffer from lack of precision with variations in urine ionic strength, and dye-based methods may suffer from interference with exogenous and endogenous urine substances. The Folin phenol (Lowry), Coomassie brilliant blue, and Ponceau S methods have been recommended as being particularly precise for urine samples (Peterson et al. 1969; Dilena et al. 1983; Finco 1997; Newman and Price 1999).

Qualitative assessment (identification of the proteins excreted) is necessary to determine if low-level proteinuria has resulted from glomerular or tubular malfunction. Concomitant elevation of albumin and one or more of the low molecular weight proteins (which are freely filtered by the normal glomerulus) indicates that the proteinuria has resulted from decreased tubular reabsorption of proteins, while albumin elevation alone or concurrently with a high molecular weight protein (normally excluded from the filtrate by the glomerulus) indicates primary glomerular injury (Peterson et al. 1969; Finn and Porter 1998; Guder et al. 1998; Umbreit and Wiedemann 2000). Commonly used filtered low molecular weight proteins include retinol-binding protein (Price 2000, 2002; Aleo et al. 2002, 2003), α_2- or β_2-microglobulin (Viau et al. 1986; Loeb 1998; Finn and Porter 1998; Price 2000, 2002), and cystatin C (Finn and Porter 1998; Herget-Rosenthal et al. 2001; Uchida and Gotoh 2002).

LIMITATIONS OF THE METHOD

Because the commercially available dipsticks are designed for human use, they are inaccurate for a number of parameters in animal urine. The specific gravity reagents are completely inaccurate in all species. Dipsticks which use a glucose oxidase method for urine glucose can show a false positive result in species such as the dog and mouse with high urinary ascorbate levels or in urine contaminated with hypochlorite used as a disinfectant (Finco 1997; Loeb and Quimby 1999). For all glucose detection methods, administration of formulations that contain glucose or other metabolizable sugars in quantities should be avoided, as they may transiently overwhelm tubule reabsorption mechanisms and generate false positive test results.

Protein detection on the strips is based on a bromphenol blue method that is most sensitive for albumin (Newman and Price 1999), which means that proteinuria that does not result primarily from an increased albumin excretion may not be detected by this method. Furthermore, since these tests are designed for human urine, false positives are frequent in species like the dog, whose normal urine protein levels are just above the lower limits for humans (Finco 1997), and dipstick tests are invariably positive in male rats and mice which have normal high proteinuria (Loeb and Quimby 1999).

The sensitivity of the quantitative protein assays to the protein(s) of interest depends in large measure on which protein is used to generate the standard curve. Albumin is most commonly used because it is the most abundant protein in urine and while it is adequate for most methods (Dilena et al. 1983), it will in general underestimate the abundance of many other proteins of interest in urine (Guder and Hofmann 1992).

Qualitative assessment of urine proteins other than albumin is hampered in many species by the lack of suitable immunoassays; with the exception of cystatin C, commerically available antibodies do not cross-react with the animal proteins (Loeb and Quimby 1999; Uchida and Gotoh 2002). Assessment of urinary retinol excretion has been shown to be a sensitive index to retinol-binding protein excretion in the rat (Aleo et al. 2002, 2003).

MODIFICATIONS OF THE METHOD

Point-in-time samples from laboratory rodents may be obtained by taking advantage of the fact that these animals frequently urinate when they are handled or shortly after being removed from their home cage.

A skilled, quick, and prepared operator with a ready small container or plain microcapillary tube may be able to obtain a small sample in the first instance (Loeb and Quimby 1999); in the second, the animal can be placed in a small confined space on plastic food wrap and observed carefully for urination, as it has been shown that most rodents will urinate within 20 min after removal from their home cages. The sample thus generated can be collected by micropipette (Kurien and Scofield 1999).

For repeated point-in-time urine samples from rats over short periods of time (1–2 weeks after the surgery), the urethra or the ureter can be cannulated. Cannulated rats can also be used to collect accurate and complete timed urine samples (Mandavilli et al. 1991; Horst et al. 1988).

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Electrolyte Excretion

Since one of the kidney's primary functions is maintaining electrolyte and mineral homeostasis in the face of fluctuating dietary intake and body needs, examination of plasma and urine electrolyte levels will provide some insight into renal function. Because of the large functional mass of the kidney, alteration of plasma electrolyte levels are usually not detected until the effect on renal functional is significant (pathologic). In contrast, urine electrolyte levels examined with knowledge of plasma levels and dietary intake can serve as an extremely sensitive

index to the effect of drugs or chemicals on the functional state of the kidney.

In animal studies, the diet can be carefully controlled and thus the intake (and hence the plasma electrolyte levels) can be assumed to be fairly constant. Provided there are no sources of significant electrolyte loss resulting from the experimental manipulations (e.g., vomiting, diarrhea, salivation), urine electrolyte levels will reflect effects on either GFR (determines the filtered load) or tubular secretion or reabsorption (determines the final urine electrolyte composition).

Fractional Excretion Methods

PURPOSE AND RATIONALE

Fractional excretion (FE) is the proportion of the filtered load of any analyte that is excreted from the plasma. If both tubular function and plasma electrolyte values are normal, increases in electrolyte FE values clearly reflect a decrement in GFR. With tubular malfunction, the direction of the change in FE values depends on the net direction of electrolyte transport (i.e., FE will increase for electrolytes that are primarily reabsorbed and will decrease for secreted electrolytes) (Finco 1997; Stockham and Scott 2002).

PROCEDURE

FE assessments can be performed in any species, as it requires only carefully timed complete urine collections and a concurrent assessment of GFR (see Sect. 1.1.1 and section "Urinalysis"). FE will be unitless if the urine collection period is expressed in minutes, thus (Finco 1997):

$$\mathrm{FE} = \frac{\text{urine electrolyte concentration (mmol/l)} \times \text{urine (ml/collection period)}}{\mathrm{GFR(ml/min)} \times \text{plasma electrolyte concentration (mmol/l)}}$$

To eliminate the need for both complete timed urine collections (difficult to do in most animals) and concurrent assessment of GFR, FE values are usually calculated based on point-in-time urine collections by using creatinine excretion during the same time period as an estimator of GFR (Finco 1997).

Animals are placed in appropriately sized metabolism cages for an appropriate period of time to allow collection of an adequate volume of urine (see section "Urinalysis" for methods). At the midpoint of the collection period a blood sample is obtained under appropriate anesthesia (note: the use of CO_2 for anesthesia will falsely elevate plasma potassium levels and render the method inaccurate) for determination of electrolyte and creatinine levels.

Plasma and urine electrolytes and creatinine are determined by standard methods (Durst and Siggard-Andersen 1999; Newman and Price 1999; Scott et al. 1999a). FE is calculated from the results as outlined below (Stockham and Scott 2002):

$$\mathrm{FE} = \frac{\text{urine electrolyte concentration} \times \text{plasma creatinine concentration}}{\text{urine creatinine concentration} \times \text{plasma electrolyte concentration}}$$

LIMITATIONS OF THE METHOD

Because there are multiple transporters for most electroytes and solutes, FE will only reflect effects on the net tubular transport of solutes, and will most reliably highlight effects on the major transport pathway for any given solute. Effects on minor pathways usually do not alter FE but may contribute to inaccuracies in these methods. Because the major tubular transporter pathways are often localized to a single nephron segment, FE methods can sometimes be used to identify the specific segment affected by a test compound.

The inherent inaccuracy in the use of creatinine clearance as an estimator of GFR has been discussed previously (see section "Assessment of GFR by Plasma Chemistry"). If the Jaffe method for creatinine is used, the investigator must be aware of potential interference due to endogenous chromogens (the

error is magnified in species where these chromogens are present in higher concentration in the plasma than in the urine (dog, mouse) (Finco 1997; Dunn et al. 2004) or possibly the test compound (Sonntag and Scholer 2001). Additionally, plasma electrolyte levels may fluctuate as the result of eating or due to diurnal rhythms (Finco 1997). The impact of inaccuracies can be minimized by consistent timing of urine collection, fasting of animals before and during urine collection, and the inclusion of a concurrent untreated (vehicle control) group in all studies.

In order for FE values to be unitless, all analytes must be converted to their SI unit (or, if necessary, molar) equivalents. The table below lists some conversion factors for some of the more commonly used analytes.

Analyte	Conventional unit	Conversion factor	SI unit
Albumin	g/dL	10	g/l
Ammonia (as NH_3)	µg/dL	0.587	µmol/l
Ammonia (as NH_4)	µg/dl	0.5543	µmol/l
Ammonia (as nitrogen)	µg/dl	0.7139	µmol/l
Bicarbonate	mEq/l	1.0	mmol/l
Calcium	mg/dl	0.25	mmol/l
Creatinine	mg/dl	88.4	µmol/l
Galactose	mg/dl	55.506	µmol/l
Glucose	mg/dl	0.0555	mmol/l
Glutathione	mg/dl	0.032	mmol/l
Magnesium	mg/dl	0.411	mmol/l
Manganese	ng/ml	18.2	nmol/l
Phosphorus	mg/dl	0.323	mmol/l
Potassium	mEq/l	1.0	mmol/l
Retinol	µg/dl	0.0349	µmol/l
Sodium	mEq/l	1.0	mmol/l
Urea nitrogen[a]	mg/dl	0.357	mmol/l
Uric acid (urate)	mg/dl	59.48	µmol/l
General metric conversion (proteins)	mg/dl	0.01	g/l

[a]Conversion is to *urea* in mmol/l. See "Assessment of GFR by Plasma Chemistry."

Data from the following sources: http://www.globalrph.com/conv_si.htm; http://www.soc-bdr.org/rds/authors/unit_tables_conversions_and_genetic_dictionaries/e5196/index_en.html

MODIFICATIONS OF THE METHOD

FE of sodium (FE_{Na}) is most commonly used to assess tubular function, and to differentiate between prerenal and tubular causes of azotemia. Increases in FE_{Na} have been detected with acute tubular necrosis, while decreased FE_{Na} usually indicates decreased tubular flow rates resulting from decreased renal blood flow (Espinel 1976). However, in some cases where acute tubular necrosis or tubular malfunction is accompanied by decreased renal perfusion or tubular flow, FE_{Na} will decrease; conversely, FE_{Na} may be increased in cases where prerenal azotemia is accompanied by systemic acid–base imbalances and/or altered excretion of other electrolytes (Nanji 1981).

FE of magnesium (FE_{Mg}) has been shown to be the most sensitive index in detecting low-level tubular injury in humans (Barton et al. 1987; Futrakul et al. 1999; Kang et al. 2000; Oladipo et al. 2003). FE_{Mg} is also directly correlated to the magnitude of peritubular blood flow and serves as a sensitive marker for the presence of renal interstitial fibrosis (Deekajorndech 2007). Increased FE of calcium (FE_{Ca}) has also been shown to serve as a sensitive index of effects on renal function unrelated to overt renal injury (Lam and Adelstein 1986; Tuso and Nortman 1992; Eliott et al. 2000). The transporters for both Ca and Mg reside in the distal convoluted tubule and connecting tubule and are regulated by alterations in systemic pH; thus, concomitant increases or decreases in both FE_{Mg} and FE_{Ca} may be indicative of the presence of systemic metabolic acidosis or alkalosis, respectively (Nijenhuis et al. 2006).

FE of urea (FE_{Ur}) may be more useful than the FE_{Na} in distinguishing between prerenal and renal azotemia in humans, as it appears to more reliably reflect renal perfusion and does not change with diuretic administration, which can falsely increase FE_{Na} (Kaplan and Kohn 1992; Carvounis et al. 2002; Pépin et al. 2007). It thus may also be useful in making this distinction in animal models. Changes in the FE of urea also reflect changes in urine flow rates (in general, these values move parallel to each other because decreased tubular flow results in increased urea reabsorption) and can be used as an estimate of this parameter (Goldstein et al. 1969; Finco 1997). The methods used to detect urea in urine are the same as those used in serum (Newman and Price 1999).

FE of anions (ammonium, bicarbonate) can be used to determine the potential mechanism underlying systemic acid–base imbalances (Rothstein et al. 1990; Kim et al. 2001).

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Quantitative Electrolyte Excretion

PURPOSE AND RATIONALE

Fractional excretion methods are useful in that they can identify alterations in analyte excretion with relative ease, as they require only point-in-time assessment of analyte concentration and do not require complete and timed urine collection. Greater sensitivity, however, is obtained with quantitative evaluation of electrolyte excretion. This method is more difficult, requiring carefully timed, complete urine collection, and because plasma levels are not evaluated there needs to be reasonable assurance that the changes in excretion seen do not result from alteration of the filtered load of the analyte in question (e.g., plasma levels and GFR remain consistent). In animal studies, where the diet is carefully controlled, these factors are not usually of concern.

PROCEDURE

The method can be used on any species, although rats are most commonly used. Animals are usually fasted but allowed access to water; if anesthetized animals are used, intravenous administration of balanced electrolyte solutions (e.g., lactated Ringer's solution) or 0.9% NaCl solution can be administered by intravenous infusion to ensure that plasma volume (and thus GFR) and electrolyte levels are maintained.

Urine is collected by any appropriate method (see "Urinalysis") over a specified period of time. If possible, the urinary bladder should be emptied before collection of test urine is initiated and at the end of the collection period; this can be accomplished in dogs by catheterization and in rodents by handling or manual expression of the bladder. If metabolism cages are used, special care must be taken to avoid evaporation of urine and contamination of the vials with feces or drinking water, as accurate determination of volume is important. The volume of urine collected is determined gravimetrically, and urine electrolytes are measured by standard methods (Durst and Siggard-Andersen 1999; Scott et al. 1999b).

Electrolyte concentrations need to be converted to their SI equivalents before calculation of electrolyte excretion (see "Fractional Excretion Methods").

EVALUATION

The volume of urine collected over the time period is defined as the urine flow rate:

$$V = \text{volume collected/time period (expressed in mL/min)}$$

and electrolyte excretion (U_XV) is defined as:

$$U_XV = \{[(\text{concentration of X in urine}) \times V]/\text{body weight in g}\} \times 100(\text{expressed in mmol/min/100g})$$

As a general rule:

$$U_{Na}V + U_KV = U_{Cl}V$$

and

$$U_{Na}V + U_KV + U_{Cl}V + U_{Urea}V = \text{urine osmolality}$$

If the first equation is untrue, the assumption must be made that there are additional anions in the urine. Carbonic anhydrase inhibition (i.e., excess urinary bicarbonate) may be the reason for this finding if the ratio:

$$\frac{U_{Cl}V}{U_{Na}V + U_KV}$$

is less than 0.8.

Deviations in the second equation indicate electrolyte or nonelectrolyte "gaps" and suggest the presence of unusual solutes in urine.

Net natriuretic activity can be assessed by evaluation of the ratio:

$$\frac{U_{Na}V}{U_KV}$$

Values greater than 2.0 indicate a favorable natriuretic effect, and values greater than 10.0 indicate a potassium-sparing effect.

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Assessment of Tubular Transport Processes

Assessment of Organic Anion and Cation Transport

PURPOSE AND RATIONALE

A number of transporters for organic anions and cations are present on both the lumenal and basolateral surfaces of the proximal tubules. These are essential to the secretion and/or reabsorption of a number of endogenous substrates as well as functioning in the elimination of xenobiotics. These transporters frequently function as ion exchangers and their activity is thus bidirectional, so that the net direction of transport (i.e., secretion or reabsorption) for any given substrate depends not only on the numbers and activity of the transporters on either side of the tubule but also on the presence and concentration of competing or cotransported substrates. This feature of tubular

transport has been one of the major challenges in the development of uricosuric agents for the treatment of hyperuricemia and influences the nature and impact of drug–drug interactions (Launay-Vachera et al. 2006; Terkeltaub et al. 2006). Compounds that affect either the expression or activity of transporters, are competitive substrates for them, or act as inhibitors of transport thus may affect not only the clearance of therapeutic agents but also that of endogenous metabolites (Wright and Dantzler 2004; Launay-Vachera et al. 2006).

PROCEDURE

The choice of substrate or inhibitor used depends on the transport process or specific transporter of interest. Table 5.1 lists the most commonly used substrates and their transporters (note: most inhibitors act competitively and are themselves substrates for the transporter in question).

Transport studies can be performed in any animal model from which urine and plasma may be collected readily. Inhibitors are administered 30 min–1 h before the administration of the test substance. Substrates are usually administered intravenously, and are detected in urine and plasma most commonly by colorimetric methods. The exception is glycylsarcosine, which is either used as a [^{14}C] radiometric tracer for clearance studies or as a [11C] PET tracer for in vivo uptake studies (Ocheltree et al. 2005; Nabulsi et al. 2005)

EVALUATION

Standard clearance, fractional excretion (FE), or excretion methods are used to assess the effects (see "Assessment of GFR and RBF by Clearance Methods" and "Electrolyte Excretion" for details).

LIMITATIONS OF THE METHOD

Significant sex differences exist in renal transporter expression and activity in rodents, with activity in males being generally greater than in females (Morris et al. 2003; Sekine et al. 2006). Sex hormone regulation of the same transporter can differ across species,

Table 5.1 List of most commonly used sub-strates and their transporters

Compound	Commonest use	Net transport direction	Transporter(s) affected	Comments
Tetraethylammonium (TEA)	Substrate	Secretion	OCT1, OCT2, OCT3	Most commonly used substrate
1-methyl-4-phenylpyridinium (MPP^+)	Substrate	Secretion	OCT1, OCT2	Can be used to differentiate OCT3-mediated transport
Tributylmethylammonium (TBuMA)	Substrate	Secretion	OCT3	Can be used to differentiate OCT3-mediated transport
Urate	Substrate	Secretion or Reabsorption (species dependent)	URAT1, OAT1, OAT3	URAT1 is selective for urate in human kidney but its ortholog has other OA transport functions in other species. Net transport is reabsorption in humans but secretion in animals
p-aminohippurate (PAH)	Substrate	Secretion	OAT1, OAT2, OAT3(?), OAT4	Most commonly used substrate. Questionable substrate for OAT3
Estrone sulfate	Substrate	Secretion	OAT3, OAT4, Oatp1	Oatps are homologues of bile acid transporters in the liver
Lucifer yellow	Substrate	Secretion	OAT1	Can be detected spectrofluorometrically
Phenolsulfonphthalein (Phenol red)	Substrate	Secretion	OAT3	Clearance has traditionally been used as a surrogate for urate transport
Glycylsarcosine	Substrate	Reabsorption	PEPT1, PEPT2	Equal affinity for both PEPTs, but renal reabsorption is primarily PEPT2 mediated
Probenecid	Inhibitor		OAT1, OAT2, OAT3, URAT1	Used therapeutically and experimentally
Cimetidine	Inhibitor		OAT3	Used to correct creatinine clearance for the contribution of tubular secretion
Benzbromarone Pyrazinamide	Inhibitor		URAT1	Selective for URAT1

Table 5.2 Common gender-related differences in renal transport processes[a]

				Response to treatment with:	
Transporter	Selected substrate(s)	Species	Gender difference	Testosterone	Estrogen
Oatp[b]	Estrone sulfate Glutathione (GSH)	Rat	Male > female	Strong increase	Decrease
OAT1	Urate PAH PGE_2 Cysteine conjugates	Rat	Male > female	Increase	Strong decrease
OAT3	Urate PAH Cimetidine	Rat	Male > female	Increase	No effect
OAT2	PAH PGE_2	Mouse	Male > female		
OAT2	PAH PGE_2	Rat	Famale > male	Strong decrease	Weak increase
OCT2	TEA	Rat	Male > female	Increase	Decrease
MRP1	Lipophilic cations GSH, glucuronide & sulfate conjugates	Mouse	Female > male	Not determined	Not determined
MRP4	GSH, glucuronide & sulfate conjugates	Mouse	Male > female	Increase	No effect
SGLT1	Glucose Galactose	Rat	Female > male	Decrease	No effect

[a]Data from Morris et al. 2003; Wright and Dantzler 2004; Sabolić et al. 2006; Ljubojević et al. 2004, 2007.
[b]Note: net effect of Oatp transport is reabsorption, whereas for others the net effect is secretion.

and similar transporters can have different sex hormone regulation within a species. These must be taken into account both in designing experiments to evaluate effects on organic anion or cation transport systems as well as in evaluating the clinical relevance of those results. Some of the common gender-related differences in renal transport processes are outlined in the Table 5.2:

Many of the substrates, and all of the inhibitors, are not specific enough for accurate determination of the exact transporter affected by the test compound. Furthermore, redundancies in tubular transport processes exist that can mask the ability to detect alteration in function of any single transporter by even selective inhibitors.

MODIFICATIONS OF THE METHOD

The listed substrates and inhibitors may also be used in in vitro models such as isolated tubules or kidney slices. In the latter model, net transport is usually assessed by measuring either the disappearance of the substrate from the medium or, more commonly, the degree or rate of accumulation of the substrate over time in the tissue slice (Kirkpatrick and Gandolfi 2005).

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Evaluation of Receptor-Mediated Endocytosis

PURPOSE AND RATIONALE

Reuptake of low molecular weight proteins that are freely filtered at the glomerulus (as well as some high molecular weight proteins) occurs primarily in the proximal tubule as the consequence of receptor-mediated endocytosis. For most of these, the process is mediated through megalin binding, either directly or subsequent to their binding to cubulin (a brush border membrane associated protein that is internalized following ligand binding and complex formation with megalin). The megalin-protein complexes are internalized, forming primary endosomes. These are subsequently acidified through the action of a number of proton exchange transporters, the most crtical of which are Na^+/H^+ exchanger-3 (NHE3) and Cl-/H + antiporter-5 (ClC-5). The decrease in pH results in release of the proton exchange transporters, megalin and cubulin from the late endosomes, which are recycled back to the brush border, as illustrated in Fig. 5 (Leheste et al. 1999; Christensen 2002; Verroust 2002; Verroust et al. 2002; Guggino 2007).

In addition to its role in renal endosomal acidification, NHE3 is also critically important to the normal endocytic uptake of megalin-protein complexes. The two proteins are located in close association in the proximal tubule brush border and disruption of this association results in decreased or absent megalin mediated endocytosis (Biemesderfer et al. 1999; Gekle et al. 1999, 2004). Because the activity of NHE3 in the proximal tubule is primarily regulated by alterations in its turnover and/or trafficking to the brush border membrane (there exists a substantial intracellular pool within the proximal tubule that can rapidly exchange with the transmembrane, megalin-associated NHE3 pool), any physiologic, pharmacologic or toxic stimulus that affects the relative distribution of NHE3 within the proximal tubule cell can have a profound effect on megalin-mediated endocytosis and result in either increases or decreases in urinary low molecular weight proteins (Donnowitz and Li 2007).

PROCEDURE

A listing of endogenous substrates for megalin-mediated transport is found in Table 5.3. Several of these (listed in italics in the table) have either been used or proposed as biomarkers of renal tubular function. While the presence of either any or increased quantities of one or more of these proteins in the urine is considered evidence of alteration of renal tubular protein transport, greater sensitivity to detect detriments in renal protein transport has been shown by using fractional excretion or clearance methods (Tencer et al. 1998a).

Information from: Leheste et al. 1999; Moestrup et al. 1996; Marinó et al. 2001; Christensen 2002; Verroust 2002; Verroust et al. 2002; Gburek et al. 2003; Cutillas et al. 2004; Roald et al. 2004; Oyama et al. 2005; Meistermann et al. 2006; Wolff et al. 2006; Kobori et al. 2008.

Proteins in *italics* have been used or proposed as urinary biomarkers of altered tubular protein transport.

The proteins can be detected and quantified in plasma and urine samples by appropriately-validated ELISA methods. Mass spectroscopy methods have also been used (Meistermann et al. 2006). Assessment of urinary retinol excretion has been shown to be a sensitive index to retinol binding protein excretion in the rat (Aleo et al. 2002, 2003).

Table 5.3 List of endogenous substrates for megalin-mediated transport

Vitamin-binding proteins/ apolipoproteins	Hormones	Enzymes	Growth factors/cytokines	Miscellaneous
Apo-AI	*Angiotensinogen*	α-amylase	Angiogenin	*Albumin*
Apo-AIV	Insulin	Carbonic anhydrase	Chemokine 14	*α_1-Microglobulin*
Apo-B	Parathyroid hormone	Lipoprotein lipase	Complement factor B	α_2-HS-glycoprotein
Apo-E	Prolactin	*Lysozyme*	Complement factor C2	*β_2-Microglobulin*
Apo-J (clusterin)	Thyroglobulin	Plasminogen	Complement factor D	β_2-Glycoprotein I
Apo-H		Plasminogen activator inhibitor-1	Complement factor H	*Cystatin C*
Cubulin		Plasminogen activator inhibitor-1 (PAI-1)	Epidermal growth factor	Cytochrome c
Metallothionein		PAI-1-urokinase	IGF-binding protein-2	Fibronectin FN70
Retinol-binding protein		PAI-1-tPA	IGF-binding protein-4	Immunoglobulin light chains
Transferrin		Pro-urokinase	IGF-binding protein-5	Hemoglobin
Transthyretin			IGF-binding protein-6	Hemopexin
Transcobalamin			IGF-II	Lactoferrin
Vitamin D–binding protein			Neutrophil-activating peptide	*L-fatty-acid-binding protein*
			Pigment epithelium–derived factor	Myoglobulin
				Major urinary protein-1 (MUP-1)
				Orosomucoid
				Odorant-binding protein
				Zinc-α_2-glycoprotein

EVALUATION

Standard clearance, fractional excretion (FE) or excretion methods are used to evaluate the effects (see "Assessment of GFR and RBF by Clearance Methods" and "Electrolyte Excretion" for details).

LIMITATION OF THE METHOD

The usefulness of these assessments in animals are limited by the lack of suitable immunoassays, as in many cases, commerically available antibodies do not cross-react with animal proteins. Notable exceptions are cystatin C and β_2-microglobulin (Viau et al. 1986; Loeb and Quimby 1999; Uchida and Gotoh 2002).

MODIFICATION OF THE METHOD

More recently, the use of fluorescently labeled probe proteins (such as FITC-albumin) and multiphoton microscopy techniques have allowed evaluation of alterations in protein transport in real time (Peti-Peterdi 2005).

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Evaluation of Tubular Function by Micropuncture

PURPOSE AND RATIONALE

Micropuncture is an important method for determining the effects of drugs or chemicals on single nephron function in the intact kidney, and can provide information concerning tubular handling of a drug or chemical. Micropuncture is a highly specialized technique that requires considerable equipment and experience to be utilized properly.

PROCEDURE

Studies may be done in small rodents and dogs, and studies in various strains of rats are most common. Munich-Wistar rats are frequently used for micropuncture studies because the glomeruli are visible and accessible at the surface of the kidney (Ramsey and Knox, 1996). The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia the animals are placed on a thermostatically heated table. Following tracheotomy, the carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and for infusion of compounds, respectively. The left kidney is carefully exposed by a flank incision and immersed in a vessel containing mineral oil at 37°C. Proximal tubules are identified under a microscope on the surface of the kidney and a micropipette (glass capillary tube of 8–10 mm external diameter) is inserted. A small amount of mineral oil or wax is infused to block retrograde flow of the perfusion fluid and the cannulated tubules then are infused with saline or appropriate balanced electrolyte solution containing inulin (H^3 or flourescein isothiocyanate conjugated) and a dye solution (lissamine green or other suitable dye) to assess the volume of absorption and identify the

perfused segments, respectively. The infusion rate/volume is determined by the size of the animal. Distal collection is done by identification of the perfused segment by its dye content; a second micropipette is then inserted, a distal oil or wax block is placed and the infusion fluid can be collected for subsequent analysis (Knox and Marchand 1976; Ramsey and Knox 1996; Lorenz et al. 1999; Wang et al. 1993, 1999).

EVALUATION

Fluid reabsorption (J_v) is assessed by comparing the inulin concentration in the perfusate to that in the collected sample, thus (Wang et al. 1993):

$$J_v = V_0 - VL$$

where VL is the collected fluid volume (normalized for the length of the tubular segment over which it was collected), and:

$$V_0 = VL(INL/IN_0)$$

where INL = concentration of inulin in the collected sample, and IN_0 is the inulin concentration in the perfusate.

The net flux of the desired analyte (J_X, where X is the anlyte of interest – Na, K, HCO_3, glucose, etc.), is calculated thus:

$$J_X = V_0[X_0] - VL[X]_L$$

where $[X]_0$ is the concentration of the analyte in the perfusion fluid, and $[X]_L$ is the concentration of the analyte in the collected fluid.

MODIFICATION OF THE METHOD

Inulin conjugated to fluorescein isothiocyanate (FITC) may be used in place of the radiolabeled inulin. The inulin concentration in the sample is assessed by comparing the fluorescence in the sample to a standard curve (Lorenz and Gruenstein 1999).

This technique can be applied to distal tubules as well as to proximal tubules. Distal tubules are identified at the surface of the isolated kidney following micropuncture and perfusion of a proximal tubule as described above. Once the dye reaches the superficial portion of the distal tubule, it is blocked with oil or wax and accessed both proximally and distally as described above (Levine 1985).

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Evaluation of Tubular Function by Stop Flow Techniques

PURPOSE AND RATIONALE

This procedure is of historic value in the localization of transport processes along the length of the nephron. During clamping of the ureter, glomerular filtration is grossly reduced. The contact time for the tubular fluid in the respective nephron segments increases, and the concentration of the constituents of tubular fluid should approximate the static-head situation. After release of the clamp, the rapid passage of the tubular fluid should modify the composition of the fluid only slightly. The first samples should correspond to the distal nephron segment, the latest to glomerular fluid.

With introduction of micropuncture techniques ("Evaluation of Tubular Function by Stop Flow Techniques"), evaluation of segment-specific transport using whole kidney stop flow techniques has become less common. However, the observation that the conduct of stop flow studies in single nephrons resulted in pronounced vasoconstriction of the afferent arteriole of the affected nephron (Arendshorst et al. 1974) led to the application of this technique to the study of this phenomenon. Micropuncture stop flow methods are thus more commonly used to investigate tubuloglomerular feedback (TGF) regulatory mechanisms.

PROCEDURE

The whole kidney stop flow method was originally described by Malvin et al. (1958). Larger species (rats, dogs, and rabbits) are most commonly used. Animals are maintained under general anesthesia and intense osmotic diuresis (via IV perfusion with a hypertonic solution such as saline or mannitol) is initiated. Once diuresis has been established, a ureter is clamped for several minutes allowing a relatively static column of urine to remain in contact with the various tubular segments for longer than the usual periods of time. Thus, the operation of each segment on the tubular fluid is exaggerated. Then the clamp is released, and the urine is sampled sequentially. Small serial samples are collected rapidly, the earliest sample representing fluid that had been in contact with the most distal nephron segment.

The test substance of interest, along with inulin or another marker of glomerular filtration, is administered intravenously to equilibrium before the application of uretral occlusion. Blood samples are collected at the midpoint of the occlusion period for the determination of the test substrate and the glomerular marker (for subsequent assessment of clearance as described in "Assessment of GFR and RBF by Clearance Methods").

EVALUATION

In each sample the concentration of a glomerular marker, such as inulin, and the concentration of the substance under study are measured. The urine flow rate may be calculated by dividing the volume of sample collected by the collection interval (this is thus expressed in ml/min). Clearance of the test substance and the glomerular marker are calculated (see "Assessment of GFR and RBF by Clearance Methods"). Fractional excretion of the test substrate is calculated as described in "Fractional Excretion Methods," or by dividing the clearance of the test substrate by that of the glomerular marker (Tanaka et al. 1990).

MODIFICATIONS OF THE METHOD

An early modification of the whole kidney stop flow technique obliterates the renal pelvis dead space in dogs just prior to the ureteral obstruction. This allows stop flow experiments to be performed with less intense diuresis and still generate reliable determination of distal tubular fluid composition (Jaenike and Berliner 1960).

The clearance of endogenous substrates (e.g., urate, uric acid, electrolytes) may be used instead of those of exogenously administered compounds to assess tubular function (Shinosaki and Yonetani 1989; Tanaka et al. 1990: Shinosaki et al. 1991). Creatinine (either endogenous or exogenous) may be used in place of inulin as a marker of glomerular filtration (Young and Edwards 1964; Tanaka et al. 1990).

Stop flow techniques can be performed in isolated perfused tubular segments using micropuncture techniques (see "Evaluation of Tubular Function by Stop Flow Techniques"). The proximal and distal micropunctures are performed as described, but instead of immediately collecting fluid from the distal micropipette, pressure is applied equal to that of the perfusion pressure at the proximal pipette, until flow ceases at the infusing pipette. Sampling occurs following a period in which the perfusate remains static in the perfused tubule segment. Care must be taken, especially in mice, to prevent tubular leaking with excessive stop-flow pressures or infusion rates (Schnermann 1999).

Micropuncture techniques are used for evaluation of effects on tubuloglomerular feedback (TGF). Surface visible juxtamedullary nephrons in which the proximal segment and loop of Henle can both be identified and accessed are utilized. The proximal segment is accessed as described in "Evaluation of Tubular Function by Stop Flow Techniques," but a viscous oil is used to block the distal micropuncture site and the proximal site is used to record the stop flow pressure (SFP) just proximal to the blocked segment. The loop of Henle just distal to the stopped segment is accessed and perfused with Ringer's solution at varying rates of flow, usually over a range of 10–40 nl/min. The resulting decrease in SFP in the upstream segment of the nephron as a function of increased flow rate is plotted and the slope of the resulting line used to define the magnitude of the TGF response. Test compounds are added to the perfusate to determine their effect on the TGF response by evaluating the change in the slope of the SPF-perfusion rate line. The baseline (unperfused) SFP is be determined in the perfused nephron before initiation of perfusion; alternatively, the proximal segments of adjacent nephrons may be blocked in a similar fashion but left unperfused to serve as controls (Schnermann et al. 1973a; Wright and Schnermann 1974; Carlström et al. 2010).

As an alternative to measurement of SFP in the perfused nephron, constriction of its afferent arteriole may be visualized using a high contrast video system. The diameter of the afferent arteriole is then determined from the images and the linear relationship

between this parameter and the distal tubular perfusion rate is used as an index to the magnitude of the TGF response (Casellas and Moore 1990).

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5.1.1.3 Assessment of Glomerular Function

Urinary Protein Markers of Glomerular Function

PURPOSE AND RATIONALE

The filtration of proteins across the glomerulus is limited primarily by their molecular radius, which is a function of molecular weight. The size limitation is a function of both the pore sizes of the glomerular capillaries as well as the maintenance of a net negative charge by the glomerular basement membrane (GBM). Low level structural or functional changes in podocytes (e.g., alteration in the cytoskeleton or the number or nature of cell–cell interactions), loss of the net negative charge on the GBM, or minor alteration in the contractile states of the mesangial cells serve to increase the effective diameters of primarily the small membrane pores, while more pronounced or prolonged structural damage to the glomerulus (especially the podocytes) increases the relative numbers of large pores present on the glomerular capillaries. The net result overall is increased excretion of protein into the urine. Slight increases in effective small pore diameter resulting from the former conditions will result in the appearance of smaller molecular weight proteins (i.e., those with molecular radii very close to the normal effective pore diameter, such as albumin, and to a lesser extent, IgG) in the urine, as the normal tubular uptake pathways for small molecular weight proteins will become rapidly saturated due to the increased tubular filtered load. In contrast, significant structural or functional changes in podocytes or glomerular mesangial cells will result in a relative increase in the numbers of larger pores, resulting in the appearance of larger molecular weight proteins (Tencer et al. 1998a, 1998b; Mundel and Reiser 2010; Tossidou et al. 2010).

PROCEDURE

Time-matched urine and blood samples are collected and determination of the concentration of the analyte(s) of interest are performed as described previously (see "Assessment of GFR by Plasma Chemistry", "Urinalysis" and "Assessment of Tubular Transport Processes").

Because increased albumin excretion is almost always the consequence of glomerular injury, it is generally considered the most sensitive index to low level glomerular injury. Sensitive detection of low level increases in urine albumin is best accomplished by the use of the antibody-based methods (turbidimetric or nephelometric) that are of higher sensitivity than colorimetric methods; the human-based reagents have been validated for cats and dogs and have been used in other species. Highly sensitive semiquantitative urine dipsticks have been developed that have been shown to be adequately accurate to detect microalbuminuria in humans, but these are not adequately sensitive to detect

microalbuminuria in animals (Sawicki et al. 1989; Pugia et al. 1997; Pressler et al. 2002; Gentilini et al. 2005; Murgier et al. 2009).

Albuminuria, accompanied by the excretion of high molecular weight proteins into the urine, is diagnostic for severe alterations in glomerular structure and/or function. Increased urinary excretion of one or more of a number of high molecular weight proteins has been used as an index to glomerular injury. IgG is most commonly used for this determination, but greater sensitivity and better correlation with the clinical and histologic diagnosis was obtained using extremely large proteins (either IgM or α_2-macroglobulin) (Tencer et al. 1998b, 2000). These are detected using antibody-based methods (described in "Evaluation of Receptor-Mediated Endocytosis").

EVALUATION

While increased urinary albumin concentrations alone are indicative of glomerular injury, the sensitivity of detection of clinically significant microalbuminuria is improved by the calculation of the urinary albumin: creatinine index (ACI), as follows:

$$ACI = U_{Alb}/U_{Creat}$$

where U_{Alb} = urinary albumin concentration, and U_{Creat} = urinary creatinine concentration (Tencer et al. 1998b; Kuwahar et al. 2008).

Greater sensitivity in the detection of alterations in glomerular function, as well as an indication of the nature of the underlying injury can result from evaluation of point-in-time serum and urinary concentrations of a selected large molecular weight protein and albumin and calculation of a selectivity index (SI), as follows:

$$SI = \frac{U_{HMW_{protein}}}{S_{HMW_{protein}}} \times \frac{S_{Alb}}{U_{Alb}}$$

where $U_{HMW\ protein}$ = urinary high molecular weight protein concentration, U_{Alb} = urinary albumin concentration, $S_{HMW\ protein}$ = time-matched serum high molecular weight protein concentration, S_{Alb} = serum albumin concentration (Tencer et al. 1998b, 2000).

MODIFICATION OF THE METHOD

Exogenous administration of endogenous proteins of variable molecular weights and effective radii is theoretically the most relevant means of determining alterations in glomerular function. Such methods have been successfully performed in rats using radiolabeled myoglobin, κ-dimer, neutral horseradish peroxidase, neutral human serum albumin, and native albumin (Lund et al. 2003). If this approach is used, proximal tubular protein transport and degradation of the labeled probes must be controlled or inhibited to prevent the introduction of errors to the resulting SI determination.

LIMITATIONS OF THE METHOD

As indicated in 1.E.1.1.12.3.2., many of the antibody-based assay methods used for the assessment of high molecular weight proteins are specific for humans and do not cross-react with those from commonly used test species.

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Assessment of Glomerular Function with Probe Substrates

PURPOSE AND RATIONALE

To overcome the inaccuracies imposed by proximal tubular protein uptake and degradation on accurate assessment of glomerular filtration, probe substrates of defined effective radius, and/or net charge have been synthesized. These allow more precise determination of the relative contributions of pore size and basement membrane charge to alterations in glomerular filtration in any given state of injury. Furthermore, these tracers are designed to be essentially spherical under physiologic conditions, eliminating inaccuracy resulting from heterogeneity in three-dimensional conformation of protein substrates.

PROCEDURE

Commonly used tracer substrates include variably sized dextrans (linear polymers of glucopyranose) and Ficoll, a cross-linked copolymer of sucrose and epichlorohydrin. These are available in a wide range of molecular weights and effective radii, and can be sulfated to render them anionic without altering the effective radius.

The probe substrates are administered by IV bolus, followed by continuous infusion to anesthetized animals. Inulin or another tracer substance may be coadministered with the tracer for concurrent determination of GFR.Timed urine and plasma samples are collected for the determination of the urinary clearance of both the probe substrate and the GFR tracer (as described in "Assessment of GFR and RBF by Clearance Methods") (Andersen et al. 2000; Asgeirsson et al. 2006)

Probe substrates (both for filtration or GFR) may be either radiolabeled or conjugated with fluorochromes. The appropriate liquid scintillation or fluorometric detection methods for each probe should be used to determine the concentration in plasma and urine.

EVALUATION

If two probe substrates are used, the fractional excretion (FE or θ) of the probe substrate relative to the GFR probe substrate (e.g., inulin) is calculated as described in "Fractional Excretion Methods":

$$\theta = \frac{U_X \times P_{in}}{P_X \times U_{in}}$$

where U_x and P_x are the concentrations of the filtration probe in urine and plasma, respectively, and U_{in} and P_{in} are the concentrations of inulin (or alternate GFR probe substrate) in urine and plasma, respectively.

If only the glomerular filtration probe substrate has been administered, a sieving coefficient (θ) is calculated as follows:

$$\theta = U_X/P_X$$

where U_x and P_x are the concentrations of the filtration probe in urine and plasma, respectively.

MODIFICATION OF THE METHOD

Micropuncture techniques may be used, in which the filtrate within Bowman's capsule is sampled to determine the concentration of the probe substrate. In this case, the sieving coefficient θ is determined as an index to the degree of filtration using the concentration of the probe substrate in the arterial plasma and the filtrate (Blouch et al. 1997; Venturoli and Rippe 2005).

LIMITATIONS OF THE METHOD

All polysaccharide probe substrates tested to date tend to have higher sieving coefficients than globular proteins of equivalent molecular weight or effective radii, and thus all will overestimate glomerular permeability and be somewhat insensitive to low level glomerular functional alteration. The results obtained with Ficoll are in general better correlated with results obtained using endogenous high and low molecular weight proteins to assess changes in glomerular function, due to the fact that Ficoll is more extensively cross-liked, rendering it more rigid and spherical than dextran under shear stresses associated with fluid flow. However, Ficoll has been shown to be more deformable (and hence more readily filtered than a globular protein of comparable effective radius) under conditions of increased ionic strength; additionally, there is a significant component of diffusion of Ficoll across the glomerular basement

membrane that contributes to overall filtration under conditions of low GFR. The use of these probes to assess alterations in glomerular permeability must therefore account for these limitations and the physiologic conditions under which they are administered in vivo must be strictly controlled (Bolton et al. 1998; Andersen et al. 2000; Venturoli and Rippe 2005; Rippe et al. 2006; Asgeirsson et al. 2007).

The use of two probe substrates concurrently will require the use of two different labels. Alternatively, chromatographic techniques to separate the two substrates within a plasma or urine sample will be needed.

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5.1.1.4 Assessment of Renal Concentrating Ability

Solute-Free Water Excretion and Reabsorption

PURPOSE AND RATIONALE

Investigations of the clearance of solute-free water represent indirect methods for the evaluation of several aspects of renal function and provide information on the site and mechanism of action of agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convoluted tubule, would augment the clearance of electrolyte-free water (EC_{H2O}) during water diuresis and the reabsorption of electrolyte-free water (ETC_{H2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both EC_{H2O} and ETC_{H2O}. On the other hand, drugs that act only in the distal tubule would reduce EC_{H2O} but not ETC_{H2O}.

PROCEDURE

These tests may be performed in any species from which urine and plasma can be readily collected, although low-level changes in concentrating ability may be more readily manifest in rats than in dogs (Sharratt and Frazer 1963; Osbourne et al. 1983). The general procedure involves initially placing the animals in a metabolism cage with unlimited access to water. After collection of a urine sample (16–24 h is best), the water is withdrawn and the urine is collected for the next 12–16 h. The urine specific gravity (or preferentially, urine osmolality) is measured and compared both with the value from the hydrated animal and the mean values from the water-deprived control group (Ragan and Weller 1999).

Assessment of renal urine diluting ability is more cumbersome but can be accomplished in rats by administration of an oral dose of water by gavage representing 5% of the animal's body weight. The animals are then placed in metabolic cages and the urine is collected every 30 min for the next 2 h (Sharratt and Frazer 1963). In dogs, water diuresis can be induced by oral administration of 50 ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5% glucose solution and 0.58% NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter (Suki et al. 1965). The volume and specific gravity (or osmolality) of the collected urine is measured and the results expressed as the percent of the administered dose of water excreted during the time period. Time-matched plasma samples are collected for the determination of plasma osmolality.

EVALUATION

In addition to gross assessment of renal dilution or concentrating ability (as described above), quantitative evaluation of either osmolar clearance, freewater clearance (C_{H2O}), or solute-free water clearance (EC_{H2O}) may add additional sensitivity to the evaluation of these functions.

Osmolar clearance can be calculated as follows:

$$C_{osm} = (U_{osm} \times V)/P_{osm}$$

where C_{osm} = osmolar clearance, U_{osm} = urine osmolality (in mosm/kg water), V = urine flow (measured in ml/min) and P_{osm} = plasma osmolality (measured in msom/kg water). C_{osm} is expressed in ml/min. C_{osm} less than V indicates excretion of a dilute urine (i.e., excess water is being excreted), but C_{osm} greater than V indicates excretion of concentrated urine (i.e., excretion of excess solute).

Freewater clearance (C_{H2O}) provides an estimate of the amount of urine being excreted in excess that needed to clear solutes, and is calculated traditionally as follows:

$C_{H_2O} = V \times [(1 - U_{osm})/P_{osm}]$ (Wesson and Anslow 1952)

More accurate assessment of the ability of the kidney to appropriately regulate plasma tonicity and/or respond appropriately to antidiuretic hormone (ADH) can be made by calculation of electrolyte-free water clearance EC_{H_2O}. This formula takes into account only water needed to excrete excess "effective" osmolytes (in general, monovalent electrolytes and their associated anions) and is calculated as follows:

$EC_{H_2O} = V \times \{1 - ([Na^+ + K^+]_{urine}/[Na^+ + K^+]_{plasma})\}$, (Shoker 1994)

LIMITATIONS OF THE METHOD

In general, the sensitivity of either urine concentration or dilution tests to detect the effects of test compounds on renal function is quite low, regardless of species (Ragan and Weller 1999). Furthermore, these tests (especially the urine dilution test) may be significantly altered by extrarenal effects (e.g., vomiting, diarrhea, delayed GI absorption, altered adrenal cortical function). Calculation of osmolar, free water, and electrolyte-free water clearances require accurate assessment of urine flow rates, which will require accurate and complete collection of all urine produced during the time interval, not always easily accomplished in small animal species. Catheterized models should be utilized in this circumstance. In addition, calculated electrolyte-free water clearances may be influenced by the presence of excess effective osmolytes in either urine or plasma (e.g., in circumstances of hyperglycemia or metabolic acidosis/alkalosis) and the formula must be corrected to account for these if they are known to be present (Shoker 1994; Nguyen and Kurtz 2005).

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Assessment of Medullary Osmolarity and Blood Flow

PURPOSE AND RATIONALE

The ability of the kidney to concentrate urine depends entirely on the maintenance of the renal medullary interstitium in a hypertonic state. Hypertonicity reflects two phenomena; water-uncoupled transport of osmotically active substances (sodium and urea, primarily) into the medullary interstitium, and the trapping of those solutes in the interstitium by the vasa recta countercurrent multiplier system. The hypertonic gradient is created by the active transport of sodium into the interstitium in the ascending

loop of Henle, and a combination of passive and facilitated transport of urea located in the collecting duct; the magnitude and effectiveness of the gradient is determined by the rate of plasma flow through the vasa recta (Pallone et al. 2003; Sadowski and Dobrowolski 2003). The method described allows simultaneous assessment of both parameters.

PROCEDURE

Male Wistar rats have been used in these experiments. Under appropriate anesthesia, the femoral artery and vein are cannulated for measurement of aortic blood pressure and infusion of fluids and test compounds, respectively. The left kidney is exposed and placed in a plastic cup as for micropuncture experiments (see "Evaluation of Tubular Function by Micropuncture"), except that the dorsal surface is placed at the top of the cup, facing the operator. The ureter is catheterized and a platinum-iridium admittance electrode is inserted along the corticopapillary axis from the dorsal surface of the kidney (the probe is sized to ensure correct placement of the recording surfaces within the inner renal medulla). The capsule is incised and a needle laser-Doppler (LD) probe is inserted adjacent to the admittance probe. Animals are maintained in intravenous infusions of balanced electrolyte solution to maintain plasma volume and ensure adequate renal perfusion.

Medullary blood flow (MBF) is assessed by the LD probe, which is connected to a perfusion monitor (Periflux 4001, Perimed). The number and velocity of erythrocytes moving between the two optical fibers of the LD probe is determined. Admittance (the reciprocal of impedence) is measured between the tip of the LD probe and the admittance electrode by a conductance meter connected to both of these, following the application of a measuring current at a frequency of 24 Hz. Following the experiment, the kidney is dissected to verify that the LD and electrode have been correctly placed.

EVALUATION

Medullary blood flow (MBF) is expressed in arbitrary perfusion units (PU), based on the voltage generated by the Doppler flux of the blood cells moving beneath the LD probe (roughly, the product of cell number X velocity, see Sect. 1.1.3). By definition, a 10 V signal from the detector is considered 1,000 PU. Admittance (Y) is expressed in millisiemens (mS), and is directly related to the ionic tonicity of the tissue (primarily due to the NaCl content). Effects in treated animals are expressed relative to those in untreated controls. If desired, urine may be collected from the ureteral cannula for excretion determinations (see Sect. 1.2.1.2).

LIMITATIONS OF THE METHOD

Because MBF is expressed in arbitrary units, only relative changes within a single animal can be detected, thus each animal must serve as its own control (although results between animals in the same study can be compared with the use of a calibration algorithm). Significant tissue damage, resulting in nonfunctional nephrons, does result from the placement of the equipment and thus the technique can only be applied to the innermost medulla (where the glomeruli originate below the depth of the damaged zone). The admittance recordings reflect only the concentration of NaCl in the medulla and thus changes in tonicity resulting strictly from changes in urea concentration cannot be detected by this method.

MODIFICATION OF THE METHOD

Regional changes in medullary tonicity and the steepness of the osmotic gradient can be detected using a system of three needle electrodes that allows assessment of admittance in the outer and inner medulla separately. This arrangement cannot be coupled with the LD probe because excessive tissue damage would result (Sadowski and Portalska 1983).

An admittance electrode surrounded by a steel cannula fitted to syringe pumps may be used in place of the needle electrode. This setup allows for direct infusion of test substances into the medulla so that local effects may be monitored (Dobrowolski and Sadowski 2004).

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Diuretic Activity in Rats (Lipschitz Test)

PURPOSE AND RATIONALE

A method for testing diuretic activity of compounds in rats was described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animal compared to rats treated with a high dose of urea. The "Lipschitz-value" is the quotient between excretion by test animals and excretion by the urea control.

This method is primarily of historic interest and generally has been supplanted by more sensitive, specific, and/or mechanism-based assessments of diuretic activity. However, it is still being used in some circumstances to determine if an unknown substance has significant diuretic activity (primarily in the context of evaluating the pharmacologic activities of herbal remedies).

PROCEDURE

Male Wistar rats are most commonly used for these studies, although mice have also been used. Animals are housed (singly or in groups) in metabolic cages (see section "Urinalysis") and food and water are withheld for at least 15 h prior to the experiment. The test compound is administered by gavage in 0.9% NaCl solution to one or more groups of animals (to enable evaluation of dose responses or to compare different compounds), while the control group is given 1 g/kg urea in a similar dose volume. A separate control group is given only 0.9% NaCl solution by gavage. The total volume of fluid administered should be 25 ml/kg.

The total urine excreted is collected for 5 and/or 24 h post dosing for determination of urine volume and electrolytes. Standard methods are used to determine electrolyte concentrations (Na^+, K^+, and Cl^-) and quantitative electrolyte secretion is determined for each group (see "Quantitative Electrolyte Excretion").

EVALUATION

Urine volume (normalized to body weight and excretion time) is calculated for each group. Results are expressed as the "Lipschitz-value," that is, the ratio T/U, in which T is the response to the test compound, and U, that of urea treatment. Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found. Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect.

"Lipschitz-values" may also be calculated for electrolyte secretion. However, direct statistical comparison of these parameters (as well as urine volume) is more commonly done (Pallavi et al. 2011).

LIMITATIONS OF THE METHOD

The effects of weaker diuretic compounds may be masked by the degree of diuresis imposed by the water load in this test. Species differences in the predictive value of this test have been noted for some classes of diuretics (Heller and Ginsberg 1961).

MODIFICATIONS OF THE METHOD

Furosemide at a dose of 20 mg/kg may also be used as a reference diuretic. In this case, electrolyte excretion is also compared (as is the urinary Na^+/K^+ ratio) to determine relative natriuretic and kaliuretic potential of the test compound (Sangma et al. 2010; Suresh et al. 2010; Kondawar et al. 2011).

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5.1.2 In Vitro Methods

5.1.2.1 Isolated Perfused Tubules

PURPOSE AND RATIONALE

The various tubule segments have different functional properties. The in vitro perfusion of isolated tubule segments (Burg et al. 1966a) is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies.

PROCEDURE

After its invention by Burg et al. (1966) this technique has been used successfully in the kidney tubule segments of several species: man, rabbit, rat, mouse, hamster, snake, birds, etc. In larger species, the tubule segments can be dissected from thin kidney slices (<1 mm thickness). Usually, dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase). The segment is identified by its anatomical location and by its appearance. A 20–50X lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study. Most segments are easily identified and dissected, but the thin loops of Henle and papillary collecting ducts are damaged easily by the mechanical dissection. Dissection is usually performed at 4°C in a Ringer type solution.

For smaller species, proximal tubule segments can also be isolated from the entire kidney using in situ collagenase perfusion techniques (Tyson et al. 1990). Following anesthesia, the kidneys may be perfused via the aorta (mice) or renal arteries (rats, rabbits) for 5 min to remove the residual blood; the kidneys and associated blood vessels are then removed and the perfusion continued for an additional 15–20 min with buffer containing 180 U/ml of Type I collagenase. The cortical tissue is then removed from the medulla and the tubules isolated as described above. The viability and function of tubules so isolated are comparable to those isolated without collagenase (Rodeheaver et al. 1990).

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400X). The chamber is usually kept at 37°C, and the bath perfusate is also preheated to this temperature. The bath perfusate (usually based on Dulbecco's modified Eagle's medium) will be modified depending on the tubule segment and process of interest. The actual perfusion is performed with two sets of concentric glass pipettes, one set at the perfusion end and one at the collection end of the segment. These pipettes are manufactured with special glass forge (Greger and Hampel 1981). The outer segment of the pipette applies suction to the isolated tubule and serves as a "holding pipette"; the inner segment acts as the "perfusion pipette." The latter has a tip diameter smaller than the inner diameter of the perfused segment and is placed in the proximal end of the segment of interest and the perfusion buffer is infused at a rate of 1–20 nl/min. The collection end of the tubule segment is sucked into a holding pipette. A sylgard pipette is advanced to seal the collection site. The holding pipette at the collection site will contain mineral oil in flux measurements to prevent loss of perfused fluid. Then a collection pipette is advanced through the oil to quantitatively collect the perfusate delivered by the tubule. The length of the perfused segment (between the perfusion and collection pipettes) should be measured.

EVALUATION

A number of different assessments can be made by this method :

Flux measurements. (Schafer et al. 1974; Edwards et al. 1999). The collection rate (*V*c,nl/min) can be measured by the constant bore collection pipette by timed collections. Radioactive or other tracers can be added to either the lumen or bath fluid. Unidirectional fluxes, bath to lumen and lumen to bath, for any given substance can be quantified:

$$J_X = (V_C C_X)/L$$

where V_C = fluid collection rate (volume/time), C_X = concentration of X (in bath or collection fluid), and L = length of the perfused tubule segment.

Permeability (*P*x) can be determined by adding a non-permeant substance (usually inulin) to the perfusion buffer, as follows:

$$P_X = (Vi - Vc)L - 1[\ln(Xp\,Inc\,Xc - 1\,Inp) + 1]$$

where *L* is the length of the segment, Xp and Xc are the concentrations of *x* in the perfusate and in the

collected fluid, and *Inc* is the inulin concentration in the collected fluid.

Net fluxes of a substrate can be determined as the difference of the unidirectional fluxes or by the chemical determination of D_X (perfusate–collected fluid). This requires very sensitive methods. Electron probe analysis of Na+, K+, Ca^{2+}, Mg^{2+}, etc., has been used to determine the net transport of these ions in various tubule segments (Wittner et al. 1988). Flux studies are usually performed at low luminal perfusion rates of a few nl/min. Substances under study can be added to either the luminal and bath perfusate, and paired data can be obtained under control and experimental conditions (Burg and Green 1973; Stoner et al. 1974; Burg and Orlov, 1980; Burg and Stoner 1976; Dillingham et al. 1993).

Transepithelial electrical measurements. The collection pipette can be connected to the high impedance input of an electrometer. The voltage is referenced to the grounded bath. With identical solutions in the bath and in the perfusate and with high luminal perfusion rates (>10 nl/min), any transepithelial voltage recorded by the collection pipette (*V*te) must result from active transport of ionic substances across the tubular epithelium, with the magnitude of this voltage proportional to the degree of transport (Frömter 1984). Hence, the effectiveness of putative inhibitors of active transport can also be examined by the measurement of *V*te. According to Ohm's law, the determination of the flux of ions also requires the measurement of transepithelial resistance. Greger (1981) has introduced a method that utilizes a dual channel perfusion pipette. One channel is used for perfusion and the other for current (*I*te) injection. The current is defined by a resistor chosen such that the deflection in *V*te generated by this pulse is in the order of 10–20 mV. Transepithelial resistance (*R*te) can now be calculated from Δ*V*te and *I*te. The ratio of *V*te and *R*te is called equivalent short circuit current. It is directly proportional to active transport (Greger 1985). The measurement of *V*te and *R*te is much more efficient than flux studies for pharmacological screening, provided that the process under study produces a transepithelial voltage. Several substances can be examined in one single tubule in strictly paired fashion (Schlatter et al. 1983; Wangemann et al. 1986). The time resolution of the measurements is on the order of 1 s, whereas that of flux studies is several minutes at best.

Intracellular electrical measurements. Greger and Hampel (1981) have developed a method for the use of impalement techniques in the isolated perfused tubule. Very fine tip microelectrodes (Ø < 100 nm) are used to impale the tubule cell across the basolateral membrane. The simultaneous measurement of *V*te, *R*te, and basolateral membrane voltage (*V*bl) allows for a complete analysis of voltages and resistances (Greger 1985; Ullrich and Greger 1985). Ion selective microelectrodes can also be used in impalement studies, and the cytosolic ion activities for, for example, Na^+, K^+, Cl^- can also be determined (Greger 1985). These methods are all rather difficult to perform. They are of high relevance for the understanding of the function of a given tubule segment and for the detailed description of the mechanism of action of a drug, which in preceding studies has been shown to act in a given tubule segment.

MODIFICATIONS OF THE METHOD

Fluorescent dyes for the monitoring of Na^+, K^+, Cl^-, Ca^{2+}, and pH are more commonly used currently. These dyes can be used in the in vitro perfused tubule (Nitschke et al. 1991). The inverted microscope is equipped with an appropriate illumination and filter wheel for excitation. The emission is measured by photon counting or by a video camera.

The perfusion and collection pipettes may be used as electrodes to determine V_{te} as outlined above. The isolated tubule is solute-clamped at a high perfusion rate, and the potential difference between the perfusion and recording pipettes represents the net active transport of substrates across the tubular epithelium (Frömter and Geßner 2001).

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5.1.2.2 Isolated Perfused Kidney

PURPOSE AND RATIONALE

The isolated perfused kidney is a valuable tool for assessing effects on the function on the entire kidney (vasculature and tubules), with several caveats (see below under "Limitations of the Method"). This model can be used either in situ and/or isolated in vitro.

PROCEDURE

Kidneys from rats, dogs, rabbits, and pigs have been used. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a ventricular incision, the renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution (usually Krebs-Ringer or Krebs-Henseleit buffer that is oxygenated) circulated by a peristaltic pump. The venous cannula is introduced into the vena cava below the renal vein, and the ureter is cannulated last. The kidney is freed from the perirenal fat, not disrupting the renal capsule. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. The perfusion pressure is maintained at a level appropriate for the species in question by adjusting the speed of the perfusion pump. All procedures to be performed on the kidney should be completed within 2–4 h of isolation.

EVALUATION

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, inulin or another suitable non-reabsorbed substrate is introduced into the arterial cannula and clearance is assessed (see Sect. 1.1.2). Electrolytes are determined in urine by standard flame photometry. Fractional excretions of water, electrolytes, and test compounds are calculated (see Sect. 1.2.1.2).

LIMITATIONS OF THE METHOD

Isolated perfused dog kidneys have been reported to be less stable than those of other species, with glomerular filtration and renal flow markedly decreasing after only 1 h of perfusion. The in situ-perfused isolated dog

kidney seems to be more stable. Distal tubule functions are impaired in isolated perfused kidneys in all species. Urine acidification, concentration, and dilution functions are also abnormal, and effects on these cannot be assessed in these models (Kirkpatrick and Gandolfi 2005).

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5.1.2.3 Patch Clamp Technique in Kidney Cells

PURPOSE AND RATIONALE

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts) fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). Besides active transport and coupled transport systems, ion channels play an important role in the function of kidney cells. The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode) (Neher and Sakmann 1976; Hamill et al. 1981) allow the investigation of ion channels. In addition, the investigation of other electrogenic transport mechanisms, such as the sodium-coupled alanine transport can be studied.

PROCEDURE

The patch clamp technique can be applied to cultured kidney cells (Merot et al. 1988), to freshly isolated kidney cells (Hoyer and Gögelein 1991) or to cells of isolated perfused kidney tubules (Gögelein and Greger 1984). The latter method shall be described in more detail.

Segments of distal superficial proximal tubules of rabbit kidney are dissected and perfused from one end (see Sect. 1.2.1) (Burg et al. 1966b; Greger and Hampel 1981). The non-cannulated end of the tubule is freely accessible to a patch pipette. Under optical control (differential interference contrast optics with 400´magnification) the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously, and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell-excised mode (Gögelein and Greger 1984, 1986a).

In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 μm). In order to facilitate the tearing off, the tubules are incubated for about 5 min in 0.5 g/l collagenase (Sigma C 2139) at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and gigaseals can be obtained. It was possible to investigate potassium channels (Gögelein and Greger 1987) and nonselective cation channels (Gögelein and Greger 1986b) in these membranes.

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. On the other hand, cotransport systems can only be investigated by the whole-cell method because the transport rate of a single event is much too small to be resolved in a similar manner as single ion channel events. Consequently, cells of rabbit proximal tubules are isolated as described in detail elsewhere (Hoyer and Gögelein 1991; Heidrich and Dew 1977). After euthanasia, the kidneys are rapidly excised and placed in ice-cold

solution (mmol/l): 150 K-cyclamate, 10HEPES, 1CaCl2, 1MgCl2, pH7.4. The following steps are performed on ice: After decapsulation, superficial cortical slices of about 0.5 mm thickness are dissected and minced with a scalpel. The tissue is homogenized in a Dounce homogenizer by three strokes with a loose-fitting pestle. The homogenate is then poured through graded sieves (250, 75, and 40 μm) to obtain a population of single cells. Since the predominant tubule section of the cortex of the rabbit kidney is the pars convoluta of the proximal tubule, it can be concluded that the majority of the isolated cells in the cell suspension are of proximal tubule origin. By light microscopy, cells are identified by long microvilli distributed over the entire cell surface and can easily be discriminated from remaining erythrocytes, cell detritus, and tubular fragments.

By application of the whole-cell mode of the patch clamp technique to freshly isolated cells of convoluted proximal tubules, the sodium-alanine cotransport system could be investigated in detail (Hoyer and Gögelein 1991).

EVALUATION

In isolated perfused renal tubules, concentration response curves of drugs that inhibit ion channels can be obtained with the patch clamp technique. In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent K_m values for sodium and L-alanine can be recorded.

MODIFICATIONS OF THE METHOD

Schlatter (1993) recorded membrane voltages of macula densa cells with the fast or slow whole-cell patch clamp method. The effects of diuretics and the conductance properties of these cells were examined.

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5.1.2.4 Isolated Brush Border Membrane Vesicles

PURPOSE AND RATIONALE

Alterations in electrolyte excretion or tubular transport processes assessed in vivo (Sections "Electrolyte Excretion" and "Assessment of Tubular Transport Processes") are limited by the fact that multiple transporters may be involved in the net transport of the probe substrate or electrolyte in question. The use of isolated brush border membranes allows investigation of the mechanisms underlying the alterations by evaluating changes in the abundance or activity of specific transporters in response to specific disease states or following exposure to toxicants and drugs. Additionally, the transport kinetics for specific substrate-transporter combinations can be determined, and the ability of drugs or toxicants to act as substrates for or inhibitors of specific transporters can be evaluated in these preparations.

PROCEDURE

Brush border vesicles can be prepared from any species, but are usually prepared from male Sprague

Dawley rats using a Mg^{++} precipitation method. Animals are euthanized by exsanguination under anesthesia and the kidneys are removed immediately. All steps for the preparation of brush border membrane vesicles are carried out at 4°C. Renal cortex is homogenized for 2 min in a medium containing 250 mM mannitol in 10 mM tris-HEPES buffer (pH 7.5) using an appropriate homogenizer (Polytron or teflon-glass). MgCl or $MgSO_4$ is added to the homogenate (to generate a final concentration of 10 mM) and the mixture is stirred on ice for 20 min. The homogenate is then centrifuged for 10 min at 2,000×*g*, and the resulting supernatant was centrifuged for 20 min at 35,000×*g*. Subsequently, the supernatant is discarded and the loosely packed membrane-rich layer is gently separated from the pellet and resuspended manually in a buffer that will generate the appropriate intravesicular conditions for the experiment in question (e.g., for urate transport the most commonly used buffer is 150 mM mannitol and 2 mM $MgSO_4$ in 50 mM potassium phosphate buffer, pH 7.5). The final supernatant is centrifuged at 35,000×*g* for 15 min and the procedure is repeated twice more. The final suspension is prepared to a protein concentration of 10–20 mg/ml, which may be either kept at 4°C for immediate use or frozen and stored at −80°C until use.

The degree of purity of the brush border membrane preparation is assessed by measuring the enrichment of a marker enzyme. Either alkaline phosphatase or γ-glutamyl transpeptidase may be used for this purpose. Tenfold or greater enrichment of the activity in the preparation relative to the baseline homogenate is considered acceptable.

Rapid filtration methods are used to determine the activity of the transporter in question. After preincubation of the brush border membrane vesicle preparation for 1–2 h at 37°C in the appropriate buffer to the experimental conditions and transporter of interest (usually identical to the suspension buffer used in the final brush border membrane vesicle preparation as described above), the uptake of [^{14}C]-labeled substrate or radioactive electrolyte isotope (e.g., [^{22}Na] is used to evaluate NHE3 transport) is initiated by adding the substrate in an approximately 10X excess volume of buffer to the membrane suspension. At a timed interval (usually 10 s) after the addition of the incubation medium, uptake is stopped by the addition of a large excess (200X) volume of ice-cold (4°C) incubation buffer. The incubation mixture is passed through a 0.45 μM Millipore filter, which is then washed 2X more with 3–5 ml of the ice-cold incubation buffer. Corrections are made for the radioactivity nonspecifically bound to the vesicles or the filters by generating an identical incubation preparation to the experimental condition but which is carried out at 4°C. The radioactivity present in this sample is subtracted from that in each of the active conditions to generate the substrate uptake in the preparation (expressed as susbstrate quantity/mg of protein).

EVALUATION

Membrane transport processes that can be described by Michaelis–Menten kinetic relationships can be characterized using isolated brush border membrane vesicles. Lineweaver–Burke double-reciprocal plots of transport velocity and substrate concentration allow determination of V_{max} and K_m values from the Y and X axis intercept values, respectively (Runge et al. 2006). IC_{50} values for transport inhibitors can be calculated using least squares linear regression techniques or curve fitting software available within a number of commercially available software programs (Excel add-in, Graphpad Prism, and SigmaPlot).

MODIFICATIONS OF THE METHOD

Isolated basolateral membrane vesicles can be used in a similar fashion to evaluate transport processes (see section "Assessment of Organic Anion and Cation Transport" for a list of substrates and inhibitors). Percoll gradient density centrifugation methods are used to generate these preparations. Renal cortices are homogenized at 4°C in 12 mM Tris-HEPES buffer (pH 7.4) containing either 300 mM mannitol or 250 mM sucrose. The homogenate is diluted 1:1 with buffer and centrifuged for 15 min at 2,500×*g*. The resulting supernatant is centrifuged for 20 min at 20,000×*g*, and the upper fluffy layer of the pellet is suspended in buffer by ten strokes with the homogenizer. Percoll is then added (13% vol/vol), and 36 ml aliquots of the resulting suspension are centrifuged for 30 min at 48,000×*g*. The top 6 ml of each aliquot is discarded and the next 6 ml is removed and diluted tenfold with intravesicular buffer and washed three times at 48,000×*g* for 15 min. The final pellet is resuspended in intravesicular buffer and may be either used immediately or stored at −80°C until use. The degree of purity of the basolateral membrane preparation is assessed by measuring the enrichment of the

marker enzymes Na^+-K^+-ATPase or leucine aminopeptidase. Tenfold or greater enrichment of the activity in the preparation relative to the baseline homogenate is considered acceptable (Edwards et al. 1997; Shimada et al. 1987). Rapid filtration methods similar to those used for brush border membrane vesicle preparations are used to determine substrate uptake.

Western blot analysis may be used to determine the presence, identity, and purity of the transport proteins present in the membrane preparations, which may be useful in determining the exact uptake pathway(s) relevant to the substrate or inhibitor in question. Comparison of kinetic parameters determined in vesicle preparations where multiple transporters are present to those determined for pure transporters expressed in stably transfected renal cell lines may help to clarify the role of selected transporters in clearance of substrates or activity of inhibitors (Takahashi et al. 1998).

Replacement of Mg^{++} with propylene glycol for precipitation of brush border membrane vesicles results in the generation of preparations of equivalent purity to those generated by traditional methods, while preventing alteration of the surrounding membrane lipids which may alter transporter functions (Basivireddy and Balasubramanian 2003).

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5.2 Assessment of Renal Injury

5.2.1 Assessment of Renal Injury by Routine Urinalysis

PURPOSE AND RATIONALE

Urinalysis provides a unique opportunity to selectively and noninvasively "sample" a single target organ by examination of its end product. The levels of proteins and small molecules normally filtered, excluded from filtration, secreted, or reabsorbed by the tubules can be used as indicators of the functional status of certain nephron segments, while injury can be assessed by examination of the urinary sediment for cellular components indicative of tubular injury.

PROCEDURE

Procedures for collection of high quality urine samples and assessment of secreted analytes are outlined in section "Urinalysis." Five ml of the urine is then centrifuged at 700 g for 5 min and the sediment is examined microscopically for the presence of cells, casts, crystals, and miscellaneous components. Samples for which urine sediment evaluation is to be performed must not be frozen.

If medullary injury is supected, the urine may also be evaluated for the presence of phospholipid by thin layer chromatography or HPLC. As with other analytes, quantitative assessment of urinary creatinine and normalization of the phospholipid concentration is necessary.

EVALUATION

The presence of greater than 1–2 erythrocytes or neutrophils per high-power field confirms a positive result of dipstick analysis for blood or leukocyte esterase. Increased number of hyaline casts (small numbers are

normal in most species), or the presence of granules (cells or cellular debris) within casts may be indicative of tubular injury. The presence of renal tubular epithelial cells in urine sediment strongly indicates tubular injury (Stonard 1990; Hofmann et al. 1994; Finco 1997; Finn and Porter 1998; Newman and Price 1999).

Small numbers of lipid droplets (representing neutral lipid, lipoprotein, or phospholipid) are considered normal in dogs, cats, mice, and humans (Gross et al. 1991; Streather et al. 1993; Finco 1997). Increased excretion of lipid droplets (which may cause increased urine turbidity) may indicate glomerular injury, as does increased excretion of phospholipid and/or lipoprotein. Increased urinary phospholipid excretion has been described as antecedent finding to microscopic evidence of papillary injury in experimental renal papillary necrosis in rodents. Unlike the profile seen with glomerular injury (de Mendoza et al. 1976; Gherardi and Calandra 1982; Neverov and Nikitina 1992), concomitantly increased levels of lipoprotein have not been described following papillotoxins; conversely, increased urinary sphingomyelin is detected in renal papillary necrosis (Thanh et al. 2001) but elevated levels are not detected in the urine humans with nephrotic syndrome (Mimura et al. 1984). The level and nature of the excreted lipid can be determined by thin-layer chromatography and comparison to known standards (Mimura et al. 1984; Thanh et al. 2001).

LIMITATIONS OF THE METHOD

The dipstick reagent for blood detects hemoglobin but cannot distinguish between free hemoglobin (possibly due to intravenous hemolysis) or intact erythrocytes and also cross-reacts with myoglobin (present in urine as the consequence of muscle injury). Examination of the sediment is needed to determine if erythrocytes are present in urine. Addition of ammonium sulfate to the urine (which precipitates hemoglobin but not myoglobin) can help differentiate the two pigments (Graff 1983).

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5.2.2 Assessment of Renal Injury by Urine Proteins

PURPOSE AND RATIONALE

The combination of qualitative and quantitative assessment of the normally excreted urine protein can serve as a reliable indicator of the nephron segment(s) affected by the injury. Proteins may also appear in the urine as the consequence of their release from damaged nephrons. Many of these are unique to the kidney; those that are not are either too large to pass an intact glomerular filter or have not been shown to circulate in plasma. Furthermore, several of these are both upregulated and shed to the urine, thus increasing their sensitivity as injury biomarkers.

PROCEDURE

Twenty-four hour urine collection is preferred for best accuracy, although point-in-time samples can be used. Urine samples are collected from the species in question by appropriate methods (see "Urinalysis"). Total protein and creatinine are determined by appropriate methods and a urine protein:creatinine ratio is calculated ("Urinary Protein Markers of Glomerular Function").

Most of the proteins used as biomarkers of renal injury are measured by immunometric methods. Newer methods based on proteomics technology (concentration of proteins by acetone precipitation or ultracentrifugation, separation by 2-day gel electrophoresis, or chromatographic techniques with subsequent identification and quantitation by mass spectrometry) have been used experimentally (Bandara and Kennedy 2002; Chapman 2002; Thongboonkerd et al. 2002a, b).

EVALUATION

All analytes need to be converted to their SI unit equivalents prior to calculation of excretion ratios (see "Fractional Excretion Methods"), which will not be unitless in this circumstance. Protein:creatinine ratios of >1.0 are considered indicative of significant renal proteinuria in most species (Gregory 2003). Excretion of markedly elevated levels of protein (protein:creatinine ratio >5.0) is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/low-grade glomerular injury (Peterson et al. 1969; Finco 1997; Gregory 2003).

Increased albumin usually indicates glomerular injury, although it is not a specific marker for any one nephron site (Guder and Hofmann 1992; Price et al. 1996; Finn and Porter 1998). High levels of albumin in urine are invariably the result of glomerular malfunction. Low level increases in urine albumin, especially in circumstances where total urine protein excretion is not elevated ("microalbuminuria") can result either from increased glomerular filtration or decreased tubular reabsorption, and albumin values in this range must be interpreted in comparison to the excretion of other protein biomarkers. Concomitant elevation of albumin and one or more low molecular weight proteins indicates tubular malfunction, while albumin elevation alone or concurrently with a high molecular weight protein indicates primary glomerular injury (Peterson et al. 1969; Finn and Porter 1998; Guder et al. 1998; Umbreit and Wiedemann 2000). Calculation of the relative clearance of albumin and either immunoglobulin or transferrin is a useful indicator of the degree of glomerular injury (the higher the ratio, the more severe the damage) (Tencer et al. 1998b, 2000).

Elevation of any of the filtered low molecular weight proteins in urine is an indication of a primary defect in tubular uptake, either as a consequence of decreased nephron mass or competition for the endocytic pathway by a competing substrate. This is discussed in detail in "Evaluation of Receptor-Mediated Endocytosis."

Increases in the urinary levels of proteins that are either shed or secreted into the urine from the site of injury may be more sensitive than alterations in filtered proteins in detecting renal injury. A number of these are listed in Table 5.4. Most of these are early response or structural proteins and serve some protective or repair function in damaged tissue. Two of these (KIM-1 and clusterin) have recently been accepted by both the FDA and EMEA as fully validated biomarkers of tubular injury in preclinical animal studies.

LIMITATIONS OF THE METHOD

The majority of assay methods for excreted low molecular weight proteins are based in immunometric methods (gel immunodiffusion, nephelometry, or ELISA) and the antibodies do not cross-react with homologous proteins in animal urine (section "Evaluation of Receptor-Mediated Endocytosis").

Although there are published antibody-based methods for many of the kidney-specific proteins listed above, antibodies have only recently become commercially available for some of them (specifically, KIM-1, CYR-61, NGAL, and Pap A1). Where the antibodies are readily available, cross-reactivity across species has not always been established (these have in general been used only in human and/or rat). Heart- and kidney-specific isoforms of fatty acid–binding protein exist that may cross-react in some species with the LFBP antibodies; additionally, α-2 μ-globulin, a normal component of male rat urine, is homologous to K-FABP and may cross-react with the antibodies to LFBP.

Table 5.4 Endogenous renal proteins excreted in response to injury[a]

Protein	Nephron segment	Species	Expression	Comments
Fibronectin	Glomerulus (Proximal tubule?)	Human Rat	Constitutive and upregulated	Precedes microalbuminuria in diabetes Plasma contribution may be a factor with severe glomerular injury Decreased tubular proteolytic degradation may contribute to increased urinary levels With chronic glomerular injury, upregulation contributes to increased urine levels
Collagen IV	Glomerulus	Human Mouse Rat	Constitutive and upregulated	Basement membrane specific Precedes microalbuminuria in diabetes With chronic glomerular injury, upregulation contributes to increased urine levels
Liver fatty acid–binding protein (L-FABP)	"Glomerulus"	Human	Upregulated	Source is proximal tubule; upregulated and secreted in response to glomerular injury/proteinuria Small contribution (up to 3%) from serum-derived L-FABP with glomerular injury or concurrent liver injury Homologues (kidney and heart FABPs) exist in both human and rat kidney; these differ in distribution and regulation but may cross-react with antibodies to L-FABP. H-FABP in rat urine may reflect increased serum levels secondary to myocardial damage. α-2μ-globulin (normal component of male rat urine) is homologous to K-FABP and may cross-react with antibodies
α-Glutathione-S-transferase	Proximal tubule	Human Rat	Constitutive	Antibody appears to have good cross-species reactivity Highest urinary levels seen with selective straight (S_3) segment toxicants in rats Sensitive index of damage (brush border loss) in the absence of overt necrosis May be measured with either enzymatic or immunoassay
Clusterin	Proximal tubule	Rat Monkey	Upregulated	Elevated rapidly (1 h) in both acute and chronic injury; stays elevated as long as the stimulus is present Upregulation/secretion specifically indicates injury; not seen with nontoxic homologues that do not cause necrosis Some upregulation seen in medulla with oxidative stress Plasma contribution with glomerular injury a possibility Antibodies commercially available
Kidney Injury Molecule-1	Proximal tubule	Rat Mouse Human	Upregulated and Increased release	Biomarker for acute tubular necrosis/ischemia Transmembrane protein; ectodomain is shed to the urine following acute injury High degree of upregulation and/or increased shedding demonstrated in rat kidney with injury (improves sensitivity False negative results frequently seen Antibodies to rat and human exist, not commercially available
Neutrophil Gelatinase-Associated Lipocalin (NGAL)	Proximal Tubule	Human Mouse Rat	Upregulated and increased secretion	Highly upregulated (>100X) with renal injury Secreted in response to ischemia and direct nephrotoxic insult Either urine or plasma levels can be measured Equally predictive and prognostic with acute or chronic injury Protease resistant (better persistence in urine) Commercial reagents for standard analytical platforms available for human, rat and mouse
IL-18	Proximal tubule	Human Mouse	Upregulated and increased secretion	Early biomarker Upregulation in response to ischemic insult Not affected by nephrotoxicants ELISA assay not commercially available

(*continued*)

Table 5.4 (continued)

Protein	Nephron segment	Species	Expression	Comments
Cysteine-rich protein 61 (CYR61)	Proximal straight tubule	Mouse Rat	Upregulated and increased secretion	Rapid upregulation and secretion into urine (3 h post injury) with renal ischemia High heparin affinity allows concentration of the protein (using heparin-Sepharose) for detection of low levels BUT also complicates assay (necessary for accurate detection)
π- Glutathione-S-transferase	Distal tubule	Human	Constitutive	Questionable sensitivity (excretion in urine only seen after azotemia is apparent) Antibodies are very isoform-selective
μ-Glutathione-S-transferase	Distal tubule	Rat	Constitutive	Similar distribution to π-GST in humans Questionable sensitivity (excretion in urine only seen when azotemia is apparent)
Pap X 5 C10 antigen (Pap A1)	Papillary collecting ducts	Rat	Constitutive	Of several papilla-specific antigens identified, this one is most consistently released to urine after papillary toxicant administration Present in urine as complexes with Tamm-Horsfall mucoprotein Monoclonal antibody not commercially available Protein is rat specific and is of unknown identity and function

[a]Material from: Price et al. 1997; Lam et al. 1988; Kimura et al. 1989; Bomhard et al. 1990; Maatman et al. 1991; Aulitzky et al. 1992; Eti et al. 1993; Gwinner et al. 1993; Volders et al. 1993; Kananauchi et al., 1995; Nath et al. 1994; Sundberg et al. 1994a, 1994b, 1994c; Makino et al. 1995; Falkenberg et al. 1996; Usuda et al. 1998; Adhikary et al. 1999; Bruning et al. 1999; Hildebrand et al. 1999; Branten et al. 2000; Cohen et al. 2001; Hidaka et al. 2001; Huang et al. 2001; Kamijo et al. 2001; Manabe et al. 2001; Melnikov et al. 2001; Okonogi et al. 2001; Ghiggeri et al. 2002; Han et al. 2002; Ichimura et al. 2004; Muramatsu et al. 2002; Van Kreel et al. 2002a; Davis et al. 2003; Mishra et al. 2003; Mori et al. 2005; Devarajan and Williams 2007; Devarajan 2008

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5.2.3 Assessment of Renal Injury by Urine Enzymes

PURPOSE AND RATIONALE

Urinary enzyme activity provides a means to determine the presence and location of renal tubular injury as opposed to an index to the functional status of the nephron. With few exceptions (lysozyme and alanine aminopeptidase are the most notable of these), the enzymes most commonly used in these determinations are quite large (greater than 80 kDa) and thus their appearance in the urine results from leakage from damaged tubular cells, not from increased filtration or decreased uptake (Plummer et al. 1986).

Urine enzymes provide several advantages over urine protein for the assessment of tubular injury (Stonard et al. 1987; Vanderlinde 1981; Price 1982; Plummer et al. 1986; Clemo 1998; Dubach et al. 1988; Westhuyzen et al. 2003):

- Increased sensitivity – Enzyme levels in urine are frequently elevated in advance of overt evidence of renal malfunction and can be used to predict its onset
- Dose-response – The amount of enzyme activity in the urine accurately reflects the degree of tubular injury present
- Ease of analysis – Most use chromatographic assays, which have been well validated for the same enzymes present in plasma and can be performed on automated equipment
- Utility in a variety of species – Activity as opposed to antigen mass is measured, so cross-reactivity of antibodies or probes across species is not a consideration
- Repeatability – Enzyme measurements over time can be used to determine the reversibility or progression of renal lesions
- Ability to localize the injury to a specific nephron site/subcellular location – Serves as an index to the severity of the underlying injury. As a general rule, brush border enzymes indicate less severe damage than cytosolic, mitochondrial, or microsomal enzymes

For best results in determining the presence and site of nephron damage, a battery of enzymes as opposed to a single biomarker should be examined, especially since the most sensitive assay is highly variable, depending on the toxicant and species. Additionally, since many of the enzymes are not completely specific to a selected nephron segment, use of a battery of tests allows more precise localization of the site of injury (Price 1982).

The most commonly used enzymes and their segment specificity are outlined in Table 5.5:

PROCEDURE

Urine samples are collected and maintained as described in section "Urinalysis." There are some special considerations in sample collection and handling that are critical to accurate assessment of urine enzyme activity (Vanderlinde 1981; Price 1982; Plummer et al. 1986; Mueller et al. 1986, 1989; Loeb et al. 1997; Clemo 1998; Jung and Grutzmann 1988; Loeb 1998):

- Contamination of collected specimens must be carefully avoided, as enzymes present in feces, food, or bacteria can contribute significantly to the activity present in the urine
- Enzyme activity can also result from increased numbers of erythrocytes, leukocytes, or epithelial cells in the urine. Prompt centrifugation of urine to remove contaminating cells will help to mitigate this source of error, and examination of the resulting sediment will allow the investigator to discard the results from heavily contaminated samples
- Normal urine frequently contains a variety of low molecular weight substances (urea is a notable example) that can inhibit the enzymes of interest. The toxicant may also act as an inhibitor. These substances usually can be removed by dialysis, dilution, Sephadex filtration, ultrafiltration, or gel filtration

Table 5.5 Most commonly used enzymes and their segment specificity[a]

Enzyme	Nephron segment	Cellular location	Comments
N-acetyl-β-D-glucosaminidase (NAG)	Proximal tubule Papilla Glomerulus	Lysosomal	Widely used in humans (screen for nephrotoxicity) and all common toxicology species Excretion rate is consistent in a 24 h period, assessment in spot samples normalized to creatine accurately reflects 24 h excretion Increased activity usually indicates proximal tubular (PT) injury but is not specific (also seen with glomerular disease, obstructive nephropathy, and papillary injury). Concurrent elevation of a brush border enzyme increases specificity for PT injury Increased urinary activity seen with pregnancy (humans), age (rats), and gender (dogs – contribution from seminal fluid in males)
Lysozyme (muramidase)	Proximal tubule	Lysosomal	Contribution to urine levels from both plasma (small protein, readily filtered) and leakage from damaged tubules – thus not specific. Increased plasma levels from nonrenal diseases (leukemia, inflammation, or other neoplasia) may result in increased urine activity, especially with altered tubular uptake. Extremely variable secretion rate over a 24 h period limits usefulness with timed or spot urine samples Immunochemical as well as enzyme assays exist (poor cross-reactivity of antibodies across species)
β-galactosidase	Proximal tubule	Lysosomal	Elevated following some glomerular toxicants before proteinuria evident Pronounced diurnal variation in basal excretion rate in humans Increased urinary activity following testosterone treatment in mice
β-glucuronidase	Proximal tubule	Lysosomal	Contribution to urine activity from preputial gland in male rat Increased urinary activity following testosterone treatment in mice, pregnancy in humans Pronounced diurnal variation in basal excretion rate in humans
β-glucosidase	Proximal tubule	Lysosomal	Pronounced diurnal variation in basal excretion rate in humans
Alanine aminopeptidase (AAP)	Proximal tubule	Brush border Microsomes	Appears in urine earliest with acute tubular necrosis Renal and serum isoforms exist, but serum isoform is smaller (90 kDa versus 230 kDa), not normally filtered. May contribute to activity with concomitant glomerular disease Some increase in urine activity may result from nonrenal diseases Basal excretion rate higher in male rats and humans, but not in dogs; higher in rats with age
Leucine aminopeptidase	Proximal tubule	Brush border	Elevations also seen in early glomerular diseases, preceding microalbuminuria
Neutral brush border endopeptidase EC	Proximal tubule	Brush border	Assay substrate is also cleaved by leukocyte elastase; concomitant pyuria many falsely elevate activity
γ-glutamyl tranferase (GGT, γ-glutamyl transpeptidase)	Proximal tubule (particularly S_3 segment in rodents)	Brush border	Somewhat unstable in urine relative to other enzymes (even with added stabilizers), should be assayed quickly after collection Within a day variation of excretion rate noted in dogs, may limit accuracy of spot sample assessment even with creatinine correction Basal excretion rate higher in male rats Increased urinary activity seen with pregnancy
Alkaline phosphatase (intestinal var.)	Proximal tubule (S_3 segment "specific")	Brush border	Increased urine activity seen with pregnancy Use of this enzyme as a selective marker of S_3 involvement requires isoenzyme characterization (by ELISA) as well as determination of activity More resistant to shedding than other brush border enzymes, indicates more severe damage

(*continued*)

Table 5.5 (continued)

Enzyme	Nephron segment	Cellular location	Comments
α-Glutathione-S-transferase (GST) (ligandin)	Proximal tubule	Cytosol	Usually assessed by ELISA as quantity of protein but rapid assay for enzyme activity is also available (activity assay also detects μ and π isoforms)
Lactate dehydrogenase (LDH)	Entire nephron	Cytosol	Most commonly used as an indicator of distal tubule damage; not segment specific
Aspartate aminotransferase (AST)	Entire nephron	Mitochondria (Cytosol)	Enzyme levels are highest in proximal tubule (particularly convoluted segment) and thick ascending limb, tracking mitochondrial density

[a]Material from: Coonrod and Paterson 1969; Ceriotti 1976; Maruhn et al. 1977; Koenig et al. 1978; Vanderlinde 1981; Price 1982, 2002; Higashiyama et al. 1983; Nakamura et al. 1983; Davey et al. 1984; Guder and Ross 1984; Hysing and Tolleshaug 1986; Grötsch et al. 1985; Mueller et al. 1986; Gossett et al. 1987; Nishimura 1987; Stonard et al. 1987; Jung and Grutzmann 1988; Verpooten et al. 1989; Houser and Milner 1991; Taylor et al. 1992; Casadevall et al. 1995; Bedir et al. 1996; Price et al. 1996, 1997; Taylor et al. 1997; Clemo 1998; Finn and Porter 1998; Loeb 1998; Montagne et al. 1998; Ohata et al. 1998; Holdt-Lehmann et al. 2000; Lofti and Djalali 2000; Vlaskou et al. 2000; Van Kreel et al. 2002b

- The effects of dilution and pH on enzyme activity must also be considered, as these factors are highly variable in urine samples. Most enzymes are very labile to acid pH and the activity of many can be decreased substantially in concentrated urine
- Enzymes are generally less stable in urine than they are in high protein matrices such as serum or plasma (especially true for γ-glutamyl transpeptidase). Assays must be performed within 2 h of sample collection or a stabilizing substrate needs to be added to the sample, preferably during collection. Albumin, ethylene glycol, glycerol, or erythritol will satisfactorily preserve the activity of most commonly used enzymes when the samples are stored at −20°C

Either 24 h urine collection or timed urine samples collected at the same time each day is recommended, with the activity expressed per unit of time (Price 1982; Plummer et al. 1986). If the assessment is to be repeated with time, the samples should be collected over the same time period on each day because there is pronounced diurnal variation in excretion rate of some enzymes (Maruhn et al. 1977; Price 1982; Gossett et al. 1987). For spot urine samples or those where accurately timed collection is not possible, normalization of activity per unit of creatinine can be done and this has been shown to be reasonably well correlated to 24 h enzyme activity (Vanderlinde 1981; Grauer et al. 1995). Diet and age-matched controls must be included if enzyme activity is to be normalized to creatinine, to control for the effects of these variables on creatinine excretion (Plummer et al. 1986; Casadevall et al. 1995).

Most of these assays can be performed using the same automated equipment that is used for plasma, but the assays will need to be validated separately for urine to ensure that the matrix (urine versus plasma) does not interfere with the method and that the enzyme levels present in urine, either endogenously or following injury, are within the limits of linearity. Immunoassays (ELISA) are commercially available for α, π or μ -glutathione-S-transferase in the rat and human (Biotrin International, Dublin).

EVALUATION

Enzyme activity enzyme activityis expressed either per unit of time or per unit of creatinine. Normalization of enzyme activity per unit of volume or osmolality is not recommended because of the high degree of variability of these parameters (Price 1982; Plummer et al. 1986).

LIMITATIONS OF THE METHOD

5.2.3.1 General Limitations of Urine Enzyme Analysis

The correct sample handling methodologies have not been well validated for all enzymes in all species and it may require some effort on the part of the investigator to determine the need and optimal method for sample preparation.

The inherent limitations of the Jaffe method for determination of creatinine have been discussed

(Sect. 1.1.1). Factors which result in reduced excretion of creatinine without acute tubular injury (e.g., chronic renal disease in aged animals with pronounced loss of nephron mass, prerenal reduction of GFR) will also result in reduced urine creatinine and falsely elevated enzyme activity when normalized to creatinine (Price 1982; Plummer et al. 1986; Casadevall et al. 1995).

5.2.3.2 Limitations of Specific Enzymes

NAG is not specific for the proximal tubule, but also increases with papillary injury and glomerular disorders. Concomitant evaluation of one of the proximal tubule-specific brush border enzymes (LAP, GGT, or IALP) allows correct interpretation of the NAG increase. There are also significant increases in basal excretion as a function of age in rats and gender in dogs (higher in males), so age and sex-matched controls must be used.

GGT is notoriously unstable in urine and must be assayed very rapidly, even in the presence of urine stabilizers (Loeb 1998). There is high diurnal variation in baseline excretion rate in dogs that cannot be corrected in spot urine samples by creatinine normalization, so timed urine collection must be used with this enzyme in this species (Gossett et al. 1987).

LAP is also seen in the urine with very early glomerular disease (Bedir et al. 1996). The assay substrate is also a substrate for leukocyte esterase and thus increased neutrophils in the urine (pyuria) will cause a false positive result (Vlaskou et al. 2000).

Due to size limitations, most enzymes present in plasma are not filtered into the urine but there are some exceptions (e.g., lysozyme); these will be increased in the urine if tubular function (and uptake of filtered protein) is decreased. In addition, if glomerular injury accompanies tubular injury, leakage of larger molecular weight proteins may occur and plasma source enzymes that ordinarily would not be filtered may appear in urine. AST, LDH, and IALP may thus appear in urine.

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5.3 Experimental Models of Renal Disorders

5.3.1 Models for Evaluation of Uricosuric Agents

In most species, uric acid (the primary end product of purine metabolism) is further metabolized by uricase to allantoin prior to excretion. Humans have a relative deficiency of uricase and, thus, have both higher circulating and urinary levels of uric acid. In excess, high circulating levels lead to pathologic precipitation of urate crystals in varios tissues ("gout") while high urinary levels can result in the formation of urate calculi, one of the most common causes of urinary tract obstruction in humans. Evaluation of therapeutic interventions for these pathophysiologic states requires the use of relevant animal models.

5.3.1.1 Naturally Occurring Animal Models

PURPOSE AND RATIONALE

Hyperuricemia occurs naturally in only two animal models. The Dalmatian dog has excessive uric acid excretion and relatively high plasma levels. This is due to an autosomal recessive trait that results in defective activity of uricase (urate oxidase) in situ in the liver (Yü et al. 1971; Safra et al. 2005). Some authors have described defective tubular reabsorption of filtered urate in Dalmations (Kessler et al. 1959), but more recent evidence indicates that urate transport is normal (Bannasch et al. 2004). The Cebus monkey (Cebus albifrons), like humans, has a relative deficiency of uricase (Fanelli et al. 1970; Terkeltaub et al. 2006). Either model may be used to evaluate compounds that decrease urate synthesis or to evaluate the effectiveness of uricosuric drugs.

PROCEDURE

Standard clearance or fractional excretion methods (see Sect. 1.1.2 and "Quantitative Electrolyte Excretion") for uric acid are used. Urine and plasma uric acid are assessed by a colorimetric combined uricase-catalase method (Kageyama 1971).

EVALUATION

The results from groups of animals receiving test compound are compared to those of controls. Alternatively, each animal may be used as its own control in sequential experiments.

LIMITATIONS OF THE METHOD

Abnormal urate handling in the Dalmatian is a recessive trait, and thus not all Dalmatians are suitable. Animals must be screened first to ensure that urate metabolism is abnormal. Both animal models are expensive and can be difficult to obtain in adequate numbers.

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5.3.2 Artificial Animal Models

PURPOSE AND RATIONALE

In species with high uricase activity, experimental hyperuricemia can by induced by the administration of enzyme inhibitors. Allantoxanamide blocks uricase and also increases endogenously synthesized uric acid in rodents (the latter effect is inhibited by allopurinol) (Johnson and Chartrand 1978). These models can be used to test the effects of xanthine oxidase inhibitors as well as uricosuric agents (Hropot et al. 1984).

PROCEDURE

Sprague Dawley rats are treated by intraperitoneal (ip) injection of at least 150 mg/kg allantoxanamide. The

effects of this dose will last up to 6 h following administration. Standard clearance or fractional excretion methods may be used (see Sect. 1.1.2 and "Quantitative Electrolyte Excretion"), or plasma levels of uric acid may be followed over time. Urine and plasma uric acid are assessed by a colorimetric combined uricase-catalase method (Kageyama 1971).

EVALUATION

Serum uric acid concentrations are evaluated at a set point in time following test article treatment. The percentage decrease in serum uric acid levels is calculated:

$$\text{Percent decrease} = [(C_C - C_T)/(C_C - C_N)] \times 100$$

where C_C, C_N, and C_T are the uric acid concentrations (mg/dl) of control (inhibitor treated), normal (untreated), and test article (inhibitor + test article treated) groups.

MODIFICATION OF THE METHOD

Potassium oxonate or oxonic acid may be used as uricase inhibitors in rats as an alternative to allantoxamide, as the former can be toxic in high doses. Potassium oxonate at a dose of 250 mg/kg, ip is given 1 h before treatment with test compounds (Nguyen et al. 2005). Chronic (7 days or more) treatment of rats with a combination of potassium oxonate (2%) and urate (3%) in the diet results in marked hyperuricemia with renal urate crystal deposits; omission of the supplemental uric acid results in moderate hyperuricemia with no renal pathology (Stavric et al. 1969; Mazzali et al. 2001). Oxonic acid at a dose of 750 mg/kg/day results in a milder hyperuricemia in rats, which may be a more physiologically relevant model of human hyperuricemia (Khosla et al. 2005).

A homozygous knockout mouse model has been developed (UOX-/-) that is deficient in urate oxidase (uricase). Plasma and urine urate levels are markedly increased in these animals. They can be difficult to maintain, as there is high prenatal and preweaning mortality due to urate crystal nephropathy and severe diabetes insipidus (Wu et al. 1994), which can partially be ameliorated by ensuring adequate water replacement (Kelly et al. 2001). These animals have been successfully used to evaluate the pharmacologic effectiveness of uricase replacement therapies (Sherman et al. 2008).

A homozygous knockout model has been developed (GLUT9-/-) that is deficient in one of the urate transporters (SLC2A9, or GLUT9) known to be associated with clinically significant hyperuricemia in humans. There are two variants, a systemic knockout and a liver-specific knockout. The former model develops moderately increased plasma and urine urate levels, but suffers from the same mortality limitations as are seen in the UOX-/- animals. The liver specific variant develops high plasma and urine urate levels (due to decreased hepatic uptake of urate and conversion to allantoin) but does not develop nephropathy (Preitner et al. 2009; Stark et al. 2009; Woodward et al. 2009).

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5.3.3 Animal Models for Chronic Renal Failure

5.3.3.1 Partial Nephrectomy Models

PURPOSE AND RATIONALE

Subtotal nephrectomy in rodents has been used by many investigators as a model for chronic renal failure, particularly because they recapitulate many of the systemic changes such as anemia, cardiomyopathy, hypercholesterolemia, secondary hyperparathyroidism, hypertension, glomerular and arterial sclerosis, and vascular calcification that accompany this condition in humans (Shobeiri et al. 2010). The oldest and most commonly used of these models is the 5/6 nephrectomized rat model (Chauntin and Ferris 1932).

These models are commonly used to evaluate the effects of renal insufficiency on the kinetics of drugs which rely primarily on renal excretion (to predict the need for dose adjustment in patients with renal insufficiency). They may also be used to evaluate the effects of drugs intended to alter the course of renal failure or one of the comorbidities associated with chronic renal failure. The 5/6 nephrectomized rat is considered the "gold standard" model for the evaluation of erythroid-stimulating agents (erythropoietin and erythropoietin-mimetic agents).

PROCEDURE

A modification of the original procedure is more commonly used at present as it results in improved survival (Aunapuu et al. 2003; Shobeiri et al. 2010). Male Sprague-Dawley or Wistar rats weighing 230–280 g are normally used for these studies. The surgery is performed in two stages with a week's recovery between each surgical modification. Prior to the first incision, the animal is given 20 ml/kg normal saline subcutaneously. On a heated table, a midline laparatomy is performed 1 cm below the xiphoid bone cartilage and the abdomen is opened. The left kidney is isolated and brought out of the abdomen by grasping the fat at the lower pole of the kidney. The perirenal fat is bluntly dissected (sparing the adrenal gland), so that the renal artery and vein may be clearly identified. Two if the three branches of the left renal artery are ligated (decreasing the functional capacity of this kidney by ~67%) and the kidney is returned to the abdominal cavity, which is then closed with clips or sutures. Following a 1 week recovery, a second (smaller) midline laparotomy is performed starting 0.5 cm from the xiphoid bone cartilage. The right kidney is isolated and cleared of the surrounding fat (sparing the adrenal gland) to identify the renal artery, vein, and ureter. These structures are ligated, cauterized proximal to the kidney and the right kidney is then removed. Animals are allowed to recover for at least 1 week before being monitored for the onset of renal failure.

EVALUATION

Onset of azotemia (increases in serum creatinine, urea, and/or phosphorus of 2–4X over baseline) can be detected as early as 1 week postsurgically in this model, with the onset of severe changes occurring 4–8 weeks post surgery. Survivial of these models for up to 36 weeks has been described, which can be improved by the administration of low protein and low phosphorus-containing diets, prevention of secondary hyperparathyroidism (by administration of vitamin D analogs and phosphate-binding agents or by parathyroidectomy), and prevention of hypertension (Cruz et al. 2007; Shobeiri et al. 2010).

MODIFICATIONS OF THE METHOD

Similar surgical methods can be used in mice, although the poles of the remnant kidney are ablated by electrocautery as opposed to ligating two of the three renal arteries. The contralateral kidney is then removed following a recovery period of 1–3 weeks. The latter method allows smaller animals to be used and improves survival because blood loss is minimal. Onset of anemia and azotemia in this model occurs within 1 week following surgery, with survival up to 15 weeks (Gagnon and Duguid 1983; Gagnon and Gallimore 1988; Kennedy et al. 2008).

Large animal 3/4 and 5/6 nephrectomy models (rabbits and dogs) have also been described. These models use unilateral nephrectomy combined with either surgical excision or electroablation of renal tissue for consistent results. The dog model is commonly used

to evaluate effects of renal insufficiency on drug disposition, as it results in a milder degree of renal failure than is seen with the rodent models. While these models do recapitulate many of the comorbidities common to chronic renal failure in humans, rabbits appear to be resistant to the development of hyperparathyroidism, and dogs do not develop the glomerular changes seen in rodents (Brown et al. 1990; Duffee et al. 1990; Fine 1991; Vaneerdeweg et al. 1992; Toutain et al. 2000; Bas et al. 2004; Feng et al. 2010).

A 5/6 nephrectomy model in chickens has been described. An incision is made along the left side of the abdomen extending into the peritoneal cavity. The right ureter is identified and ligated just proximal to its junction with the cloaca. The left ureter and renal vein are ligated with a single suture near the middle of the left kidney. Azotemia is assessed by measuring plasma uric acid levels at intervals beginning 2–6 days post surgery. The method resulted in elevation of plasma concentration of uric acid, the major product of protein catabolism in avian plasma, to levels 2–4 times normal for periods as long as 3 weeks (Hartenbower and Coburn 1972).

Surgically altered (5/6 and 3/4 nephrectomized) rats and mice may be purchased from commercial vendors. Two such sources are Charles River Laboratories (http://www.criver.com/en-US/ProdServ/ByType/Surgical/Pages/home2.aspx) and Taconic Farms (http://www.taconic.com/wmspage.cfm?parm1=691).

A modified surgical approach has been used to reproduce acute renal failure (renal ischemia-reperfusion injury) in rats, rabbits, and pigs. Animals are anesthetized and subjected to unilateral nephrectomy with occlusion of the remaining kidney or bilateral renal occlusion using atraumatic vascular clamps before renal perfusion was reestablished. The duration of renal ischemia varies between 30 and 120 min (longer durations are needed for larger animals and bilateral occlusion models). Azotemia is detectable as early as 1 day post surgery, with recovery occurring between days 7 and 14 (Williams et al. 1997; Salehipour et al. 2010; Abreu et al. 2011).

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5.3.3.2 Immune-Mediated Glomerulonephritis Experimental Models

PURPOSE AND RATIONALE

Various immune-mediated glomerulonephritis experimental procedures have been used as models for glomerulonephritis in human beings. Most of them were developed in rats and mice, although other species (rabbits, guinea pigs) are occasionally used. The correlations between the animal model and the human disease that each one recapitulates is based on common features of the renal histopathology and disease pathogenesis, although in many cases the inciting autoantigen is not the same between the human disease state and the animal model.

These models have been used to investigate the pathologic mechanisms involved in renal injury in these disease states, as well as to evaluate the effects of experimental treatments on the course of disease. They are not as commonly used to evaluate the effects

Table 5.6 Summary of the more common experimental models of immune-mediated glomerulonephritis

Model name/ generation	Antigen or agent	Species	Strain(s)	Comments
Antineutrophil cytoplasmic antibody (ANCA) vasculitis/ passive or active	Myeloperoxidase	Rats Mice	Brown Norway Spont. Hypertensive (SHR) MPO -/-	Model for Wegener's granulomatosis (combination of crescentic glomerulonephritis and small vessel vasculitis) Antigen is the same in the rodent and human model Heterologous MPO immunization (or homologous in MPO -/- animals) needed to generate the disease
Heymann nephritis/ Passive or active	Megalin (gp330) (specifically, a 14 amino acid (aa) peptide within the second LDL receptor domain)	Rats	Sprague-Dawley	Model for human membranous glomerulonephritis Characterized by glomerular subepithelial immune complex deposits (IgG and C5–9 complexes) Original antigen was a complex of megalin and its receptor-associated protein, but later studies showed purified megalin (or the 14 aa epitope) was sufficient to induce disease. Megalin is present in rat glomeruli but not in human (corresponding human antigens are phospholipase A2 receptor, aldose reductase and Mn-superoxide dismutase) Complement fixation is required for disease progression
IgA Nephropathy/ Active	*Staphylococcus aureus* 20 AA membrane peptide	Mice	Balb/C	Model for human immunoglobulin A nephropathy Immune complexes contain both IgA and IgG, as well as complement components Antigen is the same between rodent model and human disease (associated with MRSA infection) Multiple immunizations needed to reproduce the disease
IgA Nephropathy/ NA	Various trichothecene mycotoxins (nivalenol, vomitotoxin)	Mice	Balb/C (normal and high IgA) B6C3F1	Induced by dietary exposure to low levels (~25 PPM in normal Balb/C or B6C3F1 mice Lower levels (12 PPM) result in disease in mice with high endogenous circulating IgA levels Results from reduced intestinal clearance of IgA and increased circulating levels Increased circulating IgA results in circulating IgA immune complexes which become deposited on the glomerular basement membrane
Mercuric chloride/ NA	Mercuric chloride	Rats	Brown Norway	Model for human Goodpasture's syndrome (antigens are similar) Results in the generation of anti-glomerular basement membrane antibodies (including laminin, collagen type IV, and fibronectin) Anti-myeloperoxidase antibodies have also been described Induction is by 3X weekly subcutaneous administration for 2 weeks at 2 mg/kg Onset of disease is at 14 days following the first injection Deposits contain IgG and C3 components

(*continued*)

Table 5.6 (continued)

Model name/ generation	Antigen or agent	Species	Strain(s)	Comments
Nephrotoxic serum nephritis/ passive (active in XenoMouse® model)	Anti-glomerular basement membrane antigens (primarily non-collagenous domains of type IV collagen)	Rats Mice Rabbits Guinea pigs	Multiple, but primarily Wistar-Kyoto (WKY) DDY C57/Bl6 XenoMouse®	Multiple rat strains have been used, but the Kyoto-Wistar is exceptionally sensitive. Model for human rapidly progressive glomerulonephritis and Goodpasture's syndrome XenoMouse® model generates human IgG antibodies upon immunization with human Type IV collagen epitopes, resulting in disease
Pristane-induced lupus-like syndrome/ NA	Pristane	Mice	C57Bl/6 Balb/C	Single IP injection of 500 µl of pristane induces disease Anti-DNA and anti-RNA antibodies result from apoptosis of lymphoid cells and peritoneal epithelial cells in the peritoneal cavity in a pro-inflammatory cytokine milieu Although antibodies detected 2–4 months post injection, disease onset requires ~6 months following pristane injection. Onset, magnitude and nature of the autoimmune responses are all highly variable

of renal impairment on the clearance of drugs, as tubular function is not affected until very late in the disease process.

Table 5.6 (derived from material in the references for this section) summarizes the more common models of immune-mediated glomerulonephritis.

PROCEDURE

Traditionally, these models are generated by the intravenous or intraperitoneal administration of preformed heterologous antibodies (usually immunized rabbit serum for rodent models and bovine serum for rabbit models) against the renal antigen of interest (passive models). They may also be generated by direct immunization of animals with either crude or purified preparations of the antigen of interest, usually combined with complete Freund's adjuvant to ensure a robust immune response (active models). A single dose of antibody or immunization is usually sufficient to incite disease, although repeat dosing is generally more effective and results in more severe disease.

EVALUATION

The specific disease model being used will determine the optimal time point for the initiation of experimental manipulations (see "References" for details). End points generally include evaluation of the degree of azotemia (serum creatinine and/or urea levels), magnitude of proteinuria, and histologic evaluation of the kidneys. Special stains and/or immunostains for basement membrane components, Ig and complement components, and infiltrating immune/ inflammatory cells may be incorporated in the evaluation.

Numerical data may be evaluated using appropriate statistical methods. Statistical evaluation of severity scores for histologic findings is not appropriate.

MODIFICATION OF THE METHOD

In some of the animal models listed in the table above, toxicants are used to initiate the onset of immune-mediated disease. The generation of the immune response is either due to the modification of endogenous proteins (thereby generating neoepitopes against which cross-reacting antibody responses are generated) or interfering with the normal clearance mechanisms for endogenous antibodies, resulting in their accumulation within the glomerulus and subsequent complement fixation with glomerular injury.

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Naturally Occurring Disease Models

PURPOSE AND RATIONALE

Spontaneous immune-mediated glomerulonephropathy occurs in several strains of rats and mice. These models have been used as surrogates for disease states of similar pathogenesis in humans. They may better reflect the pathogenesis of some of the immune-mediated glomerulonephropathies than the experimental models do in that the natural models tend to recapitulate the gender predilections and genetic alterations characteristic of the human conditions. The use and evaluation of these models is similar to those outlined in Sect. 3.3.1.

An overview of some of these disease models (derived from material in the references for this section) is presented in Table 5.7.

Table 5.7 Overview of some naturally occurring disease models

Disease state	Species	Strain	Comments
Crescentic glomerulonephropathy	Mouse	Kinjoh (SCG/Kj)	Model for Wegener's granulomatosis/polyarteritis nodosa in humans (BXSB/Mp x MRL/Mp-lpr/lpr)F1 hybrid strain Gender differences in incidence (females > males) Early onset of renal disease (first lesions by 9 weeks of age, overt proteinuria by 2 months of age) Anti-myeloperoxidase antibodies detected in these animals
IgA Nephropathy	Mouse	DDY	Hallmark is high circulating IgA levels due to decreased renal IgA clearance (defective IgA secretory mechanisms or secondary to altered glomerular basement membranes) Pathogenesis may be related to increased circulating TGF-β, which results in increased production of glomerular collagens I and IV and fibronectin Severity is less than is seen in immune-mediated form
Systemic lupus erythematosis (SLE) glomerulonephritis	Mouse	MRL/MP-lpr/lpr	Renal arteritis as well as glomerulonephritis Pathogenicity of the antibodies produced varies from individual to individual within the strain Defect is in apoptosis of lymphocytes Anti-DNA and anti-myeloperoxidase antibodies have been identified Sex hormone dependent; estrogen exacerbates the severity of disease
Systemic lupus erythematosis (SLE) glomerulonephritis	Mouse	(NZBxNZW)F1	Cross between two highly inbred strains: New Zealand Black (NZB/BlUmc) and New Zealand White (NZW/OuUmc) As an F1 hybrid, more heterogeneous than the MRL model and thus more closely representative of the human syndrome Six strains, with variable onset, severity and penetrance of renal disease allows selection of strain based on disease phenotype desired. Glomerular lesions and circulating "LE" cells closely resemble human SLE nephritis Glomerular lesions associated with deposits of anti-DNA antibodies Onset of disease between 4 and 6 months of age, with death by 8 months of age Sex hormone dependent; estrogen exacerbates the severity of disease while testosterone and progesterone decrease it

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5.4 Assessment of Effects on Lower Urinary Tract

5.4.1 In Vivo Methods

5.4.1.1 Micturition Studies

PURPOSE AND RATIONALE

The response of the urinary bladder to filling with increasing volumes of fluid is a common procedure for evaluating bladder function in both animals and humans. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of both voluntary actions and involuntary reflexes. These involve a number of neurotransmitter systems (glutamic acid, glycine, enkephalins, serotonin, α_1- and α_2- adrenergic, muscarininc, $GABA_A$ and $GABA_B$, and tachykinins), afferent signals from the bladder wall and urothelium, and inputs to the bladder originating in the central and peripheral nervous systems (De Groat 1975; Andersson 1993; Ferguson and Christopher 1996; Andersson and Wein 2004). In view of this complexity, in vivo models were developed for the quantitative analysis of the effects of drugs on the function of the vesicourethral complex (Maggi et al. 1986).

PROCEDURE

Assessment of effects on urinary bladder contractility and response has been performed in anesthetized rats, hamsters, guinea pigs, dogs, cats, and minipigs (Postius and Szelenyi 1983; Petersen et al., 1989: Imagawa et al. 1989; Häbler et al. 1990, 1992; Seif et al. 2004; Tai et al. 2004). A vein (usually a jugular) is cannulated for drug injection. Body temperature is kept constant by means of a heating pad maintained at 37°C. Through a midline incision of the abdomen, the urinary bladder is exposed and emptied of urine by application of a slight manual pressure. A 20 gauge needle is inserted through the apex of the bladder dome for 3–4 mm into its lumen. The needle is connected to a pressure transducer by means of polyethylene tubing (1.5mmOD and 1.0mmID) and the whole system filled with saline. The tubing is provided with an internal coaxial polyethylene tubing (0.6mmOD and 0.3mmID) inserted through a side hole and sealed by a drop of epoxy resin. The second tubing serves for intravesical infusion of fluid and is connected, through a peristaltic pump, to a saline reservoir.

Intraluminal pressure signals are delivered to an amplifier and displayed on a four channel polygraph. Warm saline-soaked cotton wool swabs are laid around the exteriorized organ to maintain its temperature and to keep it moist in experiments involving the topical application of substances on the bladder dome.

After a 15 min equilibration period at zero volume, variations in intraluminal pressure are recorded in response to continuous infusion of saline at a rate of 2.8 ml/h at 37°C for 30–40 min by means of a peristaltic pump connected to the polyethylene tubing inserted into the bladder. This infusion rate simulates the maximal hourly diuresis within the physiological range. In each preparation, the infusion is continued until micturition occurs. Micturition is referred as the emission of several drops of fluid during a sustained phasic contraction of the detrusor muscle, which is followed by return to zero or, in any case, to a value lower than that recorded just before micturition. Alternatively, the micturition reflex may be defined as the onset of high amplitude (>5 mmHg) pressure contractions in the urinary bladder in response to a distention stimulus (Oyasu et al. 1994; Lecci et al. 1988).

For both intravenous and topical administration, substances are dissolved in saline.

EVALUATION

In each experiment, the following parameters may be evaluated:

- Minimum intravesical pressure = minimal pressure recorded (index of resting bladder tone)
- Pressure threshold = intraluminal pressure value recorded just before micturition
- Volume threshold = the volume of infused saline required to obtain micturition
- Maximal amplitude of micturition contraction
- Frequency of micturition contractions
- Residual volume after micturition

The effect of substances on the compliance and responsiveness of the bladder wall is evaluated by comparing these parameters in treated animals with those of controls.

MODIFICATIONS OF THE METHOD

Rapid bolus infusion of saline into the urinary bladder, as opposed to continuous infusion, may be used to evaluate the responsiveness of the urinary bladder to distention. Volume responses in the parameters outlined above are evaluated (Lecci et al. 1988).

The urinary bladder may be catheterized in intact animals under anesthesia via the urethra, and the urethral catheter can then be attached to the pressure transduction system. Animals so treated may be recovered and used in multiple experiments. Alternatively, the effects of compounds applied intravesically can be examined, which allows elucidation of the neurotransmitters critical in generation of the micturition reflex (Lecci et al. 1988).

Animals surgically prepared, as described above, may also be recovered following replacement of the urinary bladder in the abdomen with exteriorization of the bladder cannula percutaneously. These animals may then be used to evaluate the effects of either systemically or intravesically applied compounds (Yaksh et al. 1986; Conte et al. 1988).

Ultrasonic methods of determining bladder volumes resulting in a micturition response have been used in rats (Horváth et al. 1994) and minipigs (Seif et al. 2004). These methods compare favorably with the results obtained by direct instillation of fluid into the urinary bladder, although in general volumes tend to be underestimated. Some of the techniques described have the advantage of being noninvasive and applicable to conscious, free-moving animals.

MODIFICATION OF THE METHOD

Either chemical (6-hydroxydopamine, reserpine) or surgical (section of hypogastric nerves) sympathectomy in animals produces model a of detrusor hyperreflexia and urine dropping, mimicking cystometric finding in human disease (Maggi et al. 1987).

Evaluation of micturition frequency can be evaluated in conscious animals. This can be done either by placing the animal in a restrainer over a urine collector. The collector is then secured to a Statham UC3 strain transducer, the output of which was amplified by a Gould bridge amplifier. Data are then monitored on a polygraph (Harada et al. 1992). More recently, this method has been simplified by the use of an infared photodiode sensor and matched phototransistor that can detect the appearance of urine flowing from the bottom of a metabolic cage, recording the frequency of these events. This method produces results comparable to the original cup-fore transducer methods (Argentieri and Argentieri 2002).

Effects of systemically or intravesically administered drugs on the upper urinary system may be detected during the course of cystometrography, as described by Tillig and Constantinou (1996). Additional catheters are placed into the renal pelvis (to measure renal pelvis pressures and to infuse the renal pelvis with a contrast-enhancing dye, indigo carmine). A videomicroscope is used to visualize the transport of indigo carmine infused into the renal pelvis and ureters (an index to urine transport in the upper urinary system). Standard cystometrography parameters (bladder pressure and micturition responses) were recorded during continuous infusion of the bladder with saline.

Simultaneous evaluation of bladder pressure and urine flow rates has been performed in dogs (Moreau et al. 1983), rats, and guinea pigs (Conte et al. 1991; Van Asselt et al. 1995). A single urethral catheter is used both to instill saline into the bladder and measure bladder pressure, urethral pressure, and urethral flow.

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5.4.2 In Vitro Methods

5.4.2.1 Isolated Renal Pelvis

PURPOSE AND RATIONALE

Periodic contraction of the renal pelvic wall is important to the process of concentration of urine in the renal medulla as well as the propulsion of urine from the kidney into the bladder (Santicioli and Maggi 1998; Knepper et al. 2003). The isolated renal pelvis has been used to examine the effects of compounds on this activity. Rabbits, rats, and guinea pigs have been used (Maggi and Giuliani 1991, 1992; Maggi et al. 1992a, b, c, 1994, 1995; Giuliani and Maggi 1996; Santicioli et al. 1995, Santicioli and Maggi 1997; Patacchini et al. 1998; Bigoni et al. 1999).

PROCEDURE

Following appropriate anesthesia and euthanasia, a kidney and its attached ureter are removed and placed in oxygenated Krebs solution or other appropriate balanced electrolyte solution. The renal pelvis is carefully dissected from the renal parenchyma, separated from the ureter, cut and connected to threads to record motility along the circular axis. The preparation is suspended in a 5 ml organ bath and mechanical activity

recorded by means of an isotonic transducer (load 1mN). Transmural electrical field stimulation is made by means of platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) are delivered in trains of 10 s duration at frequencies of 5–10 Hz.

Experiments commence after a 60–90 min equilibrium period after which amplitude and frequency of spontaneous activity has reached a steady state. Test compounds are added to the bath and any effects on spontaneous activity (amplitude and/or frequency of spontaneous contraction) are assessed after an appropriate incubation period (up to 60 min following the addition of the test compound). As controls, concentration response curves to noradrenaline (decreased motility) and acetylcholine (increased motility) are performed by noncumulative addition to the bath at 20 min intervals, with washout in between concentrations. Parallel preparations (subjected to the same duration of incubation but without added test or control compounds) are monitored concurrently to correct for any effects of muscle fatigue during prolonged incubation (Davidson and Lang 2000).

EVALUATION

The amplitude and frequency of spontaneous contractions are assessed and the Motility Index (MI) is calculated as follows:

$$\mathrm{MI} = \left(\sum \mathrm{amp}/5\right) \times \mathrm{F}$$

where Σamp/5 is the mean amplitude of five contractions (in mN) and F is the frequency (in min^{-1}) of those five contractions. Concentration-response curves are generated by plotting the concentration versus the MI (either raw or expressed as a percent of control, using the parallel incubation as the control) (Davidson and Lang 2000).

MODIFICATIONS OF THE METHOD

Circumferentially cut strips from the proximal renal pelvis can be used instead of the whole renal pelvis, as this portion of the renal pelvis has the highest frequency of spontaneous contraction (Zhang and Lang 1994; Lang et al. 1995; Lang and Zhang 1996; Teele and Lang 1998). In larger species, the pacemaker region (the pelvic-calyceal junction) and pelviureteral junctions can be identified, isolated, and studied separately (Kimoto and Constantinou 1990, 1991; Lang et al. 2002).

Electrical activity in isolated smooth muscle cells of the renal pelvis can be determined as an index to renal pelvic motility in situ. The frequency of slow wave generation by membrane depolarization is correlated to renal pelvic motility (Seki and Suzuki 1990).

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5.4.2.2 Propagation of Impulses in the Ureter

PURPOSE AND RATIONALE

The ureter smooth muscles are normally electrically and mechanically quiescent, with contraction resulting from electrical input from a pacemaker region in the proximal renal pelvis. However, spontaneous activity and progressive peristaltic contractions can result from activity originating independently in the ureter, and the effectiveness of impulse propagation initiated by the renal pelvic pacemaker is modulated by factors originating in the ureter itself (Santicioli and Maggi 1998; Lang et al. 2002). Suppression of action potentials at any site of the ureter will theoretically suppress the propagation of contraction and peristalsis (Weiss 1992). The isolated ureter provides a system by which effects on peristalsis in the entire ureter, or any of its segments, can be examined.

PROCEDURE

A number of species (most commonly rats and guinea pigs) have been used for whole-mount or strip preparations. Following appropriate anesthesia, the whole kidney and ureter are excised and placed in a Petri dish containing oxygenated Krebs solution for dissection. Segments (length depends on the species from which collected) representative of the proximal, middle and distal ureter may be collected. The segments are attached to a force-displacement transducer and are incubated in Krebs buffer or appropriate balance electrolyte solution gassed with 95% O_2/5% CO_2 and maintained at 32–37°C. The preparation is allowed to equilibrate for at 30–60 min, until the amplitude and frequency of spontaneous or induced contractions have stabilized, before experimental manipulation.

The frequency and amplitude of spontaneous contractions are recorded. Alternatively, contractions may be induced either by electrical field stimulation, transmural electrical simulation, the application of circumferential tension (stretch response), or by the addition of 40 mM KCl to the preparation. Once the contractions have stabilized, the test compound is added (Teele and Lang 1998; Yamamoto and Koike 2000).

EVALUATION

The frequency and amplitude of contraction are recorded, and the motility index (MI) may be calculated as outlined in E.1.4.2.1. Data are expressed as a percent of the control value and appropriate statistical analysis is performed. Concentration-response curves may be plotted and pA_2 or pD_2 values may be calculated (Arunlakshana and Schild 1959; Van Rossum 1963). Hill coefficients and EC_{50} values may also be calculated (Weiss et al. 2002).

MODIFICATIONS OF THE METHOD

Ureter preparations have been made from pig or human tissues collected from the abattoir or following

surgery, respectively. Ideally, no more than 20 min should elapse between tissue collection and incubation. Tissues are placed into cold (4°C) Krebs buffer or suitable balanced electrolyte solution and cut into rings of 0.5–1 cm in length. The rings are attached to a force transducer, suspended in an organ bath at 37°C, and allowed to equilibrate for up to 1.5 h while the frequency and amplitude of spontaneous contraction is measured. If the preparation demonstrates acceptable and stable frequency and amplitude of spontaneous contraction within this time period, it is considered suitable for experimental use. In pigs, the washout period between treatments is variable and thus the preparation is monitored for return to pretreatment baseline before the next treatment is applied. In human tissue, spontaneous contraction seldom occurs and electrical stimulation (trains of 300 ms at an interval of 200 s and impulses of 200 mA with a duration of 6 ms at a frequency of 50 H) is required. A washout period of 20 min between treatments is used (Weiss et al. 2002).

The evaluation of regional differences in response, and the effect of regional responses on adjacent regions, can be evaluated in a whole ureter preparation where each region remains physically attached but can be isolated for the application of test compounds or stimuli. The entire ureter is dissected and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the proxima (renal), middle, and distal (bladder) ends. They include a window covered with condom rubber: a small hole (about 300 mm) is made in the rubber to enable the passage of the ureter. The proximal portions of each segment are pinned to a Sylgard support, and the distal portions are connected to isotonic transducers for recording of mechanical activity. Each compartment is perfused separately and electrical field stimulation can be applied to any compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter (Meini et al. 1995).

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5.4.2.3 Isolated Urinary Bladder and Internal Urethral Sphincter

PURPOSE AND RATIONALE

Isolated urinary bladder preparations are useful for evaluating the effects of compounds on smooth muscle contractility and resting tone. Modifications allow for the evaluation of effects on specific urinary bladder regions and on inputs from either the urothelium or nervous system inputs.

PROCEDURE

Rats and guinea pigs have been used for whole bladder preparations. Following appropriate anesthesia, the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue is removed, the preparation is placed en bloc into appropriate balanced electrolyte solution (Tyrode's or Krebs buffer) bubbled with 5% CO_2 and 95% O_2. The urethra is cannulated and connected to a pressure transducer, and the bladder is filled with a volume of solution adequate to evoke a contractile response. The bladder preparation is allowed to equilibrate for 30 min before any experimental manipulations are performed. The amplitude and frequency of pressure changes are recorded (Birder et al. 1998; Drake et al. 2003).

Alternatively, contractility can be assessed visually. Surface blood vessels or externally applied markings

(India ink or carbon particles) are used as landmarks. Video image recording of the bladder preparations are made during the experimental manipulations. Still images from the video are evaluated and the change in linear distance between pairs of landmarks is used as an index of contractility (Drake et al. 2003).

EVALUATION

The values are expressed or plotted as the means ± SE and pA2 values are calculated (Arunlakshana and Schild 1959). Data are analyzed using appropriate statistical methods.

MODIFICATIONS OF THE METHOD

Isolated regions or longitudinal strips of urinary bladder from a number of species, including humans, may be used to study the effects of compounds on isolated regions. Longitudinal strips (usually from the detrusor region) are most commonly used. Following harvest of the bladder as outlined above, the region of interest is cut from the bladder wall. Ligatures are placed on both ends of the strips and one end is attached to a tissue holder and the other to a strain gauge force-displacement transducer connected to a polygraph on which isometric tension changes are recorded. Each of the strips is then placed into a tissue bath containing Krebs-Ringer solution bubbled with 95%O_2 + 5%CO_2 at 37°C. Resting tension is adjusted to 1 g during an equilibrium period of at least 2 h. The contractile and relaxant responses are measured as increases or decreases from the resting tension, and contractile responses to stimulation can also be assessed with electrical stimulation of the preparations (Burnstock et al. 1978; Hills et al. 1984; Maggi et al. 1985; Pietra et al. 1990). Strips from the trigone region can be harvested from larger species and similarly evaluated (Klarskov 1987; Thornbury et al. 1992).

Contractile responses to nervous stimulation can be assessed in whole isolated bladder preparations. The pudendal nerve remnants can be identified near the ureter and can be attached to electrodes for stimulation of the bladder. Alterations in the contractile responses by the test compound are considered to result from effects at the neuromuscular junction (Hukovic et al. 1965; Weetman 1972; Dhattiwala and Dave 1975).

Denervated urinary bladder preparations (whole or strips) can be used to determine the degree of effect on nervous system inputs. Following appropriate anesthesia, the pelvic plexus is accessed in rats by a ventral midline incision and both pelvic ganglia are obliterated by electrocauterization. The animals are allowed to recover for at least 4 days before the urinary bladder is harvested as described above; during this period, the neurotransmitters downstream from the nervous system inputs become inactive and only the autonomous inputs remain. Differences in response between the denervated urinary bladder preparation and one harvested from a sham-operated animal are considered to result from neural inputs. The urinary bladders of the denervated animals must be manually emptied on a daily basis during the 4 day recovery period (Birder et al. 1998; Brauerman et al. 2006).

Inputs from the urothelium can be assessed in isolated urinary bladder smooth muscle preparations. Following preparation of strips from the urinary bladder, the urothelium is gently peeled away from the cut surface and the strips are mounted as described above. Effects on the capsaicin-mediated nitric oxide pathways located in the urothelium can be assessed in these preparations (Birder et al. 1998). Effects on the release of neurotransmitter substances from the epithelial strips removed from these preparations can also be examined; following isolation, the epithelial strips are incubated in Krebs buffer of suitable balanced electrolyte solution. Following a 20 min equilibration period, the strips are stimulated either mechanically (by stroking with a glass rod or pinching with forceps) or electrically (using field stimulation of the incubation medium). The neurotransmitter substance of interest is analyzed in the bath solution and dose-response curves are generated (Downie and Karmazyn 1984).

Isolated proximal urethral preparations can be made from a number of species. Following appropriate anesthesia, the bladder and urethra are harvested and either circular sections (rings) or transverse strips of 2–4 mm in length are collected from the proximal urethra. These are mounted in appropriate tissue holders and connected to an isometric force transducer and immersed in gassed balanced electrolyte solutions as described above. Electrodes are introduced to stimulate relaxation in the preparations. (Andersson et al. 1983, 1992; Teramoto et al. 1997; Von Heyden et al. 1997). Similar preparations can be made from human tissue, which is obtained from male patients undergoing cystourethrectomy en bloc because of bladder cancer. Rings of tissue were taken from the membranous and supra- and infracollicular parts of the prostatic urethra (Andersson et al. 1983; Kunisawa et al. 1985).

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5.4.2.4 Effects on the Isolated Urethra

PURPOSE AND RATIONALE

The relaxation response in the urethra results in a combination of decreased intraurethral pressure, increased urethral diameter, and urethral shortening resulting in a flaring of the urethral orifice. A number of neurotransmitters and mediators are involved in this process, with each affecting a different stage of the process. (Brading 1999; Andersson and Wein 2004) The urethra is difficult to study in situ because of the large amount of surrounding connective tissue, which can be overcome by the use of a whole-mount preparation (Jankowski et al. 2004).

PROCEDURE

Female rats or guinea pigs have been used. Following appropriate anesthesia, the bladder and urethra are exposed via a lower midline incision, and a catheter is inserted in the urethral lumen, extending the entire axial length and exiting from a hole placed in the bladder dome. The urethra is secured to the tubing with sutures at both ends to maintain the correct in vivo length after dissection, and is measured. The pubic bone is then cut at a position lateral to the urethra and then separated and resected. The exposed urethra was gently removed from the ventral vaginal wall and the whole bladder-urethra unit was immediately placed in cold, oxygenated suitable balanced electrolyte solution bubbled with 21% O_2, 5% CO_2, and 74% N_2.

The catheter is removed so that the urethra may be secured inside the experimental apparatus, which provides a controlled fixed intraluminal pressure via an adjustable static fluid reservoir (the intraluminal pressure is controlled by adjusting the height of the reservoir). The mounted urethra is then enclosed in a bathing chamber filled with the same gassed, balanced electrolyte solution at 37°C. The preparation is allowed to equilibrate for at least 30 min before testing. A laser micrometer is positioned to measure urethral outer diameter at chosen locations along the axial length. Proximal, mid, and distal regional measurements were performed by positioning the laser at axial positions 25%, 50%, and 75%, respectively,

from the apex of the bladder, based on in vivo length. Both pressure and outer diameter (OD) measurements are recorded simultaneously.

The test compound of interest is added to the bath and pressure and OD measurements are obtained following a 30 min equilibration period. Pressure-diameter (P-D) responses are generated by incrementally increasing the pressure, in 2 mmHg steps, from 0 to 18 mmHg, and OD data are collected at 10 Hz over a 1 min period for each 2 mmHg step. The OD data are then averaged for each incremental increase to obtain a discrete value for each value of applied pressure.

Contractile responses can be evaluated in this preparation as well. The urethra is exposed to a fixed intraluminal pressure of 8 mmHg, which causes the tissue to be predilated and allows for a contractile response to be generated. The OD resulting from this 8 mmHg applied pressure is measured at a single axial location (i.e., proximal, mid, or distal) 30 min after pressurization. The test chemical of interest is added to the bath and the preparation is exposed for 30 min; at the end of this time, a series of 100 OD measurements (taken at 1 Hz) are collected at the same location and are averaged.

EVALUATION

The P-D response data are used to generate tissue compliance (C) as follows:

$$C = [D_{max} - D_{min}/D_{min}] \times (P_{max} - P_{min})^{-1}$$

where D_{max} and D_{min} represent the OD at the maximum (P_{max}, 18 mmHg) and minimum (P_{min}, 0 mmHg) applied pressures, respectively.

Contractile responses are assessed by determining the percent change in mean OD before and after the addition of the test compound.

MODIFICATION OF THE METHOD

With careful dissection, the pudendal nerves can be identified and left intact during the diisction of the urethra from the guinea pig. These nerves can then be attached to electrodes and alterations of effects of neural inputs can be assessed. In this preparation, contractile responses were assessed by measuring changes in isometric tension and intraluminal pressure changes were also recorded (Walters et al. 2006).

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5.4.2.5 Effects on the External Urethral Sphincter

PURPOSE AND RATIONALE

In most species, the resting pressure profile responsible for continence depends on the striated muscle present in the external urethral sphincter (Brading 1999). The external urethral sphincter shows a steady tonic discharge under resting conditions. As the bladder fills, there is initially an increase in this activity. When the rise in tension in the bladder wall leads to reflex contraction, the activity in the external urethra sphincter ceases and in remains quiescent during voiding. Parlani et al. (1992) used the external urethral sphincter of the rat as an in vitro model to evaluate the activity of drugs on the smooth and striated components of the urinary bladder outlet.

PROCEDURE

Either rats or rabbits have been used. Under appropriate anesthesia, the external urethral sphincter (Watanabe and Yamamoto 1979) is isolated from the perineal muscles and surrounding connective tissue and removed in toto. The preparation is placed in oxygenated Krebs solution, and a ring is taken from its middle region. In this area the urethra is encircled by bundles of striated muscle fibers partly interlaced with urethral smooth muscle. The rings are cut to obtain strips that are suspended in a 5 ml organ bath containing Krebs solution at 37°C. A mixture of 96% O_2 and 4% CO_2 is bubbled into the organ bath.

The preparations are connected by means of a silk thread to an isometric strain gauge under a constant load of 1 g. The contractile activity is recorded on

a polygraph. Field stimulation is carried out by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The preparations are allowed to equilibrate for at least 60 min.

Electrogenic stimulation of the preparation allows assessment of the effect of test compound on contractility. Square wave pulses are delivered at an intensity of between 10 and 60 V, a frequency of 40 Hz, and a duration between 0.1 and 1 s; using 0.5 s trains of 0.5 ms pulses at 15 s intervals. Once the amplitude of the electrically induced contractions is stable, the preparations can then be exposed to test compounds and the amplitude is again measured until stable. A 90 min rest period following washout is required between treatments (Morita et al. 2000).

EVALUATION

The contraction amplitude is expressed as a percentage of the control value. Concentration response curves can be plotted and EC_{50} values calculated if desired.

LIMITATIONS OF THE METHOD

The striated muscle of the external urethral sphincter is easily damaged during removal. If this occurs, the cells will become depolarized and refractory to electrical stimulation (Brading 1999). Testing of the preparation in the absence of drug at the beginning and the end of the experiment will control for this.

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Respiratory Function Assays in Safety Pharmacology

6

Dennis J. Murphy

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6.1 Respiratory Function Assays: General Approach

The objectives of a safety pharmacology study of the respiratory system are to determine whether a drug has the potential to produce a change in respiratory function and to establish whether this change is a liability. Such changes can result from either the primary or secondary pharmacological properties of a drug or from organ dysfunction resulting from the toxicological properties of a drug.

The respiratory system consists of two functional units, the pumping apparatus and the gas exchange unit. As such, a complete assessment of respiratory function in safety pharmacology must include an evaluation of both of these components. The pumping apparatus includes those components of the nervous and muscular systems that are responsible for generating and regulating breathing patterns, whereas the gas exchange unit consists of the lung with its associated airways, alveoli, and interstitial area that contains blood and lymph vessels and an elastic fibrous network.

The function of the pumping apparatus is to ensure the appropriate movement of gases between the environment and the central airways, which is evaluated by measuring ventilatory patterns. Ventilatory parameters must include measures of respiratory rate, tidal volume, and minute volume, since normal ventilation requires that the pumping apparatus provide both adequate total pulmonary ventilation (minute volume) and the appropriate depth (tidal volume) and frequency (rate) of breathing. If a change in these parameters occurs, inspiratory flow (mean or peak), expiratory flow (mean or peak), fractional inspiratory time

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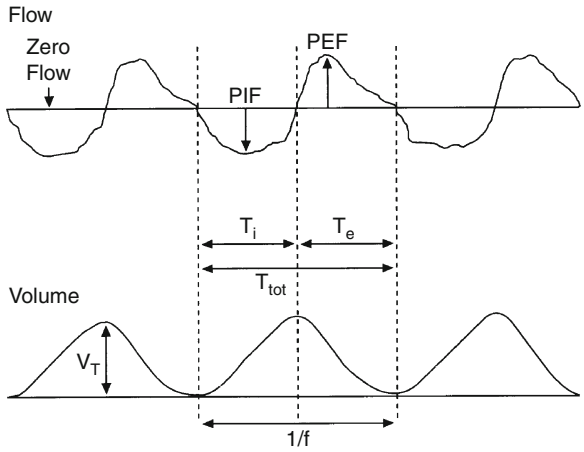
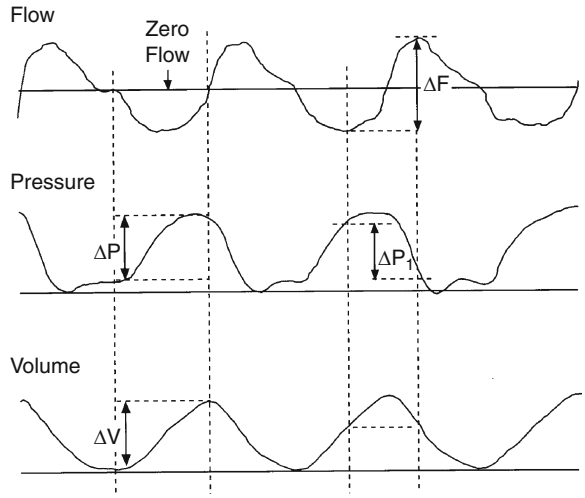


Functional Endpoints

V_E	-	Minute Volume (V_T x f)
f	-	Breathing Rate
V_T	-	Tidal Volume
PIF	-	Peak Inspiratory Flow
PEF	-	Peak Expiratory Flow
FIT	-	Fractional Inspiratory Time $\frac{(T_i)}{(T_{tot})}$

Fig. 6.1 Tracings of lung airflow and lung volume changes during spontaneous breathing in a conscious rat. Airflow was measured directly using a "head-out" plethysmograph chamber. The functional endpoints can be automatically calculated for each breath using a data acquisition and analysis software system



Functional Endpoints

Dynamic Compliance $= \frac{\Delta V}{\Delta P}$ (At zero flow points)

Dynamic Resistance $= \frac{\Delta P_1}{\Delta F}$ (At isovolume points)

Dynamic Conductance $= \frac{\Delta F}{\Delta P_1}$ (At isovolume points)

Fig. 6.2 Tracings of lung airflow, transpulmonary pressure, and lung volume changes during spontaneous breathing in a rat. Airflow was measured directly using a "head-out" plethysmograph chamber, while pleural pressure was measured using a pressure-sensitive catheter placed into the esophagus within the thoracic cavity. The functional endpoints can be automatically calculated for each breath using a data acquisition and analysis software system

(inspiratory time/total breath time), and time between breaths (expiratory pause or apnea) should be evaluated to help define the mechanism (see Fig. 6.1).

The function of the gas exchange unit is to ensure that gas which enters the airways from the environment reaches the alveoli during inspiration and is removed from the alveoli during expiration. This is accomplished by maintaining patent (open) airways and elastic recoil in the parenchyma of the lung. The function of the gas exchange unit is evaluated by measuring the mechanical properties of the lung. This is most effectively accomplished in conscious animals by obtaining dynamic measurements of airway resistance or conductance (to assess airway patency) and lung compliance (to assess elastic recoil). Airway resistance (measured as total pulmonary resistance) defines the change in pleural, airway, or transpulmonary pressure (ΔP) required to produce a defined change in lung airflow (ΔF) and is calculated as ΔP/ΔF, while conductance is calculated as ΔF/ΔP. To calculate dynamic resistance or conductance, the ΔP and ΔF are measured for each breath at the same lung volume during inspiration and expiration (usually between 50% and 70% of tidal volume) (see Fig. 6.2). By selecting isovolumetric points, the dependence of ΔP on elastic component of the lung is removed, leaving ΔP dependent only on resistance to the flow of gas in the lung and airways. Another method for assessing airway resistance involves measuring the excursions of the thorax and abdomen during breathing. During normal breathing, the thorax and abdomen move in synchrony during both inspiration and expiration. A shift from this synchronous movement to asynchronous movement can be quantified as a phase angle shift. Such phase angle shifts have been used as indexes of airway resistance (see Fig. 6.3). Dynamic compliance is calculated by measuring the differences in airway or transpulmonary pressure (ΔP) and volume (ΔV) that occur at the beginning and end of each inspiration (i.e., at zero flow points) (see Fig. 6.2). By selecting zero

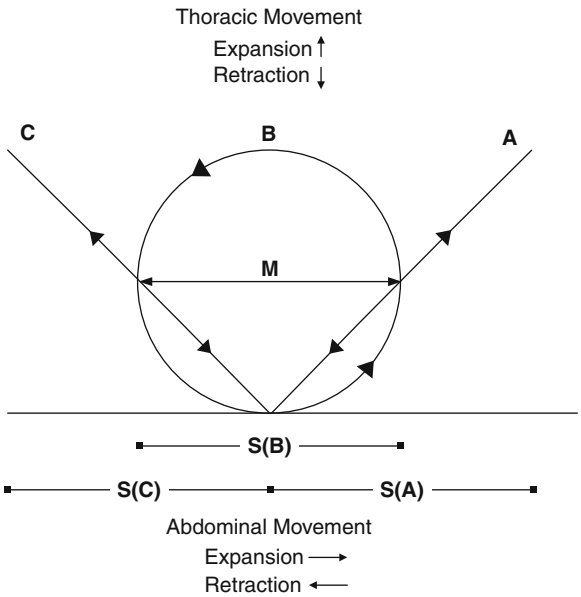


Fig. 6.3 Konno-Mead loops showing synchronous and asynchronous movements of the thorax and abdomen. Thoracic movement is plotted on the *y*-axis and abdominal movement on the *x*-axis. Phase angle (θ) defines the degree of synchrony and is calculated as $\theta = \sin^{-1}$ (M/S) where M = distance between abdomen-thorax loop intercepts when measured at one half the distance between maximal and minimal thoracic excursion and S = maximal abdominal excursion. (**a**) M/S = 0; θ = 0°—movements are synchronous. (**b**): M/S = 1; θ = 90°; movements are asynchronous. (**c**) M/S = 0(−); θ = 180°—movements are in opposite directions (paradoxical)

flow points, the dependence of ΔP on tissue and airflow movement is removed, leaving ΔP dependent only on the elastic component of the lung.

Supplemental studies in safety pharmacology are designed to investigate mechanisms of action or to help further characterize the liability of a drug effect. To help understand the mechanisms and liabilities associated with respiratory function changes, it is important to determine whether a drug-induced change in ventilation is acting through a mechanism involving the central or peripheral nervous system, and whether a drug-induced change in respiratory function has an effect on arterial blood gases.

Because the safety profiles defined by safety pharmacology studies can have a significant impact on the successful development of new therapeutic agents, it is important that the techniques and assays used in safety pharmacology studies minimize the occurrence of false negative and false positive results. For this reason, techniques that provide direct measures of respiratory parameters should be used. A direct measure is one that provides the endpoint of interest, in contrast to an indirect measure that provides a surrogate endpoint, which requires certain assumptions and/or calculations to estimate the true endpoint. Furthermore, because most drugs are intended for use in conscious patients and most anesthetics, analgesics, and sedatives can alter ventilatory reflexes, respiratory drive, and airway reactivity, safety pharmacology studies evaluating the effects of drugs on respiratory function should utilize conscious animal models. Based on the above, the respiratory function assays covered in this chapter will focus primarily on new technologies that provide direct measures of respiratory parameters in conscious animals and are considered to be most appropriate for use in safety pharmacology studies. Alternate techniques, which provide either indirect measures of respiratory function (e.g., barometric or whole body plethysmography) or use anesthetized models, have been described elsewhere (see "References") and will not be covered in this chapter.

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6.2 Respiratory Function in Conscious Rats

PURPOSE AND RATIONALE

The rat is considered an appropriate species for general use in safety pharmacology studies of the respiratory system. The physiology of the respiratory system has been well characterized in this species and much of the information on drug-induced effects on ventilatory control and mechanisms of airway disease have been obtained in the rat. The rat is also readily available from animal vendors, is easy to handle and train, has a relatively stable breathing pattern, and has the appropriate temperament for conscious respiratory measurements. Furthermore, the techniques for measuring respiratory functions in rats are well established, and the rat is commonly used in toxicology studies. Selecting a species that is used in toxicology studies provides additional supportive information including (1) pharmacokinetic data that can be used to define the test measurement intervals, (2) acute toxicity data that can be used to select the appropriate high dose, and (3) toxicology/pathology findings that can be used to help define the mechanism of the functional changes measured in safety pharmacology studies. Although this technique has been developed in the rat, it is also applicable to other small animals.

PROCEDURE

To provide a direct measure of ventilatory parameters, a head-out plethysmograph chamber is used, while measurement of pleural pressure is used to provide a direct measure of airway resistance and compliance.

6.2.1 Pleural Pressure Measurements

6.2.1.1 Catheter Placement

Pleural pressure is measured chronically in conscious rats by surgically implanting a fluid-filled polyurethane catheter (length = 10 cm; O.D. = 0.7 cm) attached to a pressure-sensitive radiotelemetry transmitter (Model TA11PA-C40, Data Sciences International, St. Paul, MN) beneath the serosal layer of the esophagus and within the thoracic cavity (see Fig. 6.4).

6.2.1.2 Surgical Procedure

Surgery is initiated by anesthetizing the rat with isoflurane (2–3%) delivered by inhalation in 100% oxygen. The surgical area is prepared by shaving the abdomen with surgical clippers and scrubbing with a Betadine® and 70% ethanol wash. Once the area has been prepared, an abdominal incision (approximately 4–5 cm in length) is made along the linea alba. The lobes of the liver (within the abdominal cavity) are refracted to expose the esophagus and the lobes gently packed against the abdominal wall using moist 2 × 2 in. Versalon® (or equivalent) squares. The esophagus is isolated approximately 2 cm below the Hiatus oesphagicus (junction with the diaphragm), a 22-gauge needle (1 in. in length with an approximate 90° bend) is inserted into the esophagus between the serosa and muscularis layers, and the needle is tunneled cranially into the thoracic cavity (see Fig. 6.4). It is important to keep the esophagus completely straight while the needle is being inserted since the needle can reemerge through the serosa layer and enter into the pleural space. This will generally lead to encapsulation of the catheter tip and result in a loss or dampening of the pressure signal. Care must be taken not to advance the needle too far up the esophagus (generally no more than 1.5 cm) since the needle may come into close contact with the heart, causing a cardiac pressure signal. Once the needle is advanced to a point approximately 1 cm beyond the diaphragm junction, the needle is removed and the catheter from the telemetry transmitter unit advanced up the channel. During this step, care must be taken not to apply pressure to the catheter for risk of damaging the fluid-filled catheter and telemetry unit. A pair of vessel cannulation forceps (5 1/8" Roboz Surgical Instruments, Rockville, MD) can be used to successfully advance the catheter without damaging the unit. Pleural pressure should be monitored during these procedures to ensure an optimal signal is obtained. Pleural pressures during isoflurane-induced anesthesia should be between approximately 8 and 20 cm H_2O. The signal may be altered by slowly moving the catheter up and down the channel in the esophagus. Once a maximal and

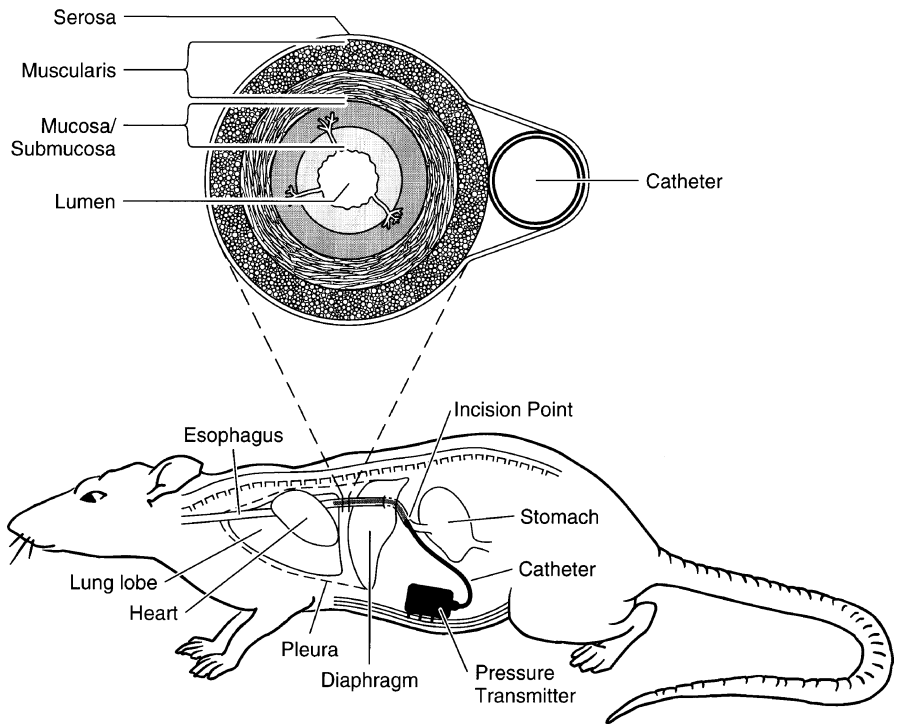

Fig. 6.4 Drawing of a rat showing placement of the pressure-sensitive subpleural catheter and radiotelemetry transmitter for chronic measurement of pleural pressure in conscious animals. The enlargement is a cross section through the esophagus showing the position of the catheter between the serosal and muscularis layers

acceptable pleural pressure signal is obtained, the catheter is secured in place using medical grade tissue adhesive (Vetbond™ or equivalent) and a small cellulose patch. The body of the transmitter unit is secured to the abdominal wall with nonabsorbable suture during the closure of the abdominal musculature. The skin layer is closed with absorbable suture and/or surgical wound clips.

6.2.1.3 Surgical Recovery and Training

If wound clips are used, they are removed approximately 8–10 days post surgery. Rats are placed in clean polycarbonate boxes with soft bedding for approximately 7–10 days, observed daily for any signs of distress, and weighed at least twice weekly. A rat may initially lose 10% of its body weight after surgery, but should begin gaining weight by day 5 post surgery. Prior to the initiation of a study, all rats must be trained to the plethysmograph chamber by placement in the chamber on three to five occasions. The first training session should be conducted prior to surgery to eliminate any rat that does not accept the chamber. The duration of time in the chamber should be at least equal to the time selected in the study. Rats can tolerate these types of chambers for up to a maximum of approximately 6 h.

6.2.2 Lung Airflow and Lung Mechanics Measurements

Changes in lung airflow are measured in conscious, restrained rats using a head-out, volume displacement plethysmograph chamber (approximately 1–3 L capacity) (Fig. 6.5). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber. A seal is made around the neck using neoprene collar (1/8 in. thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately $\pm$ 2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA) and the pressure changes converted to flow rates using a pneumotach port (1 in. diameter opening with six layers of 325 mesh stainless steel wire cloth). The analog flow signal is conditioned using a preamplifier and then converted to a telemetry (frequency) signal using a voltage analog to frequency converter (Model C12V, Data Sciences International, Inc. (DSI), St. Paul, MN). A telemetry receiver (Model RLA1020 or RPC-1, Data Sciences International, Inc., St. Paul, MN) is placed beneath the plethysmograph chamber to transmit the pleural pressure telemetry signal in parallel with the chamber flow signal to a software

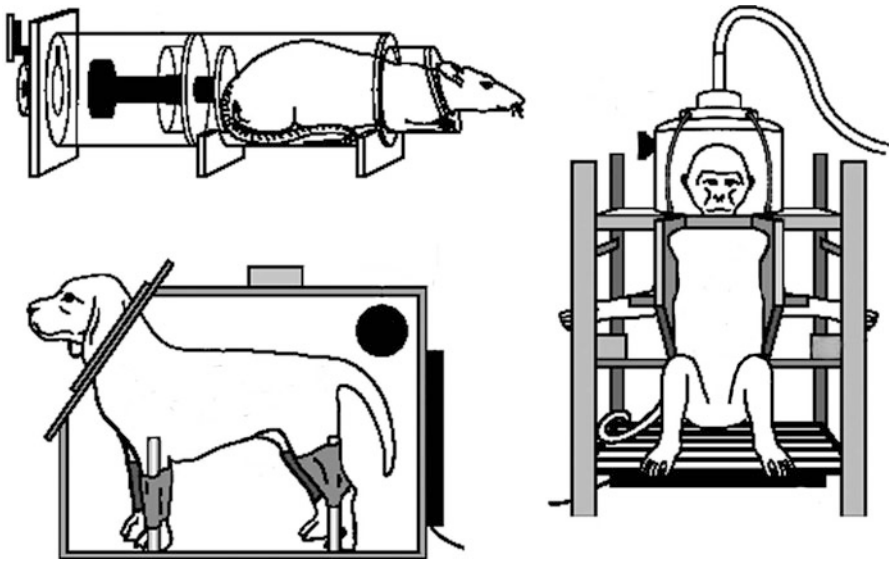

Fig. 6.5 Plethysmograph chambers for the direct measurement of ventilatory parameters. The rat and dog are in "head-out" chambers, while the monkey is in a "head-enclosed" chamber. For all chambers, an airtight seal is made around the neck using a 1/8 in. thick neoprene collar

application (e.g., DSI Dataquest ART analog system) that converts the telemetry signals into calibrated analog voltage signals and places the signals in phase by correcting for any differences in signal transmission times. The analog signals are then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory and lung function parameters (see Figs. 6.1 and 6.2).

6.3 Respiratory Function in Monkeys and Dogs: Restrained Methodologies

PURPOSE AND RATIONALE

Species other than the rat may be required to address specific study requirements. For example, the development of humanized monoclonal antibodies or other biotechnology-derived proteins requires species that have homologous target proteins and do not produce an antigenic response to the drug. In such cases, the nonhuman primate is generally required. Since both dogs and monkeys are used in toxicology studies of new drugs, either of these species may also be required to investigate pathological changes that are suspected of having an effect on the function of the respiratory pumping apparatus or gas exchange unit. Since telemetry transmitters with sufficient power to transmit two pressure signals have been developed for larger animals, the use of monkeys and dogs also has the advantage of allowing the measurement of both respiratory and cardiovascular functions. The procedure below is described for the cynomolgus monkey (*Macaca fascicularis*); however, this procedure is also applicable to the dog or other large animals.

6.3.1 Pleural and Arterial Pressure Measurements

6.3.1.1 Catheter Placements

Pleural pressure is measured chronically in conscious monkeys by surgically implanting a fluid-filled polyurethane catheter (length = 35 cm; O.D. = 1.2 mm) attached to a pressure-sensitive radiotelemetry transmitter (Model TL11M3-D70-PCP, Data Sciences International, Inc., St Paul, MN) beneath the serosal layer of the esophagus and within the thoracic cavity. Arterial pressures may also be measured by implanting a second fluid-filled polyurethane catheter (length = 35 cm; O.D. = 1.2 mm) attached to the same radiotelemetry transmitter into the abdominal aorta.

6.3.1.2 Surgical Procedure

Monkeys are premedicated with acetylcholine (0.025 mg/kg, i.m.) and flunixin meglumine

(1 mg/kg, i.m.). Anesthesia is induced with ketamine (10 mg/kg, i.m.) and maintained with isoflurane (1–3%) delivered by inhalation in 100% oxygen. The monkey is then placed in semi-dorsal recumbency, scrubbed, and draped for aseptic surgery. A feeding tube is passed down the esophagus and into the stomach. A midline laparotomy is performed and the body of the telemetry unit is sutured to the abdominal wall. Two silastic retaining beads are placed 5 cm from the tip of one of the pressure catheters, and two retracting sutures are placed in the stomach just below the cardia. The esophagus is identified at the point where it passes through the diaphragm and an anchoring suture is pre-placed at this site. A small incision is made through the serosal layer of the esophagus and a modified groove director is inserted between the serosal and muscularis layers and advanced cranially through the diaphragm along the dorsolateral aspect of the esophagus, using the feeding tube as a guide. The pressure catheter is advanced into the pleural cavity until the beads are located at the pre-placed suture and the groove director is withdrawn. The pleural pressure is visually verified and a negative deflection is confirmed with each respiratory effort. The catheter is either retracted or advanced until a maximal change in pressure (>4 mmHg) was obtained. The catheter is secured using the pre-placed suture.

To monitor arterial blood pressure, a 4 cm incision is made over the right femoral region and the femoral artery is isolated. A trocar is used to pass the blood pressure catheter from the abdomen to the femoral incision. An arterotomy is made in the femoral artery and the catheter introduced 10 cm into the artery and secured using standard techniques.

6.3.1.3 Surgical Recovery and Training

After surgery, the monkeys are allowed to recover for at least three weeks prior to the start of the study. All monkeys are observed daily for signs of pain or distress, and body weights are obtained at least weekly. All monkeys should be acclimated to handling, chair restraint, and the helmet used for respiratory measurements on at least three occasions prior to the start of each study. In addition, prior to implantation of the telemetry device, each monkey should be acclimated to the restraint chair and helmet for approximately 60 min on at least six occasions to ensure that the animals have the appropriate temperament.

6.3.1.4 Lung Airflow and Lung Mechanics Measurements

Changes in lung airflow are measured in conscious, restrained monkeys using a restraint chair equipped with a clear plastic helmet that seals around the neck and isolates the head from the rest of the body (see Fig. 6.4). The helmet is adapted to serve as a volume displacement plethysmograph by attaching a pneumotachometer (Model 4500A, Hans Rudolph, Inc., Kansas City, MO). A bias flow of approximately 5 L/min of room air is used to ensure that all monkeys have an adequate air supply for breathing. The helmet is cylindrical and has a height of 13.5 cm, a diameter of 20 cm, and an internal volume of approximately 4.2 L. Room air is pulled into the helmet through the pneumotach attached to the upper side of the helmet and is exhausted through six evenly spaced openings around the base of the helmet to ensure uniform flow. A vacuum system is attached to the output line of a six-port manifold at the top of the helmet using flexible plastic tubing (I.D. = 1.27 cm) with the openings at the base of the helmet connected to the manifold using flexible plastic tubing (I.D. = 0.635 cm).

Pressure changes within the helmet are measured using a differential pressure transducer with a sensitivity of approximately ± 2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA). The analog flow signal is conditioned using a preamplifier and then converted to a telemetry (frequency) signal using a voltage analog to frequency converter (Model C12V, Data Sciences International, Inc., St. Paul, MN). The restraint chairs are positioned in close proximity to individual telemetry receivers (Model No. RMC-1, Data Sciences International, Inc., St. Paul, MN) to ensure signal transmission. The pleural pressure signal is transmitted in parallel with the chamber flow signal to a software application (e.g., DSI Dataquest ART analog system) that converts the telemetry signals into calibrated analog voltage signals, and places the signals in phase by correcting for any differences in signal transmission times. The analog signals are then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory and lung function parameters (see Figs. 6.1 and 6.2).

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6.4 Respiratory Function in Monkeys and Dogs: Non-restrained Methodologies

PURPOSE AND RATIONALE

Methods currently available for measuring ventilatory parameters in conscious monkeys and dogs utilize a head-out or head-enclosed plethysmograph chamber, or a face mask equipped with a pneumotachograph attached to the snout of the animal. These methods require restraint and, consequently, allow for only short, periodic measurements. Because of these limitations, respiratory inductive plethysmography (RIP) was recently adopted for use as a possible method for continuous monitoring of respiratory parameters in non-restrained dogs for extended periods of time. A similar methodology is currently being developed for monkeys. However, a validation of this methodology in the monkey has not yet been published. RIP utilizes straps containing inductive coils placed around the thorax and abdomen to measure lung volume changes. A continuous, low-voltage electrical current is passed through the inductive coils and current changes, which are proportional to the changes in length of the inductive coil straps, are produced by the expansion and contraction of the thorax and abdomen during breathing. Changes in the inductive current are used to continuously monitor changes in the circumference of the thorax and abdomen and analysis software can be used to calculate volume changes and breathing rate. Linear measurements of both thorax and abdomen are obtained based on the principle that lung expansion consists of two degrees of freedom of motion. These motions are independent and consist of the circumferential expansion and elongation of the lung, the latter of which is caused by depression of the diaphragm and is measured by expansion of the abdomen. Impedance technology combined with telemetry has also been used to measure thoracic movement during breathing in dogs. This methodology, however, does not include separate measurement of abdominal movement and, consequently, its application for assessing ventilator and airway resistance measurements in conscious, non-restrained dogs and monkeys is limited.

A comparison with a standard method for measuring ventilation (facemask with attached pneumotachograph) demonstrated that ventilatory parameters (tidal volume, respiratory and minute volume) can be accurately measured in the conscious dog and, importantly, that changes in posture do not have a significant effect on tidal volume measurements. The absence of postural effects allows this methodology to accurately assess ventilatory parameters in ambulatory subjects. RIP expands current methodologies in that it allows for continuous monitoring of ventilatory parameters over extended periods of time. This added capability will allow for respiratory monitoring during both the awake and sleep states, which is significant since control of respiratory drive differs between the awake and sleep states and drug-induced effects such as sleep apnea or sleep-disordered breathing can have adverse health consequences. Combined with cardiovascular telemetry, this methodology will also allow for the combined monitoring of cardiovascular and respiratory parameters in conscious, non-restrained dogs.

PROCEDURE

6.4.1 Animal Acclimation

Non-restrained dogs are acclimated to wearing a custom-fit jacket, which contains the inductive coil straps and an accelerometer, by placing the jacket on each animal for increasing periods of time up to a target time of approximately 26 h. Acclimation to the jacket on at least one occasion for a period of 4–6 h and a second occasion for 20–24 h is considered an appropriate acclimation schedule, as indicated by the acceptance of the animal of the jacket and no overt evidence of animal stress. Only approximately 10% of dogs are rejected because of poor behavior related to acceptance of these procedures.

6.4.2 Ventilatory Measurements

Ventilatory parameters and an index of airway resistance (phase angle) can be obtained by measuring

thoracic and abdominal excursions using RIP. Respiratory parameters obtained using RIP involve fitting dogs with a custom-fit jacket equipped with inductive sensor straps woven into the jacket to fit around the chest and abdomen to detect chest and abdomen excursions. Respiratory data are transmitted wirelessly from a transmitter inside the jacket to a receiver for input into a data acquisition and analysis system (e.g., Data Sciences International, St. Paul, MN or EMKA Technologies, Falls Church, VA). The analysis software sums the thoracic and abdominal waveforms for each breath to calculate respiratory rate, tidal volume, minute volume, inspiratory and expiratory times, and flows and apneic times (times between breaths). The phase angle (θ) is determined for each breath by plotting the abdominal movement on the abscissa and the thoracic movement on the ordinate to produce figures that are commonly referred to as Konno-Mead loops. The phase angle is calculated using the following equation: $\theta = \sin^{-1}(M/S)$, where θ is the phase angle, M is the distance between the intercepts of the thoracic-abdominal loop on a line drawn parallel to the *x*-axis and which is placed at one-half the distance between the maximal and minimal thoracic excursion, and S is the maximal abdominal excursion (see Fig. 6.3).

The distance between the inductive straps must be adjusted such that one strap is located at the level of the fourth to fifth rib and the other located just caudal to the ribcage. These locations provided for stable measurements, as strap adjustments are not required for measurement periods of up to 24 h. Volume measurements should be monitored during the adjustment of inductive strap tensions to ensure maximal sensitivity. Certain behaviors (e.g., eating/drinking, barking, jumping, panting) noted in non-restrained dogs can produce abnormal volume waveforms that are excluded from data analysis. These behaviors are relatively infrequent and generally account for less than approximately 5% of the total recording time over a 24 h period.

To calibrate volume measurements of the RIP system, a qualitative diagnostic calibration (QDC) and a fixed volume calibration are performed each time the jacket is placed on an animal. QDC computes a calibration factor (k) that defines the proportional relationship between abdominal and thoracic volumes using breaths of constant tidal volume. Fixed volume is used to calculate the total volume amplification factor (M) and uses a known tidal volume that is obtained using a pneumotachograph attached to a facemask that fits over the snout of the dog. Analog flow waveforms from the pneumotachograph are detected by a differential pressure transducer (e.g., Model MP-45, Validyne Engineering, Northridge, CA). The calibration procedure involves (1) fitting the animal with a jacket containing the inductive straps and placing the animal on a table in a prone (standing) position, (2) allowing the animal to take normal breaths for a period of approximately 5 min and calculating the ratio of the standard deviations of the abdominal (ΔV_{AB}) to thoracic (ΔV_T) excursions (ratio = k), (3) placing a face mask with a calibrated pneumotachograph on the animal's snout, and (4) adjusting the amplifier gain until the pneumotachograph and RIP system tidal volume values are identical (amplifier multiple = M). The calibrated tidal volume (ΔV_{cal}) is then calculated using the equation $\Delta V_{cal} = M[(K\Delta V_T) + \Delta V_{AB}]$.

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6.5 Distinguishing Central from Peripheral Nervous System Effects of Drugs

PURPOSE AND RATIONALE

Many drugs stimulate or depress ventilation by selective interaction with the central or peripheral nervous system. Thus, distinguishing a central from peripheral site of action is an important part of characterizing the

mechanism of a drug-induced change in ventilation. Noninvasive methods for evaluating the central and peripheral effects of drugs in conscious animals have been developed and involve measuring effects of the test drug on agents known to selectively stimulate ventilation by activating either central or peripheral mechanisms. An assay developed by Murphy et al. (1995) provides a simple, noninvasive method for distinguishing the central from peripheral nervous system effects of respiratory depressant drugs in conscious rats. This technique, however, can also be applied to larger animals such as the dog or monkey. The procedure involves exposing rats for 5 min to an air mixture containing 8% CO_2 (central chemoreceptor stimulant) followed by an intravenous bolus injection of 300 μg/kg sodium cyanide (peripheral chemoreceptor stimulant) and comparing the changes in minute volume and mean inspiratory flow (respiratory drive) before and after drug treatment. Morphine sulfate (3 mg/kg, intravenous), an opioid analgesic that depresses ventilation through a central mechanism, and carotid body denervation (peripheral depressant) were used to initially develop this procedure. The central respiratory depressants phenobarbital (200 mg/kg), xylazine (3 mg/kg), L-2-phenylisopropyladenosine (L-PIA) (1 mg/kg), and gamma-hydroxybutyric acid (GHBA) (300 mg/kg) were subsequently given intravenously to confirm the validity of this procedure. Using this assay, a centrally acting respiratory depressant can be identified by its inhibition of CO_2-induced stimulation of minute ventilation and enhancement of the NaCN-induced stimulation of mean inspiratory flow, whereas a peripherally acting respiratory depressant can be identified by its lack of effect on CO_2-induced stimulation of minute ventilation and its inhibition of NaCN-induced stimulation of mean inspiratory flow.

PROCEDURES

6.5.1 Gas and Sodium Cyanide Exposures

Rats are exposed to gas mixtures using a two-chambered plethysmograph. The body of the animal is enclosed in a head-out chamber that is used to measure ventilatory parameters, while the head is enclosed in a cylindrical plastic headpiece that is attached to the front of the body chamber. A neoprene collar (1/8 in. thick) placed around the neck of the animal is used to seal the plethysmograph chamber and separate the head and body chambers. Specific gas mixtures are delivered from compressed gas tanks to the head chamber at a rate of 2 L/min. All animals are acclimated to the chamber by placement in the chamber for at least three 15-min periods on three separate occasions with compressed air flowing through the head chamber at a rate of 2 L/min. Prior to drug treatment, a catheter is inserted into a tail vein for administration of sodium cyanide. The catheter is exteriorized from the plethysmograph chamber through an opening that is made airtight by sealing with clay. Tail vein catheterization is accomplished using a 1/2 to 3/4 in. butterfly needle (23–25 gauge) attached to an extension set with syringe attachment. The gas mixtures contain either normal breathing air (21% O_2, 79% N_2) or elevated CO_2 (8% CO_2, 21% O_2, 71% N_2). All animals are first exposed to the normal air mixture for 5–10 min and then to the elevated CO_2 mixture for 5 min. The mean value for the 5–10 min exposures to air and high CO_2 are calculated for minute volume and the difference used to quantify the stimulatory effect of CO_2. Following CO_2 exposure, rats are exposed to the normal air mixture until the ventilatory parameters return to normal (generally 5–10 min). Sodium cyanide (300 μg/kg) is then administered as a bolus injection using the tail vein catheter. The peak change in mean inspiratory flow is measured during the 1–3 min period of ventilatory stimulation following the sodium cyanide injection.

6.5.2 Measurement of Ventilatory Parameters

Ventilatory parameters are measured using a head-out, volume displacement plethysmograph chamber (approximately 1–3 L capacity). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber (see Fig. 6.4). A seal is made around the neck using neoprene collar (1/8 in. thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately ± 2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA) and the pressure changes converted to flow rates using a pneumotach port (1 in. diameter opening with six layers of 325 mesh stainless steel wire

cloth). The analog flow signal is conditioned using a preamplifier and then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory parameters. The values for minute volume and mean inspiratory flow are determined for each breath and average values calculated for every 0.1 min. The mean inspiratory flow (a measure of respiratory drive) is calculated by dividing tidal volume by inspiratory time and minute volume is calculated by obtaining the product of tidal volume and respiratory rate (see Fig. 6.1).

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6.6 Continuous Measurement of Expired CO_2

PURPOSE AND RATIONALE

Changes in ventilatory parameters help define the mechanism and potential cause of a respiratory disorder, whereas changes in the partial pressure of arterial CO_2 ($PaCO_2$) define the physiological consequences, or liability, of a ventilatory change. A ventilatory disorder resulting in a decrease in $PaCO_2$ is defined as hyperventilation, whereas an increase in $PaCO_2$ is defined as hypoventilation. Obtaining samples of arterial blood for CO_2 analysis by needle puncture or arterial catheterization during ventilatory measurement is not considered practical since the acute insertion of the catheter or the restraint procedure needed for needle insertion and blood collection can often interfere with respiratory measurements. This is especially true for smaller animals. Furthermore, a blood sample only assesses blood gas status over the short time period that the sample is taken. Measuring changes in the peak concentration of expired CO_2 (end-tidal CO_2) during each breath has been developed as an alternative method for monitoring arterial CO_2 tension in humans and larger animals. End-tidal CO_2 measurements can be performed noninvasively and can be used for the continuous monitoring of arterial CO_2 during ventilatory measurements in conscious animals. With the use of microcapnometry, Murphy et al. (1994) developed a technique that could monitor end-tidal CO_2 in conscious rats during ventilatory measurements. The development of this technique in rats is important as the rat is a model commonly used for assessing ventilatory function in safety pharmacology. This assay was validated by showing that the changes in end-tidal CO_2 were sensitive to changes in ventilation, are sensitive to drug-induced respiratory stimulation or depression, and are quantitatively (linearly) related to changes in arterial CO_2 and O_2 tensions and arterial blood pH.

PROCEDURES

6.6.1 Ventilatory Measurements

Ventilatory parameters are measured using a head-out, volume displacement plethysmograph chamber (approximately 1–3 L capacity). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber (see Fig. 6.4). A seal is made around the neck using neoprene collar (1/8 in. thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately $\pm$ 2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA) and the pressure changes converted to flow rates using a pneumotach port (1 in. diameter opening with six layers of 325 mesh stainless steel wire cloth). The analog flow signal is conditioned using a preamplifier and then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory parameters. The values for tidal volume, respiratory rate, and minute volume are determined for each breath and average values calculated for every 0.1 min.

6.6.2 End-Tidal CO_2 Measurement

End-tidal CO_2 (peak expired CO_2) was measured for each breath with a microcapnometer (Microcapnometer, model 0151-003 L, Columbus Instruments, Columbus, OH, USA). This

microcapnometer is uniquely suited for measurement of expired CO_2 in rats and other animals with low minute volumes (200–400 mL/min) since it utilizes relatively low flow rates (5 or 20 cc/min) compared with the standard capnometers that require 150–200 cc/min. This capability is achieved by using a low-pressure, high-velocity principle for analyzing the sampled gas. Airflow to the microcapnometer is maintained at 20 mL/min and is collected through a Teflon catheter (I.D. = 0.76 mm). Carbon dioxide concentrations are measured spectrophotometrically using standard infrared gas sensor technology and the values are expressed as percentage of total dry air volume. Average values for end-tidal CO_2 are calculated and expressed as numerical output every 0.1 min by the microcapnometer. The waveform and numerical data can be acquired by a computer system for storage and analysis. The microcapnometer is calibrated using an analyzed gas mixture containing 5% CO_2 and 95% O_2.

To convert the percentage CO_2 values measured by the microcapnometer (in dry air and at room temperature) to partial pressure values of CO_2 present in the alveoli (P_ACO_2), the following formula is used:

$$P_ACO_2(\text{mmHg}) = \%CO_2 \times [\text{BP}(\text{mmHg}) - P_AH_2O(\text{mmHg})]$$

where BP is the barometric pressure measured at room temperature and P_AH_2O is the partial pressure of H_2O in alveolar gas at body temperature (39°C) and saturated with water.

End-tidal CO_2 is monitored in conscious rats by using a mask that is fitted to the snout of the rat. Since rats are obligate nasal breathers, enclosure of the mouth is not necessary. The distal tip of the nasal mask has an opening (I.D. = 3.5 mm) for breathing through and an attachment site for the tube connecting to the microcapnometer. The mask is held in place with a harness that fits over the head of the animal, and air samples are collected from the tip of the mask. To ensure a comfortable and tight seal around the snout, the inner edge of the plastic mask is coated with silicone foam (Life care®, Lafayette, CO, USA). Dead space associated with the mask is approximately 0.06 cc. All animals should be acclimated to the plethysmograph chamber, harness, and mask on at least five occasions prior to end-tidal CO_2 measurements.

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Safety Pharmacology in Metabolism Pharmacology

7

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7.1 General Considerations

Safety pharmacology is defined as those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above (ICH-guideline S7A 2001). Safety pharmacology in metabolism pharmacology is of secondary importance in comparison to the safety pharmacology of vital functions (safety pharmacology core battery: cardiovascular, respiratory, and central nervous systems) and therefore listed under "Follow-up and Supplemental Safety Pharmacology Studies (ICH-guideline S7A 2001: Sect. 2.8.2)." In this section of the guideline, the subjects of safety pharmacology in metabolism pharmacology are listed under the topics of "gastrointestinal system" (ICH-guideline S7A 2001: Sect. 2.8.2.3) and "other organ systems" (ICH-guideline S7A 2001: Sect. 2.8.2.4).

In this chapter, pharmacological methods are described for the characterization of candidate compounds on their effects on the gastrointestinal functions (gastric acid secretion, bile secretion, exocrine pancreatic secretion, gastrointestinal motility) and their gastrointestinal injury potential. In addition, there are pharmacological methods described with respect to elucidate the undesirable effect potential of compounds on intermediary carbohydrate and lipid metabolism resulting in hypo- or hyperglycemia, hyperlipidemia, and insulin resistance, which could finally provoke life-threatening hypoglycemia or on the long-run diabetes, atherosclerosis, and obesity.

In principle, every pharmacological assay, which is described in detail by Vogel (2008), can be used for the safety pharmacological characterization of a candidate

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compound with respect to safety pharmacology in metabolism pharmacology (Vogel (2008): Activity on the gastrointestinal tract (Chapter J), antidiabetic activity (Chapter K), anti-obesity activity (Chapter L), and anti-atherosclerotic activity (Chapter M). Here, in this part of the book, selections of these assays are presented which primarily meet the ICH guideline (ICH-guideline S7A 2001) and which are appropriately adapted to the characterization of candidate compounds with a different primary indication for the assessment of their pharmacological side effect potential on metabolism pharmacology.

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7.2 Gastrointestinal System

7.2.1 General Considerations

If a candidate compound with a totally different primary indication causes additionally an inhibition of gastric acid secretion, this finding may be assessed as additionally beneficial and therefore does not represent a safety concern, irrespective whether these pharmacological effects occur in the pharmacological dose range for the primary pharmacological effect or at suprapharmacological doses as usually used for safety pharmacological studies of a candidate compound. The opposite situation that a candidate compound for a different primary indication causes additionally a stimulatory effect on gastric acid secretion represents always a safety concern due to the risk for induction of gastrointestinal ulcers.

On the other hand, an unrealized antisecretory effect on gastric acid secretion, which might occur at suprapharmacological doses as used for toxicity studies, can become obvious during cancerogenicity studies in rats, resulting in the finding of carcinoids (ECL-cell proliferation) due to the long-lasting increase in gastric pH with subsequently elevated gastrin levels, which functionally and tropically control gastrointestinal enterochromaffin-like cells (ECL cells). This connection between gastric pH, gastrin level, ECL-cell proliferation, and gastric carcinoids has first been demonstrated for the proton pump inhibitor omeprazole (Arnold et al. 1986; Creutzfeldt et al. 1986; Ekman et al. 1985).

Table 7.1 Four main gastrointestinal functions investigated by appropriate test methods for detection of the side effect potential of new drug candidates

Function	Assay/test
Gastric secretion	Pylours-ligated rat
Gastric-intestinal injury	Gastrointestinal ulceration in rats
Transit time	Propulsive gut motility in mice or rats (charcoal)
Gastric emptying assay	Stomach emptying in mice or rats (phenol red)

An acute stimulatory effect on gastric acid secretion as well as a direct effect on reduction of gastric mucus or bicarbonate secretion may finally result in gastric ulcers. An ulcerogenic side effect potential is always a safety issue of a candidate compound and should be carefully investigated. Since nearly 100 years, it has well been known that nonsteroidal anti-inflammatory drugs (NSAID) cause gastric ulcerations, but their molecular mode of action, the inhibition of the cyclooxygenase (COX), the key enzyme in prostaglandin (PGG_2, PGH_2) synthesis, has first been proposed since the early 1970s (Vane 1971). In the meanwhile, different isoforms of the COX enzyme have been identified (Smith et al. 1996). The constitutionally expressed isoenzyme COX-1 represents the dominant isoform in gastric mucosa. To get rid of the ulcerogenic side effect potential of NSAIDs, more selective inhibitors for COX-2 have been developed (Kurumbail et al. 1996; Wolfe 1998).

In the following chapters, a selection of pharmacological methods in gastroenterology is presented which primarily meet the ICH guideline (ICH-guideline S7A 2001) and exceed present practice. To date, only four main tests appear to be widely used by pharmacologists to study gastrointestinal functions in safety pharmacology (Table 7.1; Harrison et al. 2004).

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7.2.2 Gastric Acid Secretion (Gastric Ph Measurement)

7.2.2.1 Gastric Acid Secretion in Pylorus-Ligated Rats

PURPOSE AND RATIONALE

The secretory potential of a candidate compound might be under safety aspects much more problematic due to its ulcerogenic potential compared to the antisecretory potential of a candidate compound with a different primary indication.

A simple and reliable method for the measurement of gastric acid secretion and production of gastric ulcers in the rat based on ligature of the pylorus has been published by Shay et al. (1945). Gastric acid secretion can be stimulated by histamine, carbachol, or gastrin. Candidate compounds with antisecretory potential inhibit stimulated gastric acid secretion. The secretory potential of a candidate compound can be studied during basal conditions without administration of a secretagogue.

PROCEDURE (MODIFIED AFTER Shay et al. 1945)

This study is performed in conscious rats with a body weight of 150–170 g. Food is withdrawn 16 h before beginning of the study with water available ad libitum. Following pylorus ligation, performed under ether anesthesia, the candidate compound is administered intraperitoneally (i.p.) or intraduodenally (i.d.). Gastric acid secretion is either studied under basal conditions or stimulated by subcutaneous (s.c.) injection of a secretagogue. The secretagogue is injected again 1 h later. Three hours after the beginning of the experiment, the animals are killed, the stomach excised, and the accumulated gastric juice collected. The three different secretagogues employed are histamine (2×20 mg/kg s.c.), desglugastrin (2×400 µg/kg s.c.), or carbachol (2×40 µg/kg s.c.).

EVALUATION

The volume of the collected gastric juice is measured. Acid concentration is measured by titration against 100 mM NaOH to an endpoint of pH 7. Total acid output (mmol H^+/3 h) is calculated, and percent inhibition of the treated rat group is calculated against the control group. Using various doses, dose-response curves can be established for gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0% corresponds to no and 100% to maximal stimulated gastric acid output.

CRITICAL ASSESSMENT OF THE METHOD

The pylorus-ligated rat has been proven to be a valuable method to evaluate the secretory (vs basal secretion) as well as the antisecretory potential (vs stimulation with histamine, gastrin, or carbachol) of a candidate compound with various secretory or antisecretory mechanisms of action.

For the safety pharmacological evaluation, this method allows the administration of necessary high doses by i.d. or i.p. administration probably as suspension (methylcellulose) irrespective of solubility issues of the candidate compound.

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7.2.2.2 Gastric Acid Secretion in Anesthetized Stomach-Lumen Perfused Rats

PURPOSE AND RATIONALE

Originally, Gosh and Schild (1958) introduced a method for the continuous recording of gastric acid secretion in the stomach-lumen perfused anesthetized rat. In this model, gastric acid secretion can be stimulated by histamine, carbachol, or gastrin. Candidate compounds can be pharmacologically characterized for their gastric acid antisecretory potential during stimulated gastric acid secretion.

PROCEDURE (MODIFIED AFTER Gosh and Schild 1958)

Gastric acid secretion in anesthetized rats with a body weight of 300–350 g is used. The animals are fasted for 18 h prior to the experiment with free access to water. Anesthesia is induced by ketamine (20 mg/kg) plus pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h for maintaining constant anesthesia). Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The jugular veins are then exposed and cannulated with polyethylene tubes beveled at the tip. The abdomen is opened through a midline incision. The esophagus and pylorus are ligated, and a double lumen perfusion cannula is inserted and fixed in the forestomach. The stomach is perfused continuously with warm (37°C) saline at a rate of 1 mL/min. The perfusate is collected at 15-min periods and its acid concentration measured. Histamine (10 mg/kg/h), desglugastrin (100 µg/kg/h), or carbachol (30 µg/kg/h) are administered by i.v. infusion into the jugular vein after a basal period of 45 min. Ninety minutes after the onset of the secretagogue infusion, acid output has reached a stable plateau (Fig. 7.1). As soon as acid secretion has reached a plateau, candidate compound or standard is injected intravenously.

EVALUATION

The perfusate is collected at 15-min periods, and its acid concentration measured by titration against 100 mM NaOH to an endpoint of pH 7 and acid output

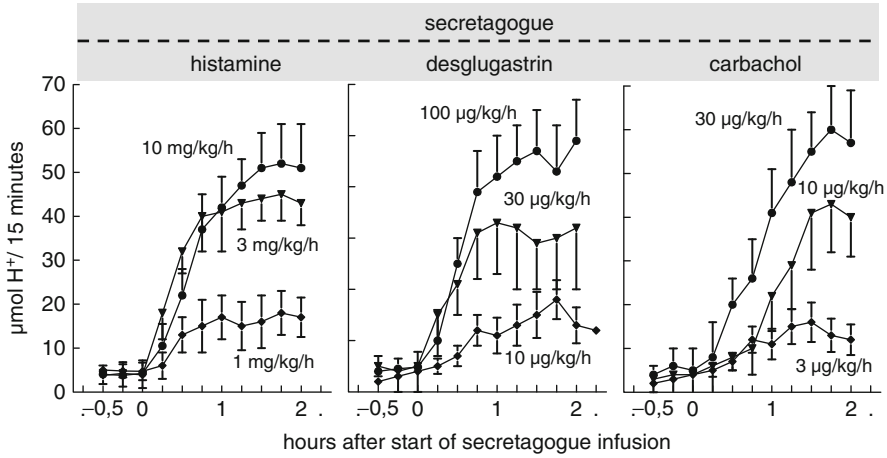

Fig. 7.1 Dose dependency of secretagogue infusion on gastric acid output in stomach-lumen perfused rats. Values are mean ± SEM, $n = 4–6$

(μmol H^+/15 min) are calculated. Using various doses of the candidate compound and of a standard, dose-response curves can be established, and activity ratios with confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

For the specific pharmacological assessment of inhibitors of gastric acid secretion, like H_2-blockers, anticholinergics, H^+/K^+-ATPase inhibitors, this method reveals valid results with respect to the antisecretory potential of the candidate compound. Limitations of this methods with respect to safety pharmacological assessment of candidate compounds are (1) only parenteral administration of the candidate compound, preferentially i.v., is feasible and should be preferred and (2) only antisecretory putative side effects can be investigated; this method is of limited relevance to study secretory side effect potential of candidate compounds.

For safety pharmacological assessment of a candidate compound with a totally different inherent primary indication, its antisecretory potential at suprapharmacological doses has to be studied. Therefore, whether this method can be used for the safety evaluation depends on the solubility of the candidate compound for i.v. administration of suprapharmacological doses.

As different pathways of stimulation are exclusively initiated by histamine, gastrin, or carbachol, it is possible to estimate the potential interaction of the candidate compound with the secretory pathways of acid secretion (Herling and Weidmann 1996): Candidate compounds affecting:

1. The H_2-receptor inhibit histamine- and gastrin-stimulated gastric acid secretion.
2. The gastrin receptor inhibit only gastrin-stimulated gastric acid secretion.
3. The muscarinic receptor inhibit only carbachol-stimulated gastric acid secretion.
4. Carboanhydrase activity inhibit gastric acid secretion, irrespective of the kind of stimulation.
5. H^+/K^+-ATPase (gastric proton pump) inhibit gastric acid secretion, irrespective of the kind of stimulation.

MODIFICATIONS OF THE METHOD

Burn et al. (1952) described the evaluation of substances, which affect gastric secretion using perfusion of the stomach in anesthetized cats.

Lawrence and Smith (1974) described the measurement of gastric acid secretion in the rat by conductivity. The stomach of an anesthetized rat is continuously perfused with 2 mL/min of an isotonic (0.308 M) glucose solution at 37°C. The conductance of a solution depends on the total ion concentration and is therefore not specific for hydrogen ions. Since hydrogen ions have an equivalent conductance nearly five times greater than any other ion found in gastric juice and since they are secreted in a far greater concentration than other ions, conductivity measurements can be regarded as a relatively specific measure of hydrogen ions. Using Mullard conductivity cells (type E 791/B) and a commercially available meter (Phillips PW 9501), simultaneous measurements in six rats were performed.

Gallo-Torres et al. (1979) described in detail a method for the bioassay of antisecretory activity in the conscious rat with acute gastric fistula with additional collection of the biliary and pancreatic secretion by means of a catheter in the common bile duct. The gastric secretions are collected by gravity via a cannula in the most gravity-dependent site of the glandular stomach.

Larsson et al. (1983) described studies in the acutely vagotomized rat. Truncal vagotomy is performed under ether anesthesia by cutting the dorsal and ventral branches of nervus vagus just below the diaphragm. The pylorus is then ligated, and a polyethylene catheter (PP 200) is inserted into the duodenum, close to the pylorus. Each animal is placed in a modified Bollman cage and is allowed to recover at least 1 h before the experiment. Gastric juice is collected by free drainage in 30 min samples.

Herling and Bickel (1986) showed that gastric acid secretion in stomach-lumen perfused rats can be stimulated in vivo on the subreceptor level by IBMX (phosphodiesterase inhibitor) and forskolin (nonreceptor activation of the adenylate cyclase). H^+/K^+-ATPase inhibitors and H_2-antagonists show, according to their different modes of action, also a different inhibitory profile in this assay.

Hammer et al. (1992) used anesthetized female Sprague-Dawley rats weighing 200–320 g. After insertion of a tracheal cannula, a 3-mm silicon tubing is placed through the mouth and advanced to the stomach. The tubing is tied to the esophagus at the neck. A 4-mm drainage tube is inserted into the stomach through a laparotomy incision and an incision in the duodenum and ligated in place at the pylorus.

Gastric perfusate (0.9% saline at 37°C) is collected on ice every 5 min for titration to pH 7.0.

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7.2.2.3 Gastric Acid Secretion in Conscious Dogs (Chronic Heidenhain-Pouch Fistula in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Sect. 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. The preparation of a chronic gastric pouch, as described by Heidenhain in 1878, is one of the classic techniques in experimental surgery. This model has much contributed to the understanding of the physiology and pathology of the stomach and to modern techniques of abdominal surgery in man. The surgical technique has been described again in detail by deVito and Harkins (1957). A preparation of chronic denervated pouches in the rat has been described by Alphin and Lin (1959). Both preparations can be used as pharmacological models for testing antisecretory drugs.

PROCEDURE

The principle technique is demonstrated in Fig. 7.2. Dogs weighing 15–20 kg are fasted 24 h preoperatively. The abdominal surgery is performed during appropriate anesthesia (in former times by, e.g., 30 mg/kg pentobarbital sodium; nowadays, more appropriate by inhalation with halothane or isoflurane). The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with a surface disinfectant (e.g., Zephiran®-70% alcohol). Sterile drapes are applied to cover the whole surgical field. A midline linea alba incision from xiphoid to umbilicus provides excellent exposure and ease for closure. As the posterior sheath is divided, the large ventral fat pad present in dogs should be excised completely. A self-retaining retractor is applied, and the stomach is palpated for the absence of food. Then, the spleen is displaced, wrapped in warm, moist pads and laid on the ventral wall below the incision.

The stomach is pulled into the operative field. The greater curvature is held at multiple points so that the stomach is stretched out and the line of incision for the pouch is selected. The pouch should be made from the corpus of the stomach so that true parietal cell juice can be obtained. A line projected from the incisura angularis perpendicular to the proximal lesser curvature will generally fall across the junction between corpus and antrum. Appropriate division of the gastric branches of the right gastroepiploic artery at the lower end of the proposed line of transsection clears the greater curvature for 1–2 cm. The gastroepiploic artery itself should be sectioned at this site and a long rent formed on the adjacent omentum, else the omentum vessels tend to tear during subsequent manipulations.

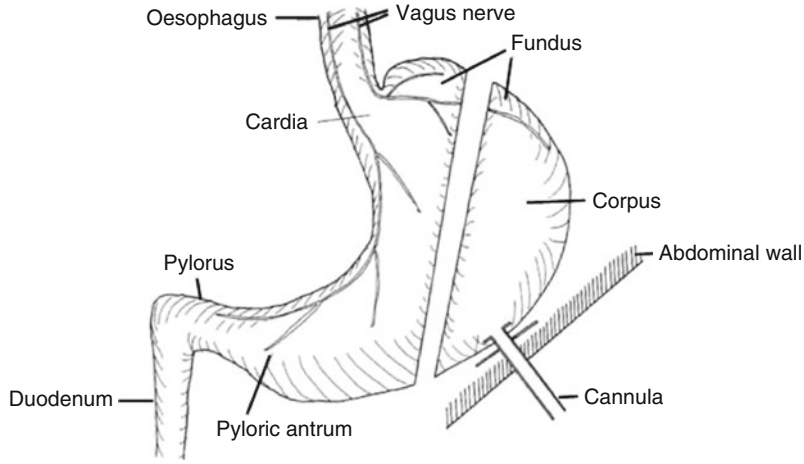

Fig. 7.2 Technique of Heidenhain-pouch fistula in dogs

An index finger is then inserted through this defect dorsal to the stomach to emerge higher on the greater curvature through the gastrosplenic ligament at the upper end of the proposed line of transsection. This portion of the greater curve is cleared for 1–2 cm. Von Petz clamps with their staplings are used to control bleeding and to avoid leakage of gastric content. The stomach should be kept stretched and flattened while the clamps are applied. After division between the staples, any bleeding is controlled, and the cut edges of the main stomach and pouch are then oversewn with continuous sutures of black silk. The suture should be of an inverting type. Surprisingly, leakage or excessive adhesions are not a problem when serosal apposition is neglected.

The pouch so formed is about 30% of the corpus volume and provides adequate secretory volume for further studies (Fig. 7.2). A cannula, made of stainless steel, 7 cm long with a beveled flange threaded at the other end is placed in the most ventral portion of the pouch through a small incision in the anterior wall. A single purse string of silk holds it in place. A double sheet of omentum is then wrapped about the pouch and the cannula before being pulled through the abdominal wall, about 3 cm to the left of the midline subcostally. It is important that the cannula be held snugly by fascia; otherwise, it will readily pull out of the pouch and abdominal wall. The linea alba is closed with a continuous suture of silk and the skin with subcuticular stitches of chromic catgut. On the outside of the cannula, a stainless steel jacket is screwed. The cannula is always open so that secreted gastric juice does not accumulate within but is drained from the pouch.

Before recovery from anesthesia, the dog receives 500 mL 5% glucose in saline intravenously. The same volume is given for 3 days postoperatively together with oral fluid ad libitum. From the fourth day onward, normal food is given. A period of 7–10 days is required for full recovery from the operation. Special care has to be taken for each animal being kept separately in a suitable cage.

For pharmacological studies, food is withdrawn 18 h prior to the experiment with water ad libitum. The animals are placed in Pawlow stands during the experiment of gastric secretion measurement, and a tube is fitted to the cannula to collect the gastric juice from the pouch for measurement of volume and acidity by titration. To test the secretory potential of a candidate compound, which might represent a direct safety concern, the candidate compound is studied under basal (nonstimulated conditions) and administered orally or by i.v. injection or infusion, and the gastric acid secretion from the pouch is measured in intervals of 15 or 30 min. The values are compared to the predrug secretion values and to a respective control group.

For testing the antisecretory potential, gastric acid secretion is stimulated either by i.v. infusions of histamine (0.1 mg/kg/h), carbachol (10 μg/kg/h), or pentagastrin (8 μg/kg/h). When stimulated gastric acid secretion has reached a stable plateau (after 1.5 h), the candidate compound is administered orally or by i.v. injection, and secreted fluid is collected at 15- or 30-min intervals and analyzed for free HCl.

EVALUATION

The secreted volume per time interval is measured. An aliquot is used for the determination of acidity by

titration against 100 mmol/L NaOH, and total acid output per time interval is calculated. The effect on volume and HCl secretion at 15- or 30-min intervals after administration of the test compound is compared with the control values. Mean inhibition of stimulated gastric acid secretion can be calculated according to the formula:

$$\text{Mean inhibition } (\%) = -\left(\left(\left(\frac{-\frac{\text{SAO}_{\text{postdrug}}}{\text{N}_{\text{postdrug}}}}{\text{AO}_{\text{predrug}}}\right) * 100\right) - 100\right)$$

$\text{SAO}_{\text{postdrug}}$ = sum of acid output per 30 min after compound administration

$\text{N}_{\text{postdrug}}$ = number of 30 min collection intervals after compound administration

$\text{AO}_{\text{predrug}}$ = acid output prior compound administration

In addition to the total acidity of the secreted juice also pepsin, total activity can be determined by appropriate enzymatic methods.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described a simplified method for isolation of a portion of the stomach as compared to the original method of Heidenhain (1878).

Gastric motility can be measured by balloon manometry of the Heidenhain pouch in the conscious dog. The animals are deprived of food for 18 h before the experiment, but water is allowed ad libitum. A latex balloon, connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB), is introduced through the fistula cannula into the accessory stomach. Changes in intragastric pressure are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of gastric motor activity. Secretin inhibits gastric motility dose dependently. After injection of gastrin or gastrin analogues, a dose-dependent increase of pressure is noted over a wide dose range.

Jacobson et al. (1966, 1967) studied gastric secretion in relation to mucosal blood flow by an antipyrine clearance technique in conscious dogs with vagally denervated gastric fundic (Heidenhain) pouches. A vagally denervated fundic pouch is so constructed that the entire arterial blood supply is delivered by the splenic artery. A noncannulating transducer (electromagnetic flowmeter) and a hydraulic occluder were implanted on the vessel.

The Heidenhain pouch preparation was used by Carter and Grossman (1978) and Kauffman et al. (1980) to study the effect of luminal pH on acid secretion evoked by topical and parenteral stimulants and the effect of topical and intravenous 16,16-dimethyl prostaglandin E_2 on gastric bicarbonate secretion.

Baker (1979) and Roszkowski et al. (1986) developed a modified Heidenhain dog pouch preparation for collecting gastric juice exclusively from the pouch during experimental periods but allowed the pouch to be an integral part of the gastrointestinal tract during nonexperimental periods. The pouch is prepared using conventional techniques, but instead of being fitted with a simple cannula through the abdominal wall, a three-way cannula is used which provides passage between the exterior orifice, the pouch, and the main body of the stomach. By inserting an appropriate adapter, passage is available only to the pouch and not to the main stomach or vice versa.

The Heidenhain pouch technique in dogs has been used for preclinical evaluation of various drugs, such as:

- A histamine H_2 antagonist by Uchida et al. (1993).
- Dual histamine H_2- and gastrin-receptor antagonists by Kawanishi et al. (1997).
- A 5-HT_4-receptor antagonist by Bingham et al. (1995).
- Another 5-HT_4-receptor antagonist by Wardle et al. (1996).
- Inhibition of motilin-induced phase III contractions by pentagastrin by Yamamoto et al. (1994).
- Peptide YY by Zai et al. (1996).
- Reversible K^+-competitive inhibitors of the gastric H^+/K^+-ATPase by Parsons et al. (1995).
- The antiulcer agent SWR-215 by Kataoka et al. (1997).
- A selective gastrin-/CCK-B-receptor antagonist by Yuki et al. (1997).
- Descroix-Vagne et al. (1993) used Heidenhain pouch preparations in cats and rabbits to study the effect of perfusion at pH 5.5 on acid and pepsin secretion.
- For identification of the KCNQ1 protein as the K^+-channel colocalized with the H^+/K^+-ATPase at the

apical membrane of the gastric parietal by studying the gastric acid inhibitory potential of the tool compound 293B (inhibitor of KCNQ1) cell by Grahammer et al. (2001).

CRITICAL ASSESSMENT OF THE METHOD

Due to the surgical procedure, the connections of the autonomic nervous system of the isolated pouch are interrupted from those of the main stomach. Therefore, basal gastric acid secretion from the pouch, which based mainly on the parasympathetic activity, is reduced.

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7.2.2.4 Effect of Candidate Compounds with Antisecretory Potential on Serum Gastrin Levels

PURPOSE AND RATIONALE

It is known from long-lasting and potent gastric acid inhibition caused, e.g., by the H^+/K^+-ATPase inhibitor omeprazole that the total acid blockade initiates a gastric antral feedback mechanism resulting in an excessive hypergastrinemia (Arnold et al. 1986; Creutzfeldt et al. 1986; Larsson et al. 1986) which is believed to cause diffuse endocrine cell hyperplasia, characterized as carcinoids, in the gastric corpus after 2 years of treatment (cancerogenicity study) in the rat (Ekman et al. 1985).

PROCEDURE

Groups of 10–15 rats weighing 90–110 g are treated daily for 10 weeks with the candidate compound (omeprazole as standard at doses of 10 or 30 mg/kg p.o.). After treatment for 2, 4, 7, and 10 weeks, blood samples are collected under ether anesthesia by retroorbital puncture. Gastrin is determined by a commercially available radioimmunoassay kit. At the end of the study of 10 weeks, the animals are studied for their gastric acid output using the pylorus ligation (Shay technique).

EVALUATION

Serum gastrin levels are determined as pg/mL. Statistical differences ($p < 0.05$) are calculated using appropriate statistical methods.

MODIFICATIONS OF THE METHOD

Katz et al. (1987) described a 5-day test to predict the long-term effects of gastric antisecretory agents on serum gastrin in rats.

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7.2.3 Bile Secretion

7.2.3.1 Bile Secretion in Mice

PURPOSE AND RATIONALE

The effect on bile secretion of a candidate compound can be studied in mice by weighing the gall bladder filled with bile. This simple method was first published by Litvinchuk (1976). With respect to the safety assessment of candidate compounds, a decreased bile secretion (compound-induced cholestasis) predominantly represents a safety issue.

PROCEDURE

Groups of ten mice weighing 15–20 g are used. Food, but not water, is withdrawn 24 h prior to the experiment. The test compound or the control solution is administered subcutaneously or orally. After 1 h, the animals are sacrificed and bled from the carotid artery. Laparotomy is performed, the liver exposed, and a no. 75 silk ligature is tied around the cystic duct, which is detached from the bile ducts and removed from the peritoneal cavity. If a large volume of bile has been accumulated, the full gall bladder is removed together with the bile ducts. The isolated gall bladder is weighed on a suitable balance; after which the contents are removed, the gall bladder walls are washed with distilled water and dried on filter paper, and the organ is weighed again. The difference in weight of the full and the empty gall bladder indicates the quantity of bile secreted during a measured time. The concentration of cholates, bilirubin, and cholesterol in the bile can be determined.

EVALUATION

The average of secreted bile in groups of ten treated mice is compared with the average value of the control group using appropriate statistical methods.

CRITICAL ASSESSMENT OF THE METHOD

The method has the clear advantage of simplicity but does not measure the true bile excretion since the outflow from the bile bladder during the test period is neglected.

MODIFICATIONS OF THE METHOD

Sterczer et al. (1996) studied the effect of cholagogues on the volume of the gallbladder in healthy dogs fasted for 24 h by two-dimensional ultrasonography. The volume was measured immediately before the administration of each test substance and at 10-min intervals for 120 min thereafter.

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7.2.3.2 Bile Secretion in Anesthetized Rats

PURPOSE AND RATIONALE

In contrast to other animals, rats do not possess a bile bladder. Therefore, cannulation of the bile duct in rats can be used as a suitable model to measure choleretic (increased bile production) or cholestatic (decreased bile production) side effect potential of drug candidates. If the test compound reduces bile production, it is recommended to investigate a putative hyperlipidemic side effect potential of the drug candidate by its influence on total blood cholesterol and triglycerides in appropriate experimental methods.

In addition, this method of the bile fistula rat can be used for ADME profiling of drug candidates with respect to a hepatobiliary elimination potential (high first-pass effect) (Herling et al. 2002).

PROCEDURE

Bile secretion is studied in anesthetized bile fistula rats, which are anesthetized by an intraperitoneal injection of ketamine (20 mg/kg) plus pentobarbital sodium (60 mg/kg), tracheotomized, and one jugular vein per rat is cannulated for intravenous administration (bolus injection or infusion of the drug candidate). Anesthesia is maintained for up to 7 h by subcutaneous infusion of pentobarbital sodium (adjusted to the anesthetic depth of the individual animal; about 24 mg/kg/h). Body temperature is monitored with a rectal probe thermometer, and temperature is maintained at 37°C by means of a heated surgical plate.

After laparotomy, the common bile duct is cannulated in the upper half with polyethylene tubing, and bile is collected every 30 min up to 7 h. The drug candidate is administered at an appropriate dose by bolus injections intravenously into the jugular vein 1 h after finishing surgery or by intraperitoneal administration of a 1% carboxymethylcellulose suspension, if not adequate soluble for an intravenous formulation. The volume of excreted bile per 30 min is determined gravimetrically (difference between tube weight without and with bile per collection period) with the assumption that 1 g is equivalent to 1 mL of bile. According to our experience, bile flow is stable for up to 3 h (200–300 μL/30 min) and can decline later due to the interruption of the enterohepatic circulation of bile acids; if not, the secreted bile is reinjected into the ileum.

For ADME purposes, the concentration of the parent test compound in the bile is measured by appropriate analytical methods, and total compound excretion is calculated from the secreted volume and the measured concentration of the test compound in the bile of each sampling interval.

For determination of the side effect potential of a candidate compound on choleresis or cholestasis, groups of at least six rats are used for control (vehicle control) and treated groups (rats receiving one dose of the test compound per group). For ADME purposes, smaller groups are sufficient ($n = 3$–4) to determine hepatobiliary elimination of the test compound. Ideally, the analytical method includes the determination of major metabolites appearing in the bile.

EVALUATION

Mean values (μL bile/30 min) for each group are calculated and compared to that of the control group. If bile flow is affected by the test compound detailed analysis of the bile with respect to cholesterol, bile

acids and phospholipids should be performed to elucidate the underlying mechanism.

For ADME purposes, the amount of hepatobiliary-eliminated compound plus metabolites are calculated per collecting interval, and total excreted amount over the whole experiment can be calculated and can be set into relation to the total administered dose per animal.

MODIFICATIONS OF THE METHOD

Several authors tested the choleretic activity of plant extracts and essential oils (De la Puerta et al. 1993; Peana et al. 1994; Trabace et al. 1994) and of synthetic compounds (Grella et al. 1992; Paglietti et al. 1994) in rats.

Tripodi et al. (1993) investigated the anticholelithogenic and choleretic activities of taurohyodeoxycholic acid by measurement of biliary flow and biliary solids content in rats.

Bouchard et al. (1993) induced cholestasis in rats by treatment with 17-α-ethinyl estradiol and studied the influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids.

Miki et al. (1993) investigated the metabolism and the choleretic activity of homochenodeoxycholic acid in hamsters with bile fistula.

Pesson et al. (1959) recommended the guinea pig as the best choice among the common laboratory animals to study choleretic agents.

Matsumura et al. (1996) analyzed hypercholeresis in dogs with pigment gallstones after cholate infusion.

CRITICAL ASSESSMENT OF THE METHOD

The method is simple and provides reliable results during terminal anesthetized conditions. Intraduodenal administration of the test compound, which is also reported in the literature, should be avoided due to the fact that intestinal absorption is obviously impaired during the reduced intestinal motility during anesthesia. The interrupted enterohepatic circulation should be taken into account when extrapolating the results (choleretic and cholestatic potential) to the intact organism.

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7.2.3.3 Bile Secretion in Conscious Rats (Chronic Bile Fistula Rats)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Sect. 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Most of the techniques for collection of bile in rats use restrained or anesthetized animals. Such factors as well as the surgical intervention itself may profoundly influence the results. Therefore, Remie

et al. (1990, 1991) developed a technique for a permanent double bile fistula in rats. The procedure is described in detail.

PROCEDURE

Preparation of Cannulae

Cannulae are made of silicon rubber. The proximal bile cannula, which will be inserted into the common bile duct in the direction to the liver, is 18 cm long (e.g., Silastic tubing, Dow Corning, no. 605–135; 0.51 i.d. and 0.94 o.d.) and has one square cut and one beveled end. Two silicon rings are wrapped around the cannula at 7 mm and 50 mm, respectively, from the beveled end.

The distal bile cannula, which will be inserted into the common bile duct in the direction of the gut, is made of the same material, is also 18 cm long and has one square cut and one beveled end. This cannula, however, must have a smaller tip diameter (e.g., Silastic tubing, Dow Corning, no. 605–105: 0.31 i.d. and 0.64 o.d.). To serve this purpose, the square cut end of the cannula is immersed in ether, causing the tubing to dilate. When the tubing is wide enough, a 13-mm piece of small diameter Silastic tubing is inserted. Subsequently, two silicon rings are wrapped around the cannula, one at the joint of the two tubes and the other 5 cm from the tip. The tip is then cut at a 45° angle, 7 mm from the first silicon ring.

The duodenal cannula (Silastic tubing, Dow Corning, no. 605–135) is also 18 cm long and has one square and one beveled end. An additional ring is placed 30 mm from the tip. Before the cannulae are fixed to the skull, they must be connected to a stainless steel needle bent in a 90° angle.

Anesthesia

The animal is anesthetized by inhalation with isoflurane or halothane/N_2O/O_2.

Preparation of the Crown of the Head

The head of the animal is shaved and disinfected. An incision of about 1 cm is made and the bregma exposed. Three stainless steel screws (1.0 × 4.2 mm) are mounted in the crown, two in the left and one on the right side of the bregma. The screws are tightened that approximately 2 mm is left between the skull and the head of the screws.

Double Cannulation of the Bile Duct

The abdominal wall is shaved and disinfected, and the animal is secured on the operation board with adhesive tape. A midline incision is made from the level of the pubic bones to the xiphoid cartilage. The abdomen is then opened by making an incision over the linea alba toward the sternum up to the distal part of the fourth sternebra, thus exposing the xiphoid cartilage.

Then, the intestines are lifted out and are laid next to the animal on moistened gauze. Using jeweler's forceps, the bile duct is stripped off its surrounding tissue and ligated with a 7–0 suture. The duct is placed under tension with an artery forceps for cannulation. With the aid of a microscope, a V-shaped hole is made just cranial of the first ligature with iridectomy scissors. The sterile proximal cannula is inserted into the duct. The second ligature is tied and pulled tight ensuring that the cannula is not obstructed. The bile is now flowing into the cannula. The first ligature is released and the threads are tied behind the silicon ring. The rat is then turned and the ligature reclamped, thereby putting the distal part of the duct under tension. A third ligature is loosely introduced around the duct, distal to the first ligature. Another V-shaped aperture is made between the first and third ligature for insertion of the distal bile cannula. The third ligature is tied and pulled tight. The first ligature is released from the artery forceps and tied around the second cannula behind the silicon ring. All the loose threats are cut close to the knots. The sections of the cannulae, which lie between the silicon rings, are placed kink-free in the abdominal cavity. The cannulae are fixed using 7–0 silk suture to the abdominal muscle near the xiphoid cartilage.

Cannulation of the Duodenum

After location of the place where the bile duct enters the duodenum (sphincter of Oddi), a four fine-stitch purse-string suture (7–0) is made in the wall of the duodenum at the outer border at about 1 cm proximal to the sphincter. Using a 20G needle, an incision is made inside the purse string. The cannula is inserted into the duodenum until the first, smaller silicon ring has entered the lumen, and the purse string is tightened between the first and the second ring. This cannula together with the bile cannula is placed kink-free in the abdominal cavity and anchored to the internal muscle. The abdomen is closed of resorbable sutures leaving 1 cm of the skin unclosed.

Subcutaneous Tunneling and Anchoring of the Cannulae

From the back of the neck, a slender needle holder is pushed subcutaneously through the connective tissue in caudal direction as near as possible to the skin down to the xiphoid cartilage. The cannulae are then grasped and pulled through to emerge at the crown of the head. The abdominal wall is closed completely.

With a 5-cm piece of polyethylene tubing (0.75 × 1.45 mm), the two long ends of the L-shaped stainless steel adapters are connected and the short ends inserted into the respective cannulae. The cannulae together with the tubing are fixed to the skull with acrylic glue flowing under the heads of the screws.

Postoperative Care

The animals are allowed to recover in a warm and quiet place. They reach usually preoperative weight within 2–3 days and display normal feeding and drinking behavior. Supplementation with saline besides the normal tap water may be necessary.

Collection of Bile

The animals are housed in individual metabolic cages. For bile collection, they are attached to long-swiveled PE cannulae (0.75 × 1.45 mm). A stainless steel coil is used to protect the rats from gnawing on the tubing. For continuous collection of bile, the cannula can be connected to a fraction collector.

CRITICAL ASSESSMENT OF THE METHOD

Among other applications, the method is suited to study the enterohepatic circulation of compounds. There should be a close health monitoring of the chronically prepared rats, and only those in a very good health conditions should be used for the study to avoid any misinterpretations of the results.

MODIFICATIONS OF THE METHOD

Castilho et al. (1990) studied the intestinal mucosal cholesterol synthesis in rats using a chronic bile duct-ureter fistula model. Male Wistar rats weighing 300–350 g were anesthetized with 50 mg/kg pentobarbital i.p. and submitted to a bile duct-right ureter fistula utilizing a PE-50 catheter after a right-kidney nephrectomy.

Cohen et al. (1992) reported a study in male black-tailed prairie dogs (*Cynomys ludovicianus*) weighing 1.0 ± 0.2 kg anesthetized with 20 mg/kg xylazine i.m. and 20 min later with 100 mg/kg ketamine i.m. Through an abdominal incision, the cystic duct is ligated, and gallbladder bile is aspirated. A PE-50 polyethylene cannula is inserted into the common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula is externalized, the abdominal incision closed, and the prairie dog placed in a restraining cage with access to food and water.

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7.2.3.4 Bile Secretion in Conscious Dogs (Chronic Bile Fistula in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Sect. 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Herrera et al. (1968) described a special cannula, which can be

used to obtain bile or pancreatic juice from a duodenal pouch after appropriate surgical procedures in conscious dogs.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are used. The abdominal surgery is performed during appropriate anesthesia (in former times by, e.g., 30 mg/kg pentobarbital sodium; nowadays, more appropriate by inhalation with halothane or isoflurane). The abdomen is opened through a midline epigastric incision. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the common bile duct is isolated. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodenojejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of three parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point, there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch. When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows bile to enter the duodenum in the normal manner. For collection of bile, this plug is removed and a long obturator is inserted. The latter effectively isolates the bile secretion from duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured by a further purse-string suture. The whole system is then generously wrapped in omentum, and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula. The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Daily checks of the cannula are advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 4 weeks to recover. Eighteen hours prior to the experiment, food is withdrawn but water allowed ad libitum. The long hollow obturator is inserted and bile collected for 15-min periods. After 1 h pretest time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of bile is measured at 15-min intervals, and volume and bile contents are determined from 1-mL samples. The values are compared with pretest data. The remaining bile is reinfused into the duodenum via the hollow obturator.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described several techniques for fistulae of the gall bladder and also for the fistula of the ductus choledochus in dogs.

An abdominal incision about 10 cm is made on the median line. The duodenum is pulled out, and the orifice of the large (first) pancreatic duct is found. The orifice of the ductus choledochus with the orifice of the small (second) pancreatic duct is situated on the other side of the intestine some 2 or 3 cm nearer the stomach. The ductus choledochus goes straight from the gallbladder to the duodenum; further, it lies parallel its end and is attached to the wall of the duodenum. The small pancreatic duct goes from the gland straight to the duodenum.

At the very beginning of the operation, it is useful to cut the ligamentum that goes from the liver to the duodenum, because this facilitates orientation and operating. It is necessary to cut out a piece of the intestinal wall with the orifice of the ductus choledochus. But before this, one must prepare off a little bit the intestine from the pancreas so as to be able to close conveniently and securely the hole in the intestine and divide between double ligatures the second pancreatic duct.

On the duodenum around the orifice of the ductus choledochus, an incomplete oval figure is now marked with a knife, so that the duct enters this figure through the incomplete part of the oval and has its orifice in the middle of this figure. The length of the oval is about 1.5 cm and its width 1 cm. A suture is then made on the edge of this oval, which is cut out not completely but

leaving a small bridge about 0.5 cm wide between the intestine and the oval; through the bridge, the duct enters the oval. The mucosa of this bridge must be completely destroyed with a knife.

The oval piece of the intestine is now turned with the mucosa up, and its serosa is sutured to the serosa of the intestine. The hole in the intestine is very carefully closed with two layers of sutures. Two heavy threads are then passed underneath the intestine on either side of the place of operation; they are laid through the abdominal wall and tied after the operation is over. They serve as temporary supporting sutures. The oval piece of the intestine is now sutured with the skin of the abdominal wound, and the wound is closed in the usual manner. The supporting sutures must be taken out 1 day or 2 days after the operation.

CRITICAL ASSESSMENT OF THE METHOD

There should be a close health monitoring of the chronically prepared dogs, and only those in a very good health conditions should be used for the study to avoid any misinterpretations of the results.

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7.2.4 Exocrine Pancreatic Secretion

7.2.4.1 Exocrine Pancreatic Secretion in Anesthetized Rats

PURPOSE AND RATIONALE

The effect of a candidate compound on pancreas secretion can be measured in rats with acute pancreas fistula. For safety pharmacological assessment of candidate compounds, the decrease of exocrine pancreatic secretion might be problematic due to the potential of induction of pancreatitis.

PROCEDURE

Rats weighing 150–200 g are used. Eighteen hours prior to the experiment, food is withdrawn with free access to water. The appropriate size of the study groups for the control and candidate compound consists of 5–7 animals. Anesthesia is induced by ketamine 20 mg/kg plus pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h). Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The abdomen is opened by a midline incision and the pylorus is ligated. The proximal part of the bile duct is ligated near the hepatic porta. The bile is drained via a thin polyethylene tube into the duodenum. The distal part of the bile duct with the orifices of pancreatic ducts is cannulated with another thin polyethylene tube. The pancreatic juice is collected in Eppendorf tubes, and secreted volume is measured gravimetrically or by graduated microsyringes every 15, 30, or 60 min. After a pretest period of 60 min, the test compounds are applied intravenously or intraduodenally.

EVALUATION

The secretion after injection of the test compound is compared with the pretest values. Secretin or cholecystokinin (CCK) increases pancreatic secretion volume in a dose-dependent manner and can be used as a positive standard.

MODIFICATIONS OF THE METHOD

Guan et al. (1990) inserted two separate cannulae for bile and pancreatic juice to rats under methoxyfluorane anesthesia. Both fluids were returned to the intestine. Placing the rats in modified Bollman-type restraint cages, experiments could be performed after a few days in conscious animals.

Ito et al. (1994) studied the inhibition of CCK-8-induced pancreatic amylase secretion by a cholecystokinin type-A-receptor antagonist in rats.

Niederau et al. (1989) compared the effects of CCK-receptor antagonists on rat pancreatic secretion in vivo. Output of amylase in pancreaticobiliary secretion was measured after various doses of cerulein. The effects of high cerulein doses were dose dependently inhibited by CCK antagonists.

Alvarez and Lopez (1989) studied the effect of alloxan diabetes on exocrine pancreatic secretion in the anesthetized rabbit. After a 14–15 h fasting period,

but with free access to water, rabbits weighing about 2.0 kg are anesthetized by intravenous injection of 1.0 g/kg urethane. After tracheotomy, a median laparotomy is performed; the main pancreatic duct is exposed and cannulated near its entrance to the duodenum following ligation of the pylorus and cannulation of the bile duct for deviation of bile to the exterior.

Kim et al. (1993) studied the effect of [(CH_2NH) 4,5] secretin on pancreatic exocrine secretion in guinea pigs and rats using an acute pancreatic fistula preparation.

Niederau et al. (1990) and Tachibana et al. (1996) determined pancreatic exocrine secretion in mice. Because the cannulation of mouse pancreatic duct is not possible for technical reasons, the amount of amylase was determined in vivo. Five minutes after i.v. administration of candidate compounds, mice were sacrificed and a 5 cm-duodenal loop was removed. The duodenal contents were washed out with 1.0 mL ice-cold saline and collected for amylase activity.

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7.2.4.2 Exocrine Pancreatic Secretion in Anesthetized Dogs

PURPOSE AND RATIONALE

To collect pancreatic secretion in dogs, three animal models have been developed: pancreatic fistulas, duodenal pouches (which collect the exocrine pancreatic secretion), and duodenal fistulas (through which a thin cannula is inserted into the pancreatic duct). With the exception of acute studies in anesthetized animals, pancreatic fistulas were used mainly during the first half of the twentieth century and have rather historic significance. The latter two methods, duodenal pouches and duodenal fistulas, although originally developed in the 1960s and 1940s, respectively, are still in use today (Niebergall-Roth et al. 1997).

The duct system of the canine pancreas is different from that of the human pancreas. In dogs, the main duct is the accessory pancreatic duct (Ductus pancreaticus minor). The pancreatic duct, which joins the bile duct (Ductus pancreaticus major) and forms the major duodenal papilla in man, is small and not always present in dogs.

Here, the pancreatic fistula technique in anesthetized dogs is described. The effect of exogenous hormones, e.g., secretin or gastrin, or of vagal stimulation, on exocrine pancreatic secretion can be measured in anesthetized dogs with acute pancreatic fistulas.

PROCEDURE

Beagle dogs of either sex weighing 12–20 kg are used. The animals are fasted for a 24-h period and then anesthetized using appropriate anesthetics, e.g., inhalation with isoflurane, i.v. infusion with probufol, or i.v. injection with ketamine plus pentobarbital sodium. After opening the abdomen along the midline, the pyloric sphincter is ligated and the common bile duct cannulated to prevent the entry of acid chyme and bile into the duodenum. The bile is allowed to drain. The pancreas is gently exposed and the major pancreatic duct ligated. A polyethylene tube of 2-mm diameter is inserted into the minor pancreatic duct for collection of the pancreatic juice. The left femoral vein is cannulated for continuous infusion or i.v. injection.

The pancreatic juice is collected in an ice bath in a special tapered tube with fine calibrations for measuring volumes of less than 1 mL.

At the end of each collection period, the volume is recorded, and the bicarbonate content determined titrimetrically. Furthermore, pancreatic enzymes, such as amylase, are determined in the samples. Determination of protein concentrations in the pancreatic juice can be used as endpoint since the total protein concentration is proportional to the individual enzymes (Keller et al. 1958). In a pretest period of 10 min, samples are collected every 2 min. Then, the test compound is injected intravenously and the pancreatic juice is collected every 2 min.

At the end of the animal experiment, the dog is euthanized by an overdose of barbiturate.

EVALUATION

The secretion after injection of the test compound is compared with the pretest values. Secretin increases pancreatic volume and bicarbonate secretion in a dose-dependent manner and can be ideally used as reference secretagogue.

MODIFICATIONS OF THE METHOD

Glad et al. (1996) tested the influence of gastrin-releasing peptide on acid-induced secretin release and pancreatobiliary and duodenal bicarbonate secretion in Danish country strain pigs weighing between 22 and 30 kg. The animals, starved overnight with free access to water, were premedicated with 4 mg/kg i.m azaperone and with 5 mg/kg i.p. metomidate. After 20 min, a cannula was placed in an ear vein, and 5–10 mg/kg metomidate was given i.v. followed by intubation and artificial respiration with 50% O_2 and 50% N_2O. Anesthesia was maintained with an intravenous bolus infusion of 0.53% chloralose.

Both external jugular veins were cannulated for infusion of saline or drugs. A femoral artery was cannulated for withdrawal of blood samples and recording of blood pressure. After laparotomy, the cystic duct was ligated, and the common hepatic duct and the pancreatic duct were catheterized. The duodenal segment was defined as extending from the pylorus to the ligament of Treitz. A Foley catheter was passed through the pylorus into the proximal part of the duodenum and inflated. Distal to the pylorus, the pancreaticoduodenal arteries, veins, and nerves were dissected, and a double ligature was passed under these structures and tied around the duodenum. At the ligament of Treitz, an inflated Foley catheter was placed in the distal part of the duodenum and tied with a suture around the duodenum. A catheter was placed through a splenic branch of the left gastroepiploic vein and advanced through the lienal vein to the portal vein.

The flow of pancreatic juice and bile was tested before and after the experiment by means of an intravenous bolus of 5 pmol/kg secretin. Before the experiment, the duodenum was continuously perfused at a rate of 2 mL/min for 435 min with isotonic saline containing phenol red (10 mg/L) as a marker. After drug treatment (intravenous infusion of gastrin-releasing peptide or duodenal HCl perfusion), pancreatic and hepatic secretions were collected in 15-min periods and the volumes were determined by weighing. Duodenal effluents were collected in 15-min periods and phenol red concentrations determined spectrophotometrically. Blood samples were withdrawn for determination of secretin by radioimmunoassay.

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7.2.4.3 Exocrine Pancreatic Secretion in Conscious Dogs (Chronic Duodenal Pouches in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Sect. 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Duodenal pouches and duodenal fistulas (through which

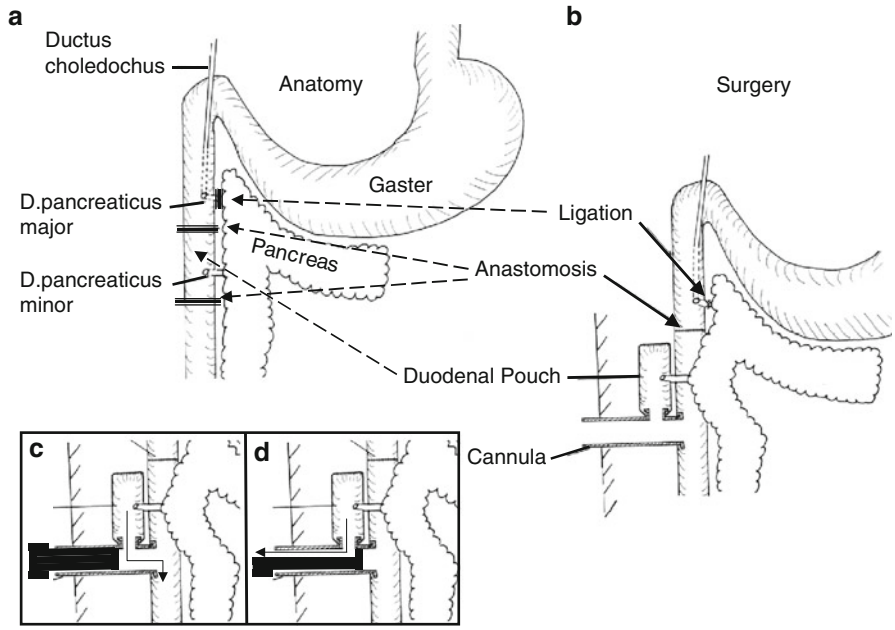

Fig. 7.3 Technique of chronic pancreatic fistula in dogs. Part **a** shows the normal anatomic situation. Part **b**: diagram of the duodenal pouch preparation. Part **c**: demonstration of the normal flow of exocrine pancreatic juice during nonassay condition. Part **d**: demonstration of the flow of exocrine pancreatic juice during study condition

a thin cannula is inserted into the Ductus pancreaticus minor, which represents the main pancreatic duct in dogs), although originally developed in the 1960s and 1940s, respectively, are still in use today (Niebergall-Roth et al. 1997). Here, the duodenal pouch technique is described by using a Herrera cannula. Herrera et al. (1968) described a special cannula, which can be used to obtain pancreatic juice or bile from a duodenal pouch after appropriate surgical procedures (Preshaw and Grossman 1965).

PROCEDURE

The principle technique is demonstrated in Fig. 7.3. Male Beagle dogs weighing 15–20 kg are used. The abdominal surgery is performed during appropriate anesthesia (in former times by, e.g., 30 mg/kg pentobarbital sodium; nowadays, more appropriate by inhalation with halothane or isoflurane). The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the Ductus pancreaticus minor, which represents the main pancreatic duct in dogs, is isolated. The proximal level of the duodenal section lies immediately distal to the opening of the common bile duct and the distal level of section lies 2.5 cm distal to the main pancreatic duct (Ductus pancreaticus minor). The duodenum integrity is restored by end-to-end duodenojejunostomy. The duodenal pouch is closed at the proximal end.

The cannula to be inserted is made of stainless steel and consists of three parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point, there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the duodenal pouch.

When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows pancreatic juice to enter the duodenum in the normal manner (Fig. 7.3, part C). For collection of juice, this plug is removed and a long obturator is inserted (Fig. 7.3, part D). The latter effectively isolates the pancreatic secretion from other duodenal contents.

The lateral limb of the cannula is inserted in the distal end of the isolated duodenal segment in which the main pancreatic duct opens. The remaining limb of the cannula inserted through a small duodenotomy and secured further by a purse-string suture. The whole system is then generously wrapped in omentum, and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula.

The cannula is left open to drain blood and secretions for 24 h postoperatively, after which time the

plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Checking the cannula daily is advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 2 weeks to recover. Eighteen hours prior to the experiment, food is withdrawn but water is allowed ad libitum. The long obturator is inserted (Fig. 7.3, part D), and pancreatic juice is collected for 15-min periods. After 1 h pretest time, the candidate compound is given either orally or intravenously.

EVALUATION

Secretion of pancreas juice is measured at 15-min intervals and volume and enzyme content determined. The values are compared with pretest data.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described details of the technique as recommended by Pavlov (1902) as well as his own modification.

Konturek et al. (1976, 1984) performed experiments with chronic gastric fistulas in cats as well as in dogs to compare the species-specific activities of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion.

Ninomiya et al. (1998) studied the effects of a cholecystokinin-A-receptor antagonist on pancreatic exocrine secretion stimulated by exogenously administered CCK-8 in conscious dogs with chronic pancreatic fistula.

Garvin et al. (1993) described distal pancreatectomy with autotransplantation and pancreaticocystostomy in dogs.

Kuroda et al. (1995) developed a new technique in dogs for pancreaticogastrointestinal anastomosis that consists of pancreatectomy using the ultrasonic dissector and implantation of the pancreatic duct into the gastrointestinal tract without suturing the pancreatic parenchyma.

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7.2.5 Gastrointestinal Injury Potential

7.2.5.1 Gastrointestinal Injury

PURPOSE AND RATIONALE

The gastrointestinal injury potential of a candidate compound is studied in rats after oral administration. Every positive finding represents a serious safety issue for a candidate compound.

Nonsteroidal anti-inflammatory agents (NASID), like indomethacin and acetylsalicylic acid (aspirin), induce gastric lesions in man and in experimental animals by inhibition of gastric cyclooxygenase (COX) resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

PROCEDURE

After a 24-h starvation period, groups of 8–10 rats weighing 150–200 g are used. The candidate compound is administered orally in 0.1% Tween 80 solution. Six hours later, the rats are sacrificed in CO_2 anesthesia, and their stomachs and intestines are removed. The stomach and gastrointestinal tract are removed. The mucosa is examined with a stereomicroscope and examined and assessed in relation to an ulceration index. The number of ulcers is noted and the severity recorded with the following scores:

0 = No ulcer
1 = Superficial ulcers
2 = Deep ulcers
3 = Perforation

For example, indomethacin 20 mg/kg orally or aspirin 50–100 mg/kg can be used as standard for the induction of ulcers.

EVALUATION

An ulcer index U_I is calculated:

$U_I = U_N + U_S + U_P \times 10^{-1}$

U_N = average of number of ulcers per animal
U_S = average of severity score
U_P = percentage of animals with ulcers

Ulcer index of treated animals is compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0% corresponds to no and 100% to maximal stimulated gastric acid output.

Instead of an ulcer index, the area of injured gastrointestinal mucosa can be measured by the following procedure. After removal of the stomach, formol saline (2% v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greater curvature, then washed in warm water, and examined under a threefold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (mm) for each animal, and the total injured area can be estimated, the mean count for each group being calculated.

MODIFICATION OF THE METHOD

Kitajima et al. (1993) studied the role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats. Four hours after subcutaneous injection of 25 mg/kg indomethacin, the rats were sacrificed after ether anesthesia, and the stomach was removed. The stomach was filled with 1.5 mL of 2% buffered formalin for 10 min and then opened along the greater curvature. The total length of the lesions was measured.

Dose and time dependency of the ulcerogenic action of indomethacin were studied by Djahanguiri (1969).

Instead of indomethacin, gastric lesions are induced by intravenous or oral doses of aspirin which can be prevented by exogenous PGE2 or PGI2 (Konturek et al. 1981). Furthermore, reserpine at a dose of 8 mg/kg i.p. or cysteamine hydrochloride at a dose of 400 mg/kg s.c. induces ulcers in rats (Tarutani et al. 1985).

Wallace et al. (1989) studied the ulcerogenic activity of endothelin in indomethacin pretreated rats using an ex vivo gastric chamber.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement and recording gastric potential difference in the rat.

CRITICAL ASSESSMENT OF THE METHOD

Absorptive and secretory changes of the gastrointestinal mucosa and microscopic damage (tight junction/brush borders) are not examined by this method.

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7.2.5.2 Gastric Ulcer in Pylorus-Ligated Rats (SHAY Rat)

PURPOSE AND RATIONALE

A simple and reliable method for production of gastric ulceration in the rat based on ligature of the pylorus has been published by Shay et al. (1945). The ulceration is caused by accumulation of acidic gastric juice in the stomach.

PROCEDURE

Rats weighing 150–170 g are starved for 48 h having access to drinking water ad libitum. During this time, they are housed single in cages with raised bottoms of wide wire mesh in order to avoid cannibalism and coprophagy. Ten animals are used per dose and as controls. Under ether anesthesia, a midline abdominal incision is made. The pylorus is ligated, care being exercised that neither damage to the blood supply nor traction on the pylorus occurs. Grasping the stomach with instruments is to be meticulously avoided, else ulceration will invariably develop at such points. The abdominal wall is closed by sutures. The test compounds are given either orally by gavage or injected subcutaneously.

The animals are placed for 19 h in plastic cylinders with an inner diameter of 45 mm being closed on both ends by wire mesh. Afterward, the animals are sacrificed in CO_2 anesthesia. The abdomen is opened and a ligature is placed around the esophagus close to the diaphragm. The stomach is removed, and the contents are drained in a centrifuge tube. Along the greater curvature, the stomach is opened and pinned on a cork plate. The mucosa is examined with a stereomicroscope. In the rat, the upper two fifths of the stomach form the rumen with squamous epithelium and possess little protective mechanisms against the corrosive action of gastric juice. Below a limiting ridge, in the glandular portion of the stomach, the protective mechanisms are better in the mucosa of the medium two fifths of the stomach than in the lowest part, forming the antrum. Therefore, lesions occur mainly in the rumen and in the antrum. The number of ulcers is noted and the severity is recorded with the following scores:

0 = No ulcer
1 = Superficial ulcers
2 = Deep ulcers
3 = Perforation

The volume of the gastric content is measured. After centrifugation, acidity is determined by titration with 100 mmol/L NaOH.

EVALUATION

An ulcer index U_I is calculated:

$U_I = U_N + U_S + U_P \times 10^{-1}$

U_N = average of number of ulcers per animal
U_S = average of severity score
U_P = percentage of animals with ulcers

Ulcer index and acidity of the gastric content of treated animals are compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0% corresponds to no and 100% to maximal stimulated gastric acid output.

CRITICAL ASSESSMENT OF THE METHOD

The "Shay rat" has been proven to be a valuable tool to evaluate the ulcerogenic or anti-ulcerogenic potential of a candidate compound independent of its mechanisms of action. However, due to the prolonged distress to the animals, the use of this method should be ethically balanced very carefully against the expected added value of the study outcome.

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7.2.6 Gut Motility

7.2.6.1 Ileal Contraction In Vitro
Isolated Ileum (MAGNUS Technique)

PURPOSE AND RATIONALE

During the profiling process of specific and safety pharmacological characterization of a candidate compound in the target-oriented research process, the side effect potential is first estimated from the binding characteristics to a receptor panel in vitro. If there is a binding to a respective receptor identified in the low molar concentration range, which is not associated to the primary mode of action of the drug candidate, there is always the question how relevant is this finding with respect to an undesired side effect potential by receptor agonism or antagonism with respect to the receptor-mediated pharmacological effect. As a first estimate, simple methods are preferred to investigate putative receptor-mediated effects. A wide variety of different receptors appear in the gut; their activation (using the drug candidate itself) or inhibition (using the respective receptor agonist together with the drug candidate) can be studied by isolated gut preparations.

The isolated ileum, as first described by Magnus (1904), is probably the most widely used model in experimental pharmacology. Magnus already studied simultaneously the spontaneous contractions of the longitudinal and circular musculature and the inhibiting effect of atropine. The method has been used for many purposes, such as the study on the effects of epinephrine (adrenaline) on the lower segments causing contraction and on the segments of the upper end causing relaxation by Munro (1951) or the study on the origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips by Paton and Zar (1968) either retaining or being denervated from Auerbach's plexus. The model is used as a basic screening procedure for spasmolytic activity, whereby an anti-acetylcholine or anticarbachol effect indicates antimuscarinic activity and an anti-$BaCl_2$ effect indicates a musculotropic, papaverine-like effect. In addition to the isolated ileum, other parts of the gut such as the isolated duodenum and colon have been used widely.

PROCEDURE

Guinea pigs of either sex weighing 300–500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine, which is then severed above the cord. The intestine is gradually removed, with the mesentery being cut away as necessary. When the colon is reached, the intestine is cut. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 2–3-cm length are cut. Preferably, the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into a 15-mL organ bath containing Tyrode solution at 37°C being oxygenated with 95% O_2/5% CO_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard, USA). A preload of 1 g is chosen. Responses are recorded on a polygraph. After a preincubation time of 30 min, the experiment is started.

The following agonists and antagonists (standards) are used (concentrations in g/mL bath fluid):

Agonist	Antagonist
Acetylcholine 10^{-7} g/mL	Atropine 10^{-8}–10^{-9} g/mL
	Scopolamine 10^{-8}–10^{-9} g/mL
Carbachol 10^{-7} g/mL	Atropine 10^{-8}–10^{-9} g/mL
Histamine 10^{-6} g/mL	Histamine antagonists, e.g., cimetidine, ranitidine, famotidine
BaCl2 10^{-4} g/mL	Papaverine 10^{-5}–10^{-6} g/mL
Serotonin (5-HT) 10^{-6} g/mL	Serotonin (5-HT) antagonists
PGE2 2×10^{-7} g/mL	PG antagonists

EVALUATION

Several methods for the quantitative evaluation of an antagonistic effect are available. One approach is the determination of pD_2 values according to van Rossum and van den Brink (1963). Acetylcholine or histamine is added in 1/2 $\log_{10}$ concentration increments until a maximum response is obtained. Control curves are recorded at 30-min intervals. After uniform control responses are obtained, the potential antagonist or the

standard is added 5 min before the concentration-response curve is reobtained. The potency of the antagonist is obtained by calculating the pD_2 value which is defined as the negative logarithm of the molar concentration of an antagonist that causes a 50% reduction or the maximal response obtained with an agonist.

MODIFICATIONS OF THE METHOD

Many modifications of the Magnus technique have been described in the literature, mainly with the isolated ileum (e.g., Koelle et al. 1950).

Okwuasaba and Cook (1980) dissected the myenteric plexus and longitudinal muscle free of the underlying circular muscle according to the method of Paton (1957) and Paton and Zar (1968) and stimulated the preparation with trains of supramaximal rectangular pulses of 1.0-ms duration at a frequency of 0.2 Hz.

Kilbinger et al. (1995) studied the influence of 5-HT_4 receptors on [^{3}H]-acetylcholine release from guinea-pig myenteric plexus.

De Graaf et al. (1983) described a fully automated system for in vitro experiments with isolated tissues. The apparatus consists of an organ bath equipped with (a) a gradient pump supplying a logarithmic concentration/time gradient of agonist; (b) pumps and valves for dispensing bath fluid, antagonist solutions, and an oxygenation gas mixture; and (c) a transducer with automatic baseline adjustment. The information coming from the preparation is fed into a minicomputer. The data of various experiments can be accumulated and Schild plots obtained.

Furukuwa et al. (1980) studied the effects of thyrotropin-releasing hormone on the isolated small intestine and taenia coli of the guinea pig.

Paiva et al. (1988) studied the role of sodium ions in angiotensin tachyphylaxis in the guinea-pig ileum and taenia coli.

Barnette et al. (1990) used electrically stimulated strips of circular smooth muscle from the lower esophageal sphincter of dogs to study the inhibition of neuronally induced relaxation by opioid peptides.

Griesbacher and Lembeck (1992) used the isolated guinea-pig ileum for analysis of bradykinin antagonists.

Hew et al. (1990) used field-stimulated (95% of maximum voltage, 0.1 Hz, 0.5 ms) guinea-pig ileum, bathed in physiological salt solution at 37°C in the presence of 1 mM mepyramine for determination of histamine-H_3 bioresponse. Reduction of contractile response by the test substance (>50% relative to control 0.3 mM R-a-methylhistamine) indicates possible histamine-H_3 agonism. At a test concentration where no significant activity is seen, ability to inhibit (>50%) R-α-methylhistamine-induced contractile reduction indicates antagonistic activity.

Feniuk et al. (1993) used the guinea-pig isolated ileum, vas deferens, and right atrium to characterize somatostatin receptors. Transmural electrical stimulation was applied to guinea-pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Radimirow et al. (1994) investigated opioid effects of short enkephalin fragments containing the Gly-Phe sequence on contractile responses of guinea-pig ileum after addition of 10 nM acetylcholine or after electrical stimulation.

Coupar and Liu (1996) described a simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle in rats. The preparation consists of a segment of rat ileum setup to measure the tension developed in the longitudinal muscle and intraluminal pressure developed in the circular muscle in response to transmural electrical stimulation.

Vassilev et al. (1993) exposed Wistar rats to subtoxic doses of Co^{2+} or Ni^{2+}, receiving $Co(NO_3)_2$ or $NiSO_4$ with drinking water for 30 days, and measured the changes in the contractile responses to carbachol and in the inhibitory effects of verapamil and nitrendipine on isolated smooth muscle preparations of the ileum and the trachea.

Pencheva and Radomirov (1993) and Pencheva et al. (1999) studied the effects of GABA-receptor agonists on the spontaneous activity of the circular layer in the terminal ileum of cats. Segments of the terminal ileum approximately 0.5 cm long were mounted in an organ bath along the axis of the circular layer through a cotton thread with a large knot situated at the inner part of the gut wall.

Similar preparations of cat ileum were used by Kortezova et al. (1994) and Chernaeva and Mizhorkova (1995).

Vassilev and Radomirov (1992) used an isolated preparation of rat rectum. The rectal region, 1–6 cm proximal to the anal sphincter, was removed and a 20-mm-long segment suspended in an organ bath. The influence of prostaglandins and antagonists on spontaneous mechanical activity and electrically stimulated responses was investigated.

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7.2.6.2 Transit Time In Vivo (Gut Motility) and Intestinal Secretion

Propulsive Gut Motility in Mice

PURPOSE AND RATIONALE

To study the side effect potential of a candidate compound on gastrointestinal motility, the passage of a charcoal meal through the gastrointestinal tract in mice or rats is a simple, reliable, and widely used method of safety pharmacologists.

PROCEDURE (EXEMPLARILY DESCRIBED FOR THE METHOD IN MICE)

Groups of ten mice weighing 15 g are fed an oat diet for 3 days. Eighteen hours prior to the experiment, food, but not water, is withdrawn. The animals are treated either subcutaneously 15 min or orally 60 min before administration of the charcoal meal (0.2 mL of a 4% suspension of charcoal in 2% carboxymethylcellulose solution). The mice are sacrificed after various time intervals: 5, 10, 15, 30, and 60 min. Ten animals serve as controls for each time interval. The entire intestine is immediately removed and immersed in 5% formalin to halt peristalsis, then washed in running water. The distance the meal has traveled through the intestine as indicated by the charcoal is measured and expressed as percent of the total distance from the pylorus to the cecum.

EVALUATION

The values for the treated groups with the candidate compound for each time point are compared to those for the vehicle control group by using appropriate statistical methods. By using several doses of the candidate compound, a dose-response curve can be established.

CRITICAL ASSESSMENT OF THE METHOD

The charcoal passage test can be used for evaluation of laxative activity as well as for inhibition of intestinal motility.

MODIFICATIONS OF THE METHOD

Instead of charcoal, unsubstituted Hostapermblau (CuPcB) suspended in gummi arabicum mucilage can be used.

Carmine red (15) suspended in a 1% tragacanth solution was used for measurement of small intestine transit in rats (Leng-Peschlow 1986).

Miller et al. (1981) measured the intestinal transit in the rat by the use of radiochromium (^{51}Cr). Female Sprague-Dawley rats weighing approximately 200 g were implanted with indwelling silastic cannulae in the proximal duodenum. Following a 3-day recovery period, the animals were fasted for 18 h and then treated with the test compounds. Thirty minutes later, 0.2 mL of radiochromium (0.5 mCi $Na^{51}CrO_4$) was instilled into the small intestine via the indwelling silastic cannula. Twenty-five minutes after chromium instillation, the animals were sacrificed. The small intestine was carefully removed and divided into ten equal segments. The radioactivity was determined with an automatic gamma counting system. The effect of drugs could be quantified by determining the geometric center of the distribution of chromium through the small intestine.

Shook et al. (1989) used radiolabeled chromium to measure gastrointestinal transit in mice.

Megens et al. (1989) used the charcoal test to study the in vivo dissociation between the antipropulsive and antidiarrheal properties of opioids in rats.

Lish and Peters (1957) recommended an intestinal antipropulsive test in intact insulin-treated rats providing certain advantages over the commonly used charcoal meal test for screening of synthetic antispasmodic and antipropulsive agents.

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Stomach Emptying in Rats

PURPOSE AND RATIONALE

Reynell and Spray (1956) described a method for the simultaneous measurements of gastric emptying and

intestinal transit of test substances in the rat using phenol red as marker. This simple method can also be ideally used for safety pharmacological assessment of candidate compounds on their side effect potential on gastrointestinal motility.

PROCEDURE

Rats weighing 200–300 g are starved for 24 h, with free access to water before the experiment. They are treated orally or subcutaneously with the test compound 15 min prior to oral administration by gavage of 1.5 mL 0.07% phenol red in 2% carboxymethylcellulose solution. Fifteen minutes later, the animal is sacrificed and the stomach is immediately removed. The whole stomach including the stomach content is alkalized with 1N NaOH and homogenized. The homogenate is filtered and, after precipitation of the protein with 10% trichloroacetic acid, centrifuged for 15 min at 3,000 rpm. The concentration of phenol red in the supernatant is measured colorimetrically in a photometer at 546 nm.

EVALUATION

Percentage of stomach emptying (S_e) is calculated according to the following formula:

$$S_e = 100 - \left(P_s \times P_a^{-1} \times 100\right)$$

P_s = Concentration of phenol red in the stomach (μg/mL)

P_a = Concentration of phenol red in the initial solution after addition of equal volumes of 1 N NaOH and trichloroacetic acid (μg/mL)

MODIFICATIONS OF THE METHOD

Droppleman et al. (1980) described a simplified method for assessing drug effects on gastric emptying in rats. Three milliliters of a semisolid test meal, based on methylcellulose, is given to rats fasted 24 h prior to the experiment. At a specified time following the test meal, the rats are sacrificed and laparotomized and the stomachs removed. The full stomachs are weighed on an analytical balance; they are opened and rinsed. Excess moisture is removed and the empty stomach is weighed again. The difference is subtracted from the weight of 3 mL of the test meal, indicating the quantity emptied from the stomach during the test period. Gastric motor stimulants, e.g., metoclopramide increase, and anticholinergic compounds decrease gastric emptying.

Megens et al. (1990) used phenol red as marker to measure gastrointestinal propulsion after castor oil or paraffin oil challenge in rats.

Hedge et al. (1995) studied 5-HT_4 receptor-mediated stimulation of gastric emptying in rats using a specially prepared semisolid test meal containing charcoal.

Bonnafous et al. (1995) investigated benzodiazepine-withdrawal-induced gastric emptying disturbances in rats. Rats, weighing 200–250 g, fasted for 16 h, received by gavage 2 mL of a test meal containing 1 μCi/mL of ^{51}Cr sodium chromate, 15 min after drug administration. Thirty minutes later, the animals were sacrificed by cervical dislocation. The stomach, small intestine (ten segments), and the colon were excised and placed into tubes. Radioactivity was determined by placing the tubes in a gamma counter. Gastric emptying was calculated as the percentage of total counts found in the small intestine and the colon.

Varga et al. (1995) determined gastric emptying in rats 5 min after a 3-mL intragastric load of 0.9% NaCl using phenol red as marker in order to define which bombesin receptors are involved in the delay of gastric emptying by bombesin-like peptides.

Lasheras et al. (1996) studied gastric emptying in rats. Sixty minutes after oral administration of vehicle or test compounds, the rats received by gavage 40 steel spheroids (1-mm diameter) in 2 mL 3% carboxymethylcellulose. Sixty minutes later, the animals were sacrificed and the spheroids remaining inside the stomach counted.

Yegen et al. (1996) studied the inhibitory effects of gastrin-releasing peptide on gastric emptying in rats using methylcellulose and phenol red as nonabsorbable marker.

Haga et al. (1994) studied gastric emptying in mice. Male mice, weighing 18–22 g, had free access to food and water before the experiment. The test compounds were administered orally in 10 mL/kg 0.5% methylcellulose solution. The mice were deprived of food and water and sacrificed 4 h later by cervical dislocation. The stomachs were removed and opened. The contents of the stomach were mixed with 10% trichloroacetic acid and centrifuged at 3,000 rpm for 30 min. The weight of the sediment was taken as the food remaining in the stomach.

Ding and Håkanson (1996) examined the effect of drugs on a cholecystokinin-A-receptor-mediated response by gastric emptying of a charcoal meal in mice.

Costall et al. (1987) used the guinea pig to study the influence of a 5-HT_3 antagonist on gastric emptying.

Brighton et al. (1987) used scintigraphy following indium-111-labeled meals in Beagle dogs and baboons. Indium-111-labeled polystyrene beads (500 mCi per dog) were mixed into a meal consisting of 50 g of finely crushed commercial dog food and 50 mL of milk. Images of 1-min duration were taken every 5 min for a period of 1 h using a large field of view gamma camera (ON Sigma 410).

Gullikson et al. (1991, 1993) studied gastric emptying of a solid meal in dogs.

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Stomach Emptying and Gastrointestinal Transit in Dogs

PURPOSE AND RATIONALE

Using the pharmacokinetic profile of specific and defined marker substances, e.g., paracetamol (acetaminophen), for estimating, the rate of gastric emptying has been used since decades not only in animals but also in humans (Clements et al. 1978). This study principle of the analysis of the pharmacokinetic profile of marker substances has also been used for physiological studies of the influence of different food constituents on gastrointestinal motility (Mizuta et al. 1990). This study principle can be ideally used for safety pharmacological assessment of candidate compounds on their side effect potential on gastrointestinal motility.

PROCEDURE

Groups of six male dogs were used in a crossover design (compound vs vehicle control) with a minimum of 3 days between each session. Animals received a single dose of candidate compound or vehicle. After an appropriate time (depends on known pharmacokinetic data of the candidate compound; T_{max}, C_{max}), the marker compound paracetamol (20–25 mg/kg) was administered into the stomach by gavage using rubber tubing connected to a 30-mL syringe. The tube was rinsed with 15 mL water and 10 mL air immediately after dose administration.

Blood samples of 1–2 mL were collected for pharmacokinetic analysis of paracetamol at multiple time intervals, e.g., at 0, 10, 20, 30, 60, 90 min, 2, 3, 4, 5, 6, and 7 h after administration of the marker.

Blood samples were drawn into vacutainer tubes containing EDTA. Plasma was prepared by centrifugation 1500 × g for 10 min, at 4°C within 30 min after sampling and stored at −70°C until analysis of marker level of paracetamol in the plasma. For the bioanalysis of paracetamol, plasma samples were appropriately prepared for analysis with HPLC/MS/MS.

EVALUATION

The rate of absorption of orally administered paracetamol depends on the rate of gastric emptying and is usually rapid and complete. The mean systemic availability is about 75%. The difference of t_{max} (and c_{max}) of paracetamol between vehicle-treated and candidate-compound-treated group represents a measure of changes in the rate of gastric emptying. For example, atropine delayed gastric emptying during starved and fed conditions.

MODIFICATIONS OF THE METHOD

Sjödin et al. (2011) described an elegant method of combined assessment of the rate of gastric emptying and the intestinal transit time. They used a pharmacokinetic method (double marker method) for the simultaneous measurements of gastric emptying (marker: paracetamol) and intestinal transit (marker: sulfapyridine generated from orally administered sulfasalazine by colonic bacterial flora). They investigated the influence of the nutritional state (fed vs fasting) as well as that of standard substances (atropine, erythromycin) in the dog on gastric emptying and gastrointestinal transit, respectively. Paracetamol and sulfasalazine were administered together as an oral mixture at doses of 24 mg/kg and 20 mg/kg, respectively. The pharmacokinetic profile of paracetamol was used for evaluation of gastric emptying, as mentioned above. Sulfasalazine is poorly absorbed in the stomach and small intestine but is rapidly metabolized by the bacterial flora in the colon; the colonic metabolite sulfapyridine is then absorbed in the colon, and its appearance in the blood served as a marker of gastrointestinal transit time through the small intestine. The time period of blood sampling is about 7 h for paracetamol and about 36 h for sulfapyridine.

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Enteropooling Test

PURPOSE AND RATIONALE

The enteropooling assay in rats has been developed by Robert et al. (1976) to test the diarrheogenic property of prostaglandins for prediction of this clinically relevant side effect of several synthetic prostaglandins. This method can also be used for the safety pharmacological assessment of candidate compounds on their side effect potential to induce diarrhea.

PROCEDURE

Rats weighing 190–215 g are used. The animals are fasted overnight having free access to water. The candidate compound is administered orally, and the animals, 10–12 per group, are sacrificed 1 h later. The fluid accumulation occurs in the small intestine which is cut at the pylorus and the ileocecal junction, and its contents, consisting of a thick fluid (in controls) and a very watery fluid (in prostaglandin-treated animals), are collected into a graduated test tube by milking the whole length of the small intestine with the fingers. The volume of fluid is recorded.

EVALUATION

Using various doses, dose-response curves can be established and potency ratios calculated. 16,16-dimethyl PGE_2 was found to be the most active compound and can be used as standard to estimate the diarrheogenic side effect potential of candidate compound.

CRITICAL ASSESSMENT OF THE METHOD

Some other diarrheogenic agents, like $MgSO_4$, castor oil, bile, taurocholate, and taurochenodesoxycholate,

cause enteropooling, whereas mineral oil and tragacanth are ineffective. The anticholinergic agent methylscopolamine partially counteracted the enteropooling. The assay, therefore, can be used to test the laxative or the antidiarrheal activity of drug candidates (Shook et al. 1989).

MODIFICATIONS OF THE METHOD

Beubler and Badhri (1990) used the PGE_2-induced net fluid secretion in the jejunum and colon in the rat to evaluate the antisecretory effects of antidiarrheal drugs. Polyethylene catheters were placed into the jejunum and colon, and Tyrode solution was instilled into the loops. Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution.

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7.3 Carbohydrate and Lipid Metabolism

7.3.1 General Considerations

If a candidate compound with a totally different primary indication has additionally lipid-lowering potential or causes an insulin sensitization, these findings may be assessed as additionally beneficial and therefore do not represent a safety concern, irrespective whether these pharmacological effects occur in the pharmacological dose range for the primary pharmacological effect or at suprapharmacological doses as usually used for safety pharmacological studies of a candidate compound. In contrast, if a candidate compound causes increases in plasma lipid parameters (free fatty acids, triglycerides, cholesterol), detailed analysis of the these side effects is highly recommended, because increased free fatty acids and triglycerides might induce insulin resistance and increased triglycerides and total cholesterol might be connected to an increased atherogenic risk.

The acute side effect potential on intermediary metabolism resulting in changes in blood glucose and lactate as well as in changes of free fatty acids can be investigated in rodents after single administration of candidate compounds, as these blood parameters physiologically vary quickly. However, changes of triglycerides and predominantly of cholesterol appear physiologically much slower. Therefore, the side effect potential of candidate compounds on these parameters are hardly to be detected in single dose studies as usually performed during initial safety pharmacological characterization. If there are additional hints, e.g., from toxicity studies, demonstrating putative side effects on triglyceride and cholesterol metabolism, additional multiple-dose studies in safety pharmacology in appropriate animal models to detect an atherogenic potential are necessary to characterize the side effect potential of candidate compounds.

Due to the fact that nearly most of initial safety pharmacological studies are performed in small rodents (mainly rats and mice), one has to take carefully into consideration the differences in rat physiology compared to human physiology for characterizing the side effect potential of a candidate compound on intermediary metabolism. A normal rat is night active and takes up food predominantly during the night (8–10 meals per night) and, to a smaller extent, also during the day (3–4 meals per day). Blood glucose and liver glycogen do not vary during 24 h (Gaertner 2001), resulting in a permanent prandial or postprandial state but never in a postabsorptive state. In humans, blood glucose and liver glycogen vary in dependence to meal intake during the day and decrease during the night to fasting values in the morning. In general, carbohydrate and fatty acid metabolism in laboratory animals are much more similar to that of humans compared to cholesterol metabolism. The functions of lipoprotein fractions (predominantly LDL and HDL) differ substantially between rodents and humans. Therefore, total cholesterol in blood is the most reliable marker in rats and mice, and the results from LDL and HDL cholesterol in these rodents should be interpreted very carefully with respect to their role in atherosclerosis known from human pathophysiology. With respect to cholesterol metabolism, the

guinea pig seems to be more closely to the human cholesterol metabolism compared to mice and rats (Fernandez 2001).

Interpretation of changes in blood glucose and free fatty acids should only be performed with the additional knowledge of the corresponding serum insulin levels. This is important not only in single dose studies but also in long-term studies. Candidate compounds, which cause a slight increase in free fatty acids and triglyceride in multiple-dose studies, may induce insulin resistance (normoglycemia in the presence of hyperinsulinemia), which might provoke the progression to diabetes and obesity.

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7.3.2 Acute Effects on Metabolic Blood and Tissue Parameters

7.3.2.1 Acute Effects on Metabolic Blood and Tissue Parameters in Anesthetized Rats

PURPOSE AND RATIONALE

Anesthetized rats are used for testing the side effect potential of a candidate compound on intermediary metabolism in liver, muscle, and adipose tissue with subsequent effects on metabolic blood parameters (e.g., glucose, lactate, free fatty acids, triglycerides) and insulin. The use of anesthetized rats represents more a principal assessment of the pharmacological side effect potential since the candidate compound must be administered intravenously or intraperitoneally (enteral/intestinal administration should be avoided due to the anesthesia-induced decrease in intestinal motility with subsequent impairment of enteral absorption), compared to the study in conscious rats in which the candidate compound can be studied after oral administration, which, in most cases, represents the clinical route of administration for small molecular drugs.

PROCEDURE

Metabolic blood parameters are assayed in anesthetized male rats using a modified method of glucose clamp studies in rodents (Terrettaz and Jeanrenaud 1983). Four to six rats per group (vehicle control and one dose of the candidate compound) are used. Rats are anesthetized with an intraperitoneal injection of ketamine (20 mg/kg) plus pentobarbital sodium (60 mg/kg) and tracheotomized, and one jugular vein per rat is cannulated for intravenous infusion; the other vein is prepared for collection of blood samples. Anesthesia is maintained for up to 7 h by subcutaneous infusion of pentobarbital sodium (adjusted to the anesthetic depth of the individual animal; about 24 mg/kg/h). Body temperature is monitored with a rectal probe thermometer, and temperature is maintained at 37°C by means of a heated surgical table. Blood samples for glucose analysis (10 μL) are obtained from the tip of the tail every 15 min and for lactate analysis (20 μL) every 30 min. The rats are allowed to stabilize their blood glucose levels after surgery for up to 2 h. Then, the candidate compound is injected or infused intravenously or administered intraperitoneally, respectively. Blood samples (100 μL) for detection of free fatty acids, triglycerides, and insulin are collected from a peripheral vein (e.g., jugular vein) immediately prior and every 1 or 2 h after compound administration.

At the end of the experiment, the abdomen is opened; terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1 gr) of skeletal muscle (e.g., M. gastrocnemius). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P), and ATP. In liver samples, hepatic triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P, and ATP (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA kit.

EVALUATION

The differences of the metabolic blood parameters after compound administration are calculated with respect to the predrug values and against a control group, which has received the vehicle only. The

metabolic tissue parameters at the end of the study are assessed between control and treatment group.

Two parallel experiments in normal fed (high glucose, low free fatty acids, high triglycerides, high serum insulin) and overnight starved rats (fasting blood glucose, elevated free fatty acids, low serum insulin) as well as the complete data set of metabolic blood and tissue parameters and serum insulin levels are necessary to assess the acute putative side effect potential and its putative mechanism of the candidate compound on intermediary metabolism.

MODIFICATIONS OF THE METHOD

By using rats, which are starved longer than 16 h before their use in the pharmacological experiment, fasting blood glucose is maintained exclusively by the process of gluconeogenesis, due to the very low glycogen content in the liver. Candidate compounds, which reduce blood glucose during this experimental setup, interfere with the process of endogenous glucose production (hepatic and renal gluconeogenesis) either by direct interference with an enzymatic step of gluconeogenesis or by interference with the energy generation machinery (mitochondrial function, generation of ATP). Additional reasons of a blood glucose reduction during this experimental setup could be an insulin release from the pancreatic β-cell or the interference with the insulin-signaling cascade in the insulin target tissues. Candidate compounds, which decrease free fatty acids, which are physiologically elevated during starvation, interfere with the process of lipolysis (antilipolytic activity) (Schoelch et al. 2004). Compounds, which increase free fatty acids (lipolytic activity), might have, e.g., a stimulatory β-sympathetic potential.

It is recommended to perform a parallel experiment by using normal fed rats. Candidate compounds, which increase blood glucose, might inhibit insulin release or peripheral insulin action or stimulate glycogenolysis. A putative lipolytic activity (increase in free fatty acids) is more pronounced during normal fed conditions, because free fatty acids are physiologically low due to elevated insulin levels. Fed rats can be also used for studying the effect of a candidate compound exclusively on the process of glycogenolysis. During this experimental setup, glycogenolysis is induced by an intravenous bolus injection of glucagon at a dose of 1 mg/rat. It can be assumed that the hyperglycemia induced by the glucagon injection, and which lasts for about 90–120 min, is the result of the glucagon-induced breakdown of hepatic glycogen (Herling et al. 1998, 1999).

CRITICAL ASSESSMENT OF THE METHOD

In general, pharmacological studies during anesthesia should be assessed appropriately due to the possible interaction between the test compound and the used anesthetic as well as due to the reduced tone of the autonomic nervous system. Enteral administration of the candidate compound should be avoided, because enteral absorption of the test compound might be reduced due to the impaired intestinal motility during anesthesia. With respect to the effect of the anesthetic compound itself on intermediary metabolism, the barbiturate pentobarbital sodium is the most inert anesthetic and does not cause alterations of metabolic blood and tissue parameters. In contrast, e.g., urethane (often used in former times for long-term anesthesia) as well as isoflurane (inhalation anesthetic) influences by itself substantially metabolic parameters over time (hours). In our hands, anesthesia with pentobarbital sodium does not influence intermediary metabolism or insulin secretion and is essential for obtaining reliable results during such pharmacological studies lasting for several hours. Most other anesthetics including inhalation anesthetics like isoflurane should not be used due to their inhibitory effect on insulin secretion from pancreatic β-cells and subsequent alterations of metabolic control.

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7.3.2.2 Acute Effects on Metabolic Blood and Tissue Parameters in Conscious Rats

PURPOSE AND RATIONALE

Conscious rats are used for testing the side effect potential of a candidate compound on intermediary metabolism in liver, muscle, and adipose tissue with subsequent effects on metabolic blood parameters (e.g., glucose, lactate, free fatty acids, triglycerides) and insulin after oral administration, which represents in most cases the clinical route of administration for small molecular drugs.

PROCEDURE

Rats weighing 180–240 g are kept on standard diet. Groups of eight nonfasted animals are treated orally with various doses of the test compounds suspended in 0.4% starch suspension. One control group receives the vehicle only. Blood is withdrawn from the tip of the tail immediately before and 1, 2, 3, 5, and 24 h after administration of the candidate compound. Blood glucose is determined in 10 μL blood samples collected from the tip of the tail. If pharmacokinetic data for the candidate compound are already available, when performing this test, additional blood samples (100 μL) should be taken at t_{max} by retroorbital bleeding for detection of free fatty acids, triglycerides, and insulin.

At the end of the experiment, the rats are terminally anesthetized, the abdomen is opened, and terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1 gr) of skeletal muscle (e.g., M. gastrocnemius). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P), and ATP. In liver samples, hepatic triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P, and ATP (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA.

EVALUATION

The differences of the metabolic blood parameters after compound administration are calculated against a control group, which has received the vehicle only, for the respective time points. The metabolic tissue parameters at the end of the study are assessed between control and treatment group.

Two parallel experiments in normal fed (high glucose, low free fatty acids, high triglycerides, high serum insulin) and overnight starved rats (fasting blood glucose, elevated free fatty acids, low serum insulin) as well as the complete data set of metabolic blood and tissue parameters and serum insulin levels are necessary to assess the acute putative side effect potential and its putative mechanism of the candidate compound on intermediary metabolism.

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7.3.2.3 Blood-Glucose-Lowering Activity in Conscious Rabbits

PURPOSE AND RATIONALE

The rabbit has been used since many years for standardization of insulin. Therefore, it has been chosen as primary screening model for screening of blood-glucose-lowering compounds as well as for establishing time-response curves and relative activities (Bänder et al. 1969; Geisen 1988). For the safety pharmacological evaluation of candidate compounds with a different primary indication, the rabbit is not the preferred animal species for first initial studies on metabolism pharmacology.

PROCEDURE

Groups of 4–5 mixed breed rabbits of either sex weighing 3.0–4.5 kg are used. For insulin evaluation, food is withheld overnight. For evaluation of sulfonylureas and other blood-glucose-lowering agents, the animals are on a normal diet prior to the experiment. The animals are gently placed into special restraining boxes allowing free access to the rabbit's ears.

Oral blood-glucose-lowering substances are applied by gavage in 1 mL/kg of 0.4% starch suspension or intravenously in solution. Several doses are given to different groups. One control group receives the vehicle only. By puncture of the ear veins, blood is withdrawn immediately before and 1, 2, 3, 4, 5, 24, 48, and 72 h after treatment. For time-response curves, values are also measured after 8, 12, 16, and 20 h. Blood glucose is determined in 10 μL blood samples.

MODIFICATIONS OF THE METHOD

For special purposes, the effect of blood-glucose-lowering agents is studied in glucose-loaded animals. Rabbits of either sex weighing 3.0–4.5 kg are treated either once (0.5 h after test compound) or twice (0.5 and 2.5 h after test compound) orally with 2 g glucose/kg body weight in 50% solution.

EVALUATION

Averages of the blood glucose values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Similarly, other metabolic blood parameters (e.g. lactate, insulin) are plotted versus time and compared with control values. Statistical evaluation is performed using appropriate methods.

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Geisen K (1988) Special pharmacology of the new sulfonylurea glimepiride. Arzneim Forsch/Drug Res 38:1120–1130

7.3.2.4 Acute Effects on Metabolic Blood Parameters in Conscious Dogs

PURPOSE AND RATIONALE

Since most safety studies for safety pharmacological evaluation of candidate compounds are performed in rodents, a second nonrodent species should be included to exclude any species differences in the safety pharmacological findings. Dogs are a preferred second animal species in metabolism pharmacology, because their night/day acitivity, their food intake behavior, and therefore their intermediary metabolism are obviously different to that of rodents and resemble more that of humans.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are kept on standard diet. Food is withdrawn 18 h prior to the administration of the candidate compound which is given either orally or intravenously in various doses. Control animals receive the vehicle only. Blood is collected at different time intervals up to 48 h for determination of metabolic blood parameters (e.g., glucose, lactate, free fatty acids, triglycerides) and insulin. Standard enzymatic procedures were used to determine metabolic blood parameters (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA kit.

EVALUATION

Averages of the metabolic blood parameters (e.g., glucose, lactate) are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Similarly, plasma insulin levels are plotted versus time and compared with control values. Statistical evaluation is performed using appropriate methods.

MODIFICATIONS OF THE METHOD

Continuous Blood Glucose Monitoring

A device for continuous blood glucose monitoring and infusion in freely mobile dogs was described by Geisen et al. (1981).

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7.3.3 Functional Tests

7.3.3.1 Oral Glucose Tolerance Test (oGTT) in Conscious Rats

General Consideration

Oral glucose tolerance test (oGTT) represents a simple method for estimating the effect of a candidate compound either to impair or to improve glucose tolerance. In general, impaired glucose tolerance often represents a condition of some degree of insulin resistance or impaired pancreatic insulin secretion during a glucose load. An impairment of insulin sensitivity (induction of insulin resistance) resulting in impaired glucose tolerance, which can be detected in normal animals (rarely in single dose studies but more likely in multiple-dose studies lasting for 1 or 2 weeks), represents a safety concern, since insulin resistance is associated with hyperinsulinemia and can provoke the progression to overt diabetes (type 2) and obesity. For the assessment of the glucose tolerance, the additional determination of insulin is essential. Since the commercial availability of ELISA kits using only some microliter serum for insulin determination, it has become possible also in rodents to collect appropriate blood samples for insulin determinations in parallel to blood glucose measurements during conscious conditions.

On the other hand, normal animals are insulin sensitive, and further improvement of insulin sensitivity cannot be expected. Therefore, if there are hints from other studies for a candidate compound (reduction in free fatty acids, triglycerides and insulin), to have a putative insulin-sensitizing potential, which does not represent a safety concern, appropriate studies should be performed by using insulin-resistant animal disease models (genetic models: e.g., Zucker-fatty (fa/fa) rat, Zucker Diabetic Fatty (ZDF, fa/fa) rat, diet-induced insulin resistant rats: e.g., high-fat-fed rats, high-fructose-fed rats) (Vogel 2008)

PURPOSE AND RATIONALE

oGTT in conscious rats are used to assess the side effect potential of a candidate compound to impair glucose tolerance (Fig. 7.4).

PROCEDURE

Rats weighing 180–240 g are starved overnight. Groups of eight animals are treated orally with various doses of the candidate compound suspended in 0.5% HEC or Tylose. One control group receives the vehicle only. Based on the pharmacokinetic data (t_{max}), the candidate compound is administered appropriately before the glucose load (e.g., 0.5, 1, or 2 h). Glucose is administered at a dose of 2 g/kg orally. Blood is withdrawn from the tip of the tail immediately before compound administration and immediately before glucose administration and 0.5, 1, 2, 3, and 5 h after administration of glucose. Blood glucose is determined in 10 µL blood samples collected from the tip of the tail. An additional blood sample (80–100 µL, resulting in about 40 µL serum) should be taken for insulin determinations. Standard enzymatic procedures are used to determine blood glucose (Bergmeyer 1974); insulin is determined by using a commercially available ELISA kit.

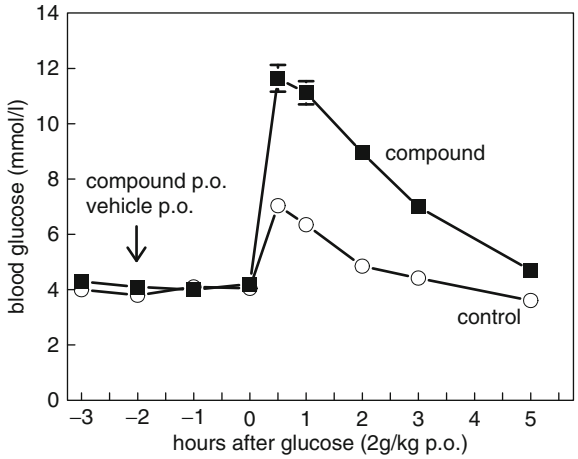

Fig. 7.4 Representative oral glucose tolerance test shown for a candidate compound, which impairs glucose tolerance. Candidate compound and vehicle are administered 2 h before the oral glucose load. Values are mean +/−SEM, $n = 8$ rats

EVALUATION

Mean values of blood glucose for each time point are calculated, and time course of control and treated groups are compared. Area under the blood glucose curve (AUC) can be calculated and compared for both groups; baseline values of blood glucose should be subtracted for the calculation of glucose AUC.

CRITICAL ASSESSMENT OF THE METHOD

An impairment of glucose tolerance can be the result of peripheral insulin resistance, an impaired insulin

secretion from the pancreatic β-cell, or both. Therefore, blood glucose levels as well as corresponding insulin levels are essential for the assessments of the glucose tolerance results. An improvement of glucose tolerance during an oGTT in normal animals can be caused by candidate compounds, which inhibit gastric emptying and thereby delay the glucose absorption from the gut.

MODIFICATION OF THE METHOD

Many authors used mice instead of rats (Gross et al. 1994; Bailey et al. 1997; Ahren et al. 2000). However, parallel insulin measurements are much more limited in mice than in rats. Also, oGTT in insulin-resistant animal disease models in mice (e.g., genetic: ob/ob, db/db, Ay) are described to assess peripheral insulin resistance (Xiao et al. 2001; Arakawa et al. 2001; Nagakura et al. 2003; Thorkildsen et al. 2003; Minoura et al. 2004). Other routes for glucose administration (e.g., intraperitoneal) are also reported (Xie et al. 2004).

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7.3.3.2 Euglycemic-Hyperinsulinemic Glucose Clamp Technique in Anesthetized Rats

PURPOSE AND RATIONALE

The euglycemic glucose clamp technique represents the "gold standard" for measuring peripheral insulin sensitivity in humans and animals. This technique was first described in humans by DeFronzo et al. (1979). In this technique, a variable glucose infusion is delivered to maintain euglycemia during insulin infusion. Whole-body tissue sensitivity to insulin, as determined by net glucose uptake, can be quantitated under conditions of near steady state glucose and insulin levels. Terrettaz and Jeanrenaud (1983) adapted this technique to anesthetized rats; Kraegen et al. (1983, 1985) developed the euglycemic glucose clamp technique for use in conscious rats.

PROCEDURE

The study can be performed as described by Terrettaz and Jeanrenaud (1983). Briefly, overnight-fasted rats are anesthetized with ketamine (20 mg/kg) plus pentobarbital sodium (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h); their temperature is kept at 37.5°C. Through left jugular and femoral vein catheters, glucose and insulin are infused. Studies last 240 min. During a 120-min baseline period, blood glucose is determined every 15 min. Then, insulin is administered as a bolus (48 mU/kg/min) for 5 min, followed by a constant i.v. infusion at 4.8 mU/kg/min for

further 115 min. Blood drawn from the tip of the tail is now measured for glucose every 5 min, and 15% D-glucose infused to maintain euglycemia at about 5 mmol/L. At 120 and at 240 min, additional blood samples are drawn from the right jugular vein and placed in K-EDTA-tubes, which are immediately centrifuged at 6°C at 5,000 rpm followed by determination of plasma FFA within 60 min; plasma aliquots are frozen for insulin determinations.

To differentiate between peripheral (predominantly skeletal muscle) and hepatic insulin sensitivity, tracer techniques can be applied (e.g., U-^{13}C-glucose; Neumann-Haefelin et al. 2004). Additionally, the rats are given a constant infusion of U-^{13}C-glucose (1 mg/kg/min) to estimate rates of glucose production and utilization (Michael et al. 2000). Every 15 min, blood samples are obtained via tail-tip bleeds for determination of glucose enrichment. Enrichments are calculated from the ratio of U-^{13}C-glucose/^{12}C-glucose during the last 30 min of the basal period and during the last 60 min of the clamp (i.e., steady state conditions). This ratio was determined by GC-MS analyses of derivatized glucose from blood samples following literature protocols (Michael et al. 2000).

Blood samples were taken at 30 min for determination of baseline insulin levels and at 180, 210, and 240 min for determination of insulin levels under hyperinsulinemia. At 120 and at 240 min, additional blood samples for determination of free fatty acids are taken. Animals were killed by pentobarbital overdose. Metabolic blood parameters are determined using standard methods (Bergmeyer 1974); insulin measurements are performed using commercially available ELISA kits.

EVALUATION

Whole-body insulin sensitivity is calculated as the mean glucose infusion rate (GIR) during the last 60 min of the clamp study.

During steady state, the total glucose appearance in the circulation (Ra) equals the rate of disappearance of glucose (Rd) and is calculated by dividing the U-^{13}C-glucose infusion rate by the steady state value of glucose enrichment. Endogenous glucose production (EGP) is calculated as follows: EGP equals Rd minus glucose infused (GIR). For each animal, two values of EGP are obtained: one during basal conditions and one during the euglycemic-hyperinsulinemic clamp. The methods for quantification of endogenous glucose production in humans are entirely reviewed by Radziuk and Pye (2002).

MODIFICATIONS OF THE METHOD

Burnol et al. (1983) and Smith et al. (1987) used the euglycemic insulin clamp technique coupled with isotopic measurement of glucose turnover to quantify insulin sensitivity in the anesthetized and conscious rats, respectively.

The effects of counterregulatory hormones on insulin-induced glucose utilization by individual tissues in rats, using the euglycemic-hyperinsulinemic clamp technique combined with an injection of 2-[1-3H]-deoxyglucose, were studied by Marfaing et al. (1991).

Lee et al. (1994) studied the metabolic effects of troglitazone on fructose-induced insulin resistance with the euglycemic-hyperinsulinemic clamp technique in rats.

Hulman et al. (1993) studied insulin resistance in the conscious spontaneously hypertensive rat with the euglycemic-hyperinsulinemic clamp technique.

Cheung and Bryer-Ash (1994) described a modified method for the performance of glucose insulin clamp studies in conscious rats under local anesthesia.

Xie et al. (1996) described an insulin sensitivity test using a modified euglycemic clamp in cats and rats. This test uses the amount of glucose required to be infused to maintain euglycemia over a 30-min period in rats and 60 min in cats following a bolus administration of insulin as the index of insulin sensitivity. Glucose levels are determined a short intervals, and variable glucose infusion is used to hold glucose levels within a few percentage points of the basal pretest glucose level. A new blood sampling technique is described that allows each insulin sensitivity test to be carried out using a total of only 0.5 mL of blood.

CRITICAL ASSESSMENT OF THE METHOD

For the safety, pharmacological assessment of candidate compounds to increase or reduce insulin resistance often long-term pretreatment periods for 1 week or longer is necessary before an effect on insulin sensitivity can be detected. Candidate compounds causing an acute effect on lipolysis or antilipolysis of adipose tissue with subsequent changes in free fatty acids normally causes also a fast effect on peripheral insulin sensitivity, which can be measured after a relatively short (16 h) pretreatment period (Schoelch et al. 2004).

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7.3.4 Multiple-Dose Studies

7.3.4.1 Effects on Metabolic Blood and Tissue Parameters in Conscious Rats (Multiple-Dose Study)

PURPOSE AND RATIONALE

For studying the side effect potential of candidate compounds on blood triglycerides and cholesterol as well as on metabolic rate, insulin sensitivity and body weight development multiple-dose studies for at least 1 week are necessary. For transcriptional active compounds like the PPAR agonist (peroxisome proliferators-activated receptor), multiple-dose studies lasting several weeks are necessary to detect the pharmacological activity. The fibrates (e.g., fenofibrate), known since decades as triglyceride-reducing drugs, are agonist of PPARα; thiazolidinediones (or glitazones, e.g., rosiglitazone, pioglitazone) are ligands of PPARγ. These agonists exhibit their full pharmacodynamic profile after they have activated the transcription of whole gene families. Agonists of PPARα predominantly induce gene expression of enzymes involved in lipid catabolism, while agonists of PPARγ predominantly induce genes which are involved in lipid anabolism (adipocytogenesis). PPARα agonists reduce blood lipids (triglyceride, free fatty acids) as well as tissue lipids in muscle and liver by inducing fatty acid oxidation in peroxisomes and mitochondria and thereby improving metabolic blood and tissue parameters. PPARγ agonists induce the permanent formation of new adipocytes in fat tissue and thereby maintain a permanent remodeling of the adipose tissue resulting in a redistribution of fatty acids from ectopic lipid accumulation in liver and muscle as well as reducing blood lipid parameters, however, at the expense of increased total fat mass and increased body weight. Their effects in normal rats are marginal but impressive in animal disease models of insulin resistance, obesity, and hypertriglyceridemia (e.g., Zucker-fatty (fa/fa) rats, Zucker

diabetic fatty (ZDF-fa/fa) rats). Various animal models resembling type 2 diabetes are used for evaluation. Details of these models are described elsewhere (Vogel 2008).

At the end of such multiple-dose studies, the animals are killed in terminal anesthesia, and maximal blood collection is possible. Therefore, not only the target metabolic parameters (e.g., glucose, lactate, free fatty acids, triglycerids, cholesterol) but also other parameters, which reflect intermediary metabolism (e.g., keton bodies, urea, uric acid), as well as safety parameters (e.g., ASAT, ALAT, AP, LDH) can be determined by clinical chemistry.

PROCEDURE

Eight rats per group (lean control, obese control, and obese dose groups) are treated with the candidate compound in various doses for up to 6 weeks. According to the pharmacokinetic profile ($t_{1/2}$), the candidate compound is administered once or twice a day by gavage. If the stability of the compound is sufficient, the candidate compound can also be administered by food admixture. Food and water consumption as well as body weight are measured at least once a week. Blood glucose is determined in 10 μL blood samples as well as for insulin (80 μL blood: 30–40 μL serum) collected from the tip of the tail and by retroorbital bleeding for detection of free fatty acids, triglycerides, and other metabolic (e.g., cholesterol, keton bodies) or safety (e.g., ASAT, ALAT, AP) parameters every 2 weeks.

At the end of the experiment, the rats are terminally anesthetized, the abdomen is opened, and terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1 gr) of skeletal muscle (e.g., M. gastrocnemius). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P), and ATP. In liver samples, hepatic triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P, and ATP (Bergmeyer 1974); insulin is determined by using a commercially available ELISA kits.

EVALUATION

The differences of the metabolic blood parameters of the treated groups are calculated against both control groups (lean and obese), which have received the vehicle only. The metabolic tissue parameters at the end of the study are assessed between the two control and treatment groups.

MODIFICATION OF THE METHOD

For the assessment of the side effect potential of the candidate compound on peripheral insulin sensitivity, multiple oGTTs can be performed during the treatment period or the animal study is finished by a hyperinsulinemic-euglycemic glucose clamp study.

Chang et al. (1983) studied ciglitazone in ob/ob and db/db mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats.

Fujita et al. (1983) investigated the effects of ciglitazone in obese-diabetic yellow KK (KK-Ay) mice and obese Zucker-fatty rats.

Diani et al. (1984) treated C5BL/6J-ob/ob and C57BL/KsJ-db/db mice for several weeks with ciglitazone and studied the morphological effects on pancreatic islets.

Fujiwara et al. (1988) performed studies in KK and ob/ob mice and Zucker-fatty rats.

Moreover, Fujiwara et al. (1991) studied the effects of CS-045 on glycemic control and pancreatic islet structure at a late stage of the diabetes syndrome in C57BL/KsJ-db/db mice.

Ikeda et al. (1990) and Sohda et al. (1990) used insulin-resistant animals (yellow KK mice, Zucker-fatty rats, and obese Beagle dogs with moderate insulin resistance).

Gill and Yen (1991) studied the effects on endogenous plasma islet amyloid polypeptide and insulin sensitivity in obese-diabetic viable yellow mice.

Hofmann et al. (1991, 1992) treated insulin-resistant KKAy mice.

Stevenson et al. (1991) studied the effects of englitazone in nondiabetic rats and found no overt hypoglycemia but an enhancement of insulin action.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activating insulin receptor kinase in genetically obese Wistar fatty rats treated with various doses of pioglitazone.

Sugiyama et al. (1990) found a reduction of glucose intolerance and hypersecretion of insulin in

Wistar fatty rats after treatment with pioglitazone for 10 days.

Tominaga et al. (1993) used the glucose clamp technique in streptozotocin-induced diabetic rats.

Yoshioka et al. (1993) found antihypertensive effects in obese Zucker rats.

Lee et al. (1994) studied the metabolic effects on fructose-induced insulin resistance in rats.

Apweiler et al. (1995) administered BM 13.09143 to lean and obese Zucker rats and performed hyperinsulinemic-euglycemic clamp studies in these animals.

Fujiwara et al. (1995) found a suppression of hepatic gluconeogenesis in long-term troglitazone-treated diabetic KK and C57BL/ksJ-db/db mice.

Lee and Olefsky (1995) studied the effects of troglitazone in normal rats with the euglycemic glucose clamp technique.

CRITICAL ASSESSMENT OF THE METHOD

The results of metabolic tissue parameters in liver and muscle must be interpreted carefully, when a hyperinsulinemic-euglycemic glucose clamp study is performed at the end of the treatment period. Under clamp conditions, these tissue parameters are mainly influenced by the hyperinsulinemic condition during the clamp study than by the compound's effect itself.

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7.3.4.2 Cholesterol Diet-Induced Atherosclerosis in Rabbits and Other Species

General Consideration

The additional anti-atherosclerotic potential of a candidate compound with a different primary indication does not represent a safety concern. In contrast, the atherogenic potential of a candidate compound, identified during safety pharmacological evaluation, represents a serious safety issue. Development of atherosclerosis needs time, and therefore, multiple-dose studies are necessary to detect a putative anti-atherosclerotic or atherogenic side effect potential of a candidate compound.

Experimental atherosclerosis was first successfully induced in rabbits by Saltykow (1908) and Ignatowski (1909). During the following years, various scientists found that dietary cholesterol was the responsible stimulus for development of atherosclerosis. Other species are also susceptible to diet-induced atherosclerosis (Reviews by Kritchevsky 1964a, b; Hadjiinky et al. 1991). A unifying hypothesis of the pathogenesis of atherosclerosis has been proposed by Schwartz et al. (1991).

PURPOSE AND RATIONALE

Rabbits are known to be susceptible to hypercholesterolemia and arteriosclerosis after excessive cholesterol feeding (supplemented with 0.3–2% cholesterol in the diet). Therefore, this approach has been chosen by many authors to study the effect of potential anti-arteriosclerotic drugs. For studying the atherogenic potential of a candidate compound, a low cholesterol concentration in the diet (0.1–0.3%) is recommended (proatherogenic).

PROCEDURE

Several modifications of the protocol have been described. Usually, male rabbits from an inbred strain, e.g., white New Zealand, at an age of 8–10 weeks are used. Body weight variation should be as low as possible. At the beginning of the experiment, blood is withdrawn from the marginal ear vein for determination of total cholesterol, total triglycerides, and blood glucose. Groups of ten animals are used for treatment with drugs or as controls. The rabbits are switched from commercial food to a diet supplemented with 0.3–2% cholesterol and kept on this regimen for a period of 10–12 weeks. One group is kept on normal diet. During and at the end of the experiment, blood is taken for analysis. Usually, cholesterol and triglyceride levels increase several fold over the original values.

The animals are sacrificed and the thoracic aorta is removed, cleaned of surrounding tissues, and longitudinally cut and opened for fixation with formaldehyde. The tissue is stained with oil red. The percentage of the intimal surface covered by the oil red positive lesions is calculated with a computerized planimeter. In animals fed a normal diet, the aorta does not show any staining whereas, in cholesterol-fed rabbits, the aorta shows severe atherogenic lesions.

EVALUATION

The areas of the aortic lesions are compared between control and treatment groups. Appropriate statistical evaluation is performed, and percent inhibition (anti-atherogenic effect) or percent increase of areas with lesions (atherogenic effect) can be calculated.

MODIFICATIONS OF THE METHOD

Shore and Shore (1976) studied two different strains of rabbits (New Zealand White and Dutch Belt) as models of hyperlipoproteinemia and atherosclerosis.

Studies of Kritchevsky et al. (1989) on experimental atherosclerosis in rabbits fed cholesterol-free diets revealed a greater influence of animal protein and of partially hydrogenated soybean oil on development of atherosclerosis than plant protein and unsaturated soybean oil.

Cockerels (Tennent et al. 1960) and turkeys (Simpson and Harms 1969) are very susceptible to cholesterol feeding and develop marked hypercholesterolemia in rather short periods. Atherosclerosis could also be induced in cockerels by high doses of estrogen without atherogenic diet (Caldwell and Suydam 1959).

Spontaneous arteriosclerosis in pigeons has been described by Clarkson and Lofland (1961).

The Japanese sea quail (*Coturnix coturnix japonica*) is highly susceptible to the rapid development of severe experimental atherosclerosis (Day et al. 1975, 1977, 1979, 1990; Chapman et al. 1976).

Out of 13 strains of mice, Roberts and Thompson (1976) selected the C57BR/cdJ and the CBA/J strain and used these strains and their hybrids as models for atherosclerosis research.

Paigen et al. (1987) described quantitative assessment of atherosclerotic lesions in mice. After 14 weeks on an atherogenic diet, C57BL/6J female mice had aortic lesions at each of the coronary arteries, at the junction of the aorta to the heart and in scattered areas of the aortic surface. The lesions increased after 9 months of atherogenic diet. Methods of evaluating the number and size of lesions were compared including sizing with a microscope eyepiece grid and computer-assisted planimetry.

Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice were described by Paigen et al. (1990).

Yamaguchi et al. (1993) found that addition of 10% linoleic acid to a high-cholesterol diet enhanced cholesterol deposition in the aorta of male ICR strain mice.

In rats, hypercholesterolemia can be induced by daily administration by gavage of 1 mL/100 g body weight of a cocktail containing in 1 L peanut oil: 100 g cholesterol, 30 g propylthiouracil, and 100 g cholic acid over a period of 7 days. The test compounds are administered simultaneously with the cocktail (Fillios et al. 1956; Lustalot et al. 1961).

Inoue et al. (1990) induced experimental atherosclerosis in the rat carotid artery by balloon de-endothelialization and atherogenic diet. A balloon catheter was introduced into the rat's carotid arteries from the iliac arteries, and the endothelium was denuded.

The hamster is susceptible to atherosclerosis. Nistor et al. (1987) fed male hamsters a hyperlipidemic diet consisting of standard chow supplemented with 3% cholesterol and 15% commercial butter for 12 months. Serum total cholesterol doubled after 3 weeks and attained a 17-fold value after 10 months. Up to 6 months, smooth muscle cells in the intima and media of the aorta as well as endothelial cells began to load with lipids. After 10 months, the affected zones looked like human atherosclerotic plaque with huge cholesterol crystal deposits, calcium deposits, and necrosis.

Especially, the hybrid hamster strain Bioä F1B (Bio Breeders Fitchburg, MA, USA) is more susceptible to dietary-induced atherosclerosis than other strains (Kowala et al. 1991). Early atherosclerotic lesions can be induced within a 3-month feeding of a cholesterol/butter-enriched diet. In these animals, simvastatin dose dependently inhibited the development of hyperlipidemia and the plaque formation by cholesterol synthesis inhibition. The histopathological examination of the aortas showed that the cholesterol-/butter-fed F1B hamster developed atherosclerotic lesions and functional changes in the aorta which are closely related to man (Schäfer et al. 1999).

Soret et al. (1976) studied the diet-induced hypercholesterinemia in the diabetic and nondiabetic Chinese hamster.

Beitz and Mest (1991) used cholesterol-fed guinea pigs to study the antihyperlipemic effects of a potentially anti-atherosclerotic drug.

Malinow et al. (1976) recommended the cynomolgus monkey as a model for therapeutic intervention on established coronary atherosclerosis.

This species was used by Hollander et al. (1978) to study the development atherosclerosis after a cholesterol- and fat-enriched diet.

Beere et al. (1992) described experimental atherosclerosis at the carotid bifurcation of the cynomolgus monkey by a cholesterol-enriched diet.

Eggen et al. (1991) studied the progression and the regression of diet-induced atherosclerotic lesions in aorta and coronary arteries on rhesus monkeys.

Howard (1976) recommended the baboon as model in atherosclerosis research because of the similarity of cholesterol metabolism and composition of the lipoproteins to man.

Kushwaha et al. (1991) determined the effect of estrogen and progesterone on plasma cholesterol concentrations and on arterial lesions in ovarectomized and hysterectomized baboons fed a high-cholesterol/high-saturated-fat diet.

Blaton and Peeters (1976) reported studies on the chimpanzee, the baboon, and the rhesus macacus as models for atherosclerosis.

Ming-Peng et al. (1990) studied high-density lipoproteins and prevention of experimental atherosclerosis in tree shrews (*Tupaia belangeri yunalis*). In contrast to rabbits, no increased lipid deposition in aortic intima after cholesterol feeding was found in tree shrews.

CRITICAL ASSESSMENT OF THE METHOD

Diet-induced hypercholesterolemia is useful only for detection of agents interfering with the adsorption, degradation, and excretion of cholesterol. Agents interfering with cholesterol biosynthesis are less probable to be detected.

The use of normal adult marmosets, a species with a lipoprotein profile similar to that of man, may be an alternative (Crook et al. 1990; Baxter et al. 1992).

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7.3.5 Acute Effect on Food Consumption

7.3.5.1 General Considerations

The additional weak anorectic effect of a candidate compound with a different primary indication does not represent a safety concern, if not representing a symptom of weak or starting toxicity. In contrast, an orexigenic potential of a candidate compound, identified during safety pharmacological evaluation, represents a serious safety issue. Compound-mediated increased food consumption (orexigenic effect) might lead to obesity, to insulin resistance, and to overt type 2 diabetes. Food and milk consumption can be measured in acute experiments in mice and rats as well as in multiple-dose studies over weeks in parallel to the measurement of body weight development and changes in body composition.

7.3.5.2 Acute Effect on Milk Consumption in Mice

PURPOSE AND RATIONALE

Milk intake is measured in acute experiments in normal or obese mice after an overnight fast. Milk intake serves as a surrogate for food consumption.

PROCEDURE

According to Bickel et al. (2004), male, fasted (24 h) mice, which are placed individually in cages having free access to sugared milk (1 mL = 3.27 kJ) in a graduated cylinder, are used to monitor the amount of milk consumed by the animals. Milk is offered at time 0 min. Cumulative milk consumption is measured at 2, 4, and 6 h after drug or vehicle administration. The experiments are performed during the light phase. During the experimental session, the animals have no access to solid feed.

EVALUATION

Average milk consumption is recorded per control, and treatment groups and can be expressed as means per time point or cumulative over the whole study period of 6 h.

MODIFICATIONS OF THE METHOD

Yamada et al. (2003) studied the interaction of the leptin-induced suppression of milk consumption with the serotonergic pathway.

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7.3.5.3 Acute Effect on Food Consumption in Rats

PURPOSE AND RATIONALE

Food intake is measured in acute experiments in normal or obese rats. Additionally, in subchronic and chronic multiple-dose experiments, body weight gain is recorded.

PROCEDURE

Female Zucker (fa/fa) rats weighing 250–350 g are maintained under standard conditions (temperature, light-dark cycle, ground rodent pellet chow, tap water). Measurements of food intake begin on day 2. The food is offered in special dishes to reduce spillage. Food intake and body weight are measured daily between 8:00 and 9:00 a.m. At this time, any spilled food from the collecting paper under the cage is gathered, air-dried if necessary, and weighed. Individual food intakes in grams are recorded. The tests compounds are either administered with the food or injected intraperitoneally. Groups of 8–10 animals are used for control or treatment with various doses of the test compound or the standard. Mazindol, 3 m/kg i.p. or 10 mg/kg p.o., can serve as standard. Treatment is continued for 7 days.

EVALUATION

Average food intake and body weight are recorded for each day. Average values of candidate compounds are compared statistically for each day with the control group. Results after oral administration have to be confirmed by parenteral route in order to exclude errors due to palatability.

MODIFICATIONS OF THE METHOD

Bickel et al. (2004) reported the use of an online-feeding monitoring system. Animals were placed individually in cages (Macrolon-cage (type 4), size: 44′ 26′ 15 cm) equipped with a device for continuous monitoring of feed consumption. This device is a container, filled with feed, and hanging on an electronic balance. The balance transmits the weight changes of the feed container continuously to a central unit; data were stored and processed at the end of the experiment. This system can measure feed consumption up to 3 weeks from >100 feeding sensors simultaneously. The feeding monitoring system and data processing hard- and software (Release V3.07-03/2001) were obtained from TSE, Technical & Scientific Equipment GmbH, Bad Homburg, Germany. Software options: The data are processed from a matrix. The matrix delivers cumulatively or sequentially feed consumption in grams (g) for given time intervals for each individual animal. Cumulative and sequential feed consumption is normalized to g feed/time/100 g body weight (bw). A program for microstructural analysis calculates the parameters for feed consumption over a given time (24 h). Parameters delivered are number of meals (Nm/time), intermeal breaks (IMB) (min), average meal size (g), and average meal duration (min). A meal was defined to be 0.5 g and the IMB 15 min. Details of this method are described by Bickel et al. (2000).

Hull and Maher and Maher and Hull (1990) used male Sprague-Dawley rats placed on a mush diet composed of equal parts of ground rodent chow and of 4% nutrient agar solution. The agar-based chow allows a more accurate measurement of food intake and has been shown to be sufficient for maintaining normal growth in rats. The rats were made hyperphagic by food deprivation for 4 h at the beginning of the dark cycle.

Mennini et al. (1991) and Anelli et al. (1992) studied the anorectic activity of various compounds in different species, such as mice, rats, and guinea pigs.

Bowden et al. (1988) used metabolism cages equipped with automated feeding monitors. Food was provided as 45 mg pellets which were singly delivered

to a feeding trough. A photodetector sensed the removal of the pellet, and the number of pellets delivered over a specified time interval was recorded.

Samanin et al. (1979) described anorexia in rats induced by the central serotonin agonist m-chlorophenylpiperazine.

Blavet et al. (1982) studied food intake in fasted rats after treatment with several typical anorexigenic agents.

Dourish et al. (1985) investigated the effects of the serotonin agonist 8-OH-DPAT on food intake in nondeprived male rats. This effect was prevented by *p*-chlorophenylalanine (Dourish et al. 1986).

The anxiolytics gepirone, buspirone, and ipsapirone increased free feeding in rats and did not inhibit feeding induced by 8-OH-DPAT (Gilbert and Dourish 1987).

Jackson et al. (1997) investigated the mechanisms underlying the hypophagic effects of the 5-HT and noradrenaline reuptake inhibitor, sibutramine, in the rat.

Simansky and Vaidya (1990) tested the anorectic action of a serotonin uptake inhibitor by measuring the volume of milk consumed by food-deprived rats.

Stevens and Edwards (1996) induced anorexia by subcutaneous injection of 5 mg/kg 5-hydroxytryptamine in Wistar rats habituated to a restricted feeding regime and tested the effects of a 5-HT_3 antagonist.

Rouru et al. (1992) investigated in genetically obese male Zucker rats the effect of subchronic metformin treatment on food intake, weight gain and plasma insulin and corticosterone levels, and somatostatin concentrations in the pancreas.

Cooper et al. (1990a, b) used nondeprived rats to study anorectic effects in a test of palatable food consumption and in nocturnal free feeding.

Cooper et al. (1990c) tested not only food consumption but also the frequency of feeding bouts and duration of individual feeding episodes.

Eberle-Wang and Simansky (1992) studied the influence on the anorectic action of CCK and serotonin by measuring the uptake of sweetened mash mixture in rats.

Voigt et al. (1995) studied the involvement of the 5-HT_{1A} receptor in CCK induced satiety by recording food intake during a 2-h test meal in food-deprived and in freely feeding rats.

Influence on postprandial satiety in rats was tested by Rosofsky and Geary (1989). Rats were given pelleted chow and water ad libitum. Near the middle of the bright phase of the light-dark cycle, pellets were removed, the animals treated, and condensed milk presented 30 min later. Milk consumption was measured at 4-min intervals for 40 min.

Rats show a dramatic and reliable reduction of food intake if they are prefed a low-protein basal diet and then offered a diet that is imbalanced in any of the essential amino acids (Leung and Rogers 1969). This anorectic response has been used by Hammer et al. (1990) to test serotonin3-receptor antagonists.

Thurlby and Samanin (1981) studied the effect of anorectic drugs on food-rewarded runway behavior.

Ferrari et al. (1992) studied the effects on anorexia induced by ACTH and immobilization in rats in an X-maze with alternate open and covered arms, each baited with laboratory chow.

Cooper et al. (1993) studied dopamine D1-receptor antagonists in rats with chronic gastric fistula, which were trained to sham feed a 10% sucrose solution in a 60 min test.

In wild rodents, hoarding of food covers the long-term alimentary need. In the laboratory, hoarding behavior does not occur in ad libitum fed rats. On the contrary, rats whose energy balance is threatened by previous food restriction hoard as soon as experimental conditions allow to do so. When such a rat gets free access to a food stock (placed outside its usual territory), it carries food into its shelter and accumulates an amount proportionate to its body weight. Fantino et al. (1980, 1986, 1988) and Nishida et al. (1990) used the reduction of the amount of food hoarded during a period of 3 h as parameter for anorectic activity of drugs.

Caccia et al. (1993) studied the anorectic effect of D-fenfluramine in the marmoset (*Callithrix jacchus*).

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Peripheral Nervous System

8

H. Gerhard Vogel*

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8.1 Tolerance of Local Anesthetics

8.1.1 General Considerations

One generally has to distinguish between surface anesthesia, infiltration anesthesia, and conduction anesthesia (Fromherz 1922; Schaumann 1938; Camougis and Takman 1971). Special local tolerance tests have been developed for each of these applications including peridural and intrathecal injections.

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8.1.2 Irritancy After Surface Anesthesia

PURPOSE AND RATIONALE

Surface anesthesia is used to anesthetize the cornea and conjunctiva of the eye and the mucous membranes in the mouth. The classical pharmacological test is the blockade of the rabbit corneal reflex as described by Régnier (1923) that has become a standard test method for evaluating local anesthetics (Fußgänger and Schaumann 1931; Ther 1953a; Quevauviller 1971;

*Deceased

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Muschaweck et al. 1986). These pharmacological methods are only partially suitable to determine the irritancy potential of local anesthetic on mucous membranes. Luduena et al. (1960) compared the mucous membrane irritancy of mepivacaine and lidocaine by the eye irritation method according to Hoppe et al. (1950) and Draize et al. (1944).

For further description of the method, see Chapters I.N (Chap. 12, "Ocular Safety Tests") and I.P (Chap. 15, "Skin Pharmacology").

PROCEDURE

For the rabbit eye irritation tests, the rabbits are placed in ventilated boxes with only the head free. Solutions of local anesthetics in 0.9% saline are prepared, and a volume of 0.2 ml is instilled into the conjunctival sac of one eye, leaving the other as control. The lower lid is retracted gently and held away from the cornea for 1 min, thus ensuring exposure to the solution.

EVALUATION

The medicated eye is examined in comparison with the control eye at 1, 2, 4, and 8 h after medication and scored according to the method of Draize et al. (1944).

Threshold irritant concentrations are calculated. The threshold irritant concentration is taken as that concentration, expressed in percent, which produces no more than a mild irritation.

According to Draize et al. (1944), injuries to the cornea, conjunctival and palpebral mucosae, and the iris are scored separately. The severity of ocular lesions has been graded in a scale of weighted scores.

8.1.2.1 Cornea

A. Opacity—Degree of density (area which is most dense is taken for reading)

Scattered or diffuse area, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4

B. Area of cornea involved

One quarter (or less) but not zero	1
Greater than one quarter, but less than one-half	2
Greater than one half, less than three quarters	3
Greater than three quarters, up to the whole area	4

Score equals A × B × 5
Total maximum = 80

8.1.2.2 Iris

Values

Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any one or all of these)	2

Score equals A × 5
Total possible maximum = 10

8.1.2.3 Conjunctivae

A. *Redness* (refers to palpebral conjunctivae only)

Vessels definitively injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3

B. *Chemosis*

Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of the lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4

C. *Discharge*

Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and considerable area around the eye	3

Score (A + B + C) × 2
Total maximum = 20

The *maximum total score* is the sum of all scores obtained from the cornea, iris, and conjunctivae.

CRITICAL ASSESSMENT OF THE METHOD

The Draize ocular irritation test is the accepted standard as part of evaluating chemical substances for eye safety. Justified by an exigency for public protection, the Draize test became a governmentally endorsed method to evaluate the safety of materials meant for the use in or around eyes. Because of discomfort to test animals that may be associated with a positive response, continued use of the Draize test has been criticized and alternative methods proposed (Durham

et al. 1992; Prinsen and Koeter 1993; York and Steiling 1998; Curren and Harbell 1998; Kulkarni et al. 2001; Wilhelmus 2001; Abraham et al. 2003; Perrot et al. 2003). With the development of alternative methods to replace the Draize test, the data generated in the Draize test are still being used as a "gold standard" against which the performance of alternative procedures is measured.

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8.1.3 Irritancy After Intradermal Injection

PURPOSE AND RATIONALE

Intradermal tolerance of local anesthetics can be used as a screening selection criterion for finding an optimal local anesthetic. Luduena and Hoppe (1952) and Luduena et al. (1960) determined intradermal irritancy by the trypan blue method according to Hoppe et al. (1950).

PROCEDURE

Albino rabbits of either sex weighing 2–3 kg are fastened securely in a supine position and the hair carefully clipped from the abdominal area with a fine-bladed clipper. The bare abdominal surface is marked off into six to eight areas of approximately 20 cm^2 each by a soft wax pencil. In preparing solutions for injections, the highest concentration is prepared first and subsequent dilutions are made therefrom in ratios of 1/2, 1/4, 1/8, 1/16, etc., in order to find the submaximal irritation range. A volume of 0.3 ml of each dilution is injected intracutaneously into randomly designed areas of the abdominal skin of the rabbit. A dose of 1.0 ml/kg of a 1% solution of trypan blue in normal saline is injected 10–20 min after the last intracutaneous injection of test solutions. The sites of injection are examined at one-half, one, and three hours after injection of trypan blue.

EVALUATION

The intensity of staining by trypan blue at the site of injection is scored as follows:

No color	0
Faint but discernible color	2
Distinct blue color throughout	4
Deep blue color throughout	8
Ischemic central area surrounded by deep blue halo	16

The scores are rated as follows:

Average score rating	
0	None
1–3	Mild
4–7	Moderate
8	Or greater marked

The threshold concentration is taken as that concentration expressed in%, which produces no more than a mild irritation.

MODIFICATION OF THE METHOD

Henn and Brattsand (1966), using the trypan blue test, reported that intradermal irritancy of mepivacaine in rabbits was less than with tetracaine, whether the solution contained adrenaline or not.

Using the trypan blue test, Luduena et al. (1972) found that racemic, (+), and (−) mepivacaine had the same intradermal irritancy.

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8.1.4 Irritancy After Subcutaneous Injection

PURPOSE AND RATIONALE

The pharmacological irritancy after subcutaneous injection is determined with the method of Bülbring and Wajda (1945) in guinea pigs and modifications thereof (Ther 1953b).

Irritancy of local anesthetics after subcutaneous injections can be determined by subcutaneous injection into the ears of rabbits (Ulfendahl 1957). This method can be used not only as a screening selection criterion for finding an optimal local anesthetic but also as test method for evaluation of production batches (Hergott 1965).

PROCEDURE

Rabbits of either sex weighing 2.5–3.5 kg are used. A volume of 0.1 ml of the test solution is injected in the outer part of the rabbit's ear avoiding hitting any blood vessels. The same volume of saline is injected into the contralateral ear. A pale discoloration of the skin appears immediately, which disappears within 1 h in the control.

EVALUATION

The injection site is inspected after 2 and 24 h. Reactions are scored as absent, slight, moderate, or marked (necrosis).

MODIFICATION OF THE METHOD

The method has been used for evaluation of several local anesthetics (Ther 1953a; Muschaweck and Rippel 1986).

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8.1.5 Irritancy After Intramuscular Injection

PURPOSE AND RATIONALE

Local irritancy after intramuscular injection anesthetics can cause irritation and necrosis in muscular tissue. Most studies are performed in rabbits (Luduena et al. 1960; Baeder et al. 1974).

PROCEDURE

Groups of three rabbits of either sex weighing 2–3.5 kg are injected intramuscularly in the gluteal muscle with 1 ml solution of the local anesthetic with and without vasoconstrictor in various concentrations. Each muscle injection site is used only once. The animals are sacrificed at 1-, 2-, or 7-day intervals. The injection sites are examined macroscopically, fixed in Zenker formalin and embedded in paraffin. Histologic preparations are made in the usual way for microscopic examination.

EVALUATION

The slides are checked for inflammatory signs, such as edema, leukocytic infiltration, or foci of coagulative necrosis.

MODIFICATION OF THE METHOD

Several studies were performed on myotoxicity of local anesthetics.

Basson (1978) and Basson and Carlson (1980) described myotoxicity after single and repeated injections of mepivacaine in the rat. Young rats received single or repeated injections of 2% mepivacaine into the tibialis anterior or extensor digitorum longus muscles. Repeated injections consisted of six injections of the anesthetic (100 µl per injection into the tibialis anterior) on different schedules, at intervals of 2 1/2 h, 24 h, or 4 days. The muscles were examined histologically for evidence of myotoxicity from 0 to 7 and 20 days after the last injection. Single injections showed that mepivacaine is a myotoxic drug, producing a lesion, which ultimately results in a degeneration and subsequent regeneration of large amounts of muscle. A similar picture was seen with repeated injections except that greater tissue destruction was noted. Long-term studies following a single injection of mepivacaine showed restoration of the original muscle structure, whereas after repeated injections, some muscles showed persisting foci of interstitial connective tissue. The damage produced by 2% mepivacaine is restored to a large extent by the regeneration of new muscle fibers.

Benoit and Belt (1972) described effects of local anesthetic agents on skeletal muscle and the correlation between local anesthetic-induced myotoxicity and disturbances of intracellular calcium distribution. The gracilis anticus and posticus muscles of albino rats were exposed to various local anesthetic agents (lidocaine, procaine, bupivacaine, cocaine, mepivacaine, or prilocaine). Clinically used concentrations of most agents appeared to be specifically destructive to skeletal muscle. Muscle regeneration from single myoblasts was found to be rapid and complete by 2 weeks. One experiment (experiment 3) was designed to assess only extent of damage but not sequence of changes. Muscles were removed 1 day after a single injection of local anesthetic. No muscle damage was observed with 0.5% lidocaine, 0.5% mepivacaine, or 2% procaine. After 2% prilocaine and 2% mepivacaine, the muscle damage was more extensive.

In another study (Benoit et al. 1980), the local anesthetic mepivacaine and various other drugs known to perturb sarcoplasmic calcium metabolism and/or sarcolemmal sodium conduction were injected into the gastrocnemius or subcutaneously over the gracilis muscle of rats. After 48 h, all of the agents which are capable of increasing the intracellular concentration of free calcium (mepivacaine HCl, quinidine gluconate, A23187, caffeine, and 2,4-dinitrophenol) produced extensive and qualitatively similar myonecrosis. To assess the influence of a calcium antagonist on local anesthetic-induced myonecrosis, verapamil HCl was administered s.c., both alone and in combination with mepivacaine. Although verapamil itself resulted in some minor surface injury, it almost completely blocked the damage produced by mepivacaine. It was concluded that the myotoxicity of local anesthetics is related to a disturbance of intracellular calcium homeostasis.

Carabot et al. (1988) tried to explain the myotoxicity by effects of mepivacaine on the microcirculation of the skeletal muscle. Mepivacaine was inoculated directly into the anterior tibialis muscle of rats. Capillaries were examined through an electron microscope, and capillary density in the damaged

area was calculated by the alkaline phosphatase technique. Degenerative changes were observed in the capillaries 1 h postinoculation. These changes were more significant at 5 days and were no longer visible after 20 days when the regeneration of the muscle fibers was almost complete. Capillary density was reduced when ultrastructural changes were most intense.

Since local anesthetics are widely used in ophthalmic surgery, several studies on extraocular muscle regeneration after local anesthetic–induced lesions were performed by Carlson and Rainin (1985), Økland et al. (1989) in rats, and by Carlson et al. (1992) in primates. In the first study, rats were given an injection of 50 µl of a local anesthetic (mepivacaine, lidocaine, or bupivacaine) into the retrobulbar space of the left eye and injection of the same volume of sterile saline into the retrobulbar space of the right eye. The point of needle entry was posterior to the eyeball. Rats were sacrificed at 3, 7, and 30 days after treatment. The eyes and orbital content were removed and fixed in Bouin's fluid for paraffin sectioning. Sections were stained with hematoxylin–eosin. All three anesthetics produced massive degeneration of the extraocular muscles. Muscle degeneration is followed by regeneration on the damaged muscle fibers. In addition to muscular damage, severe damage was also seen in Harderian glands. In another study, adult rats were given single retrobulbar injections of 50 µl of 2% mepivacaine, and the lateral rectus muscles were examined ultrastructurally from 15 min to 30 days postinjection. The lateral rectus muscle was massively damaged by exposure to the anesthetic, with membrane lesions seen as early as 15 min after the injection. Intracellular damage was followed by the phagocytic removal of the remnants of the damaged muscle fibers. The activation of satellite cells to myoblasts began during the phase of phagocytosis, and between 3 and 4 days after injection, multinucleated myotubes actively forming sarcomeres appeared. The myotoxic effect of retrobulbarly applied local anesthetics in rats seemed to be much greater than they are in primates.

In a study in rhesus monkeys, retrobulbar administration of local anesthetics resulted in a low incidence of muscle fiber lesions in the extraocular muscles closest to the site of injection. Most lesions resulted in the degeneration and regeneration of muscle fibers on the surface of the muscles, but occasionally, a massive internal lesion was seen.

Hagiwara and Ozawa (1985) investigated toxicity of local anesthetics on chick myogenic cells (mononucleated myoblasts and multinucleated myotubes) in culture. Following treatment with the drugs, myogenic cells showed some morphological changes and finally detached from the culture dishes. The toxic effect was estimated by the amount of cells detached and by the DNA and creatine kinase activity of the cells remaining on the dishes. Dibucaine was more toxic than bupivacaine, mepivacaine, tetracaine, and procaine.

Carlson et al. (1990) studied local anesthetic–induced skeletal muscle fiber degeneration and regeneration in the monkey by light microscopy and Komorowski et al. (1990) by electron microscopy. Intramuscular injections of 0.75% bupivacaine, 2% mepivacaine, or 2% lidocaine + epinephrine were given into the abductor pollicis muscles of rhesus monkeys. The muscles were examined from 2 to 28 days. Severe muscle damage, consisting of breakdown of sarcolemma and myofibrils, was seen as early as 2 h. Phagocyte-mediated fragmentation of the degenerating muscle fibers was at its peak during the third and fourth days. Myoblasts were abundant during the fourth day. Early myotubes appeared on the fifth and sixth days, and they matured during the second week. Satellite cells appeared alongside mature myotubes.

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8.1.6 Irritancy After Intraneural and Perineural Injection

PURPOSE AND RATIONALE

Nerve irritancy after intraneural and perineural injection injuries is a well-recognized complication of regional anesthesia (Selander 1993; Borgeat and Ekatodramis 2001). Kalichman et al. (1986, 1988, 1989) studied neurotoxicity of local anesthetics in rat sciatic nerve.

PROCEDURE

Female Sprague Dawley rats (250–400 g) were anesthetized by intraperitoneal injection of a mixture of sodium pentobarbital 50 mg/ml, diazepam 5 mg/ml, and 0.9% saline in volume proportions of 1:1:2. Both sciatic nerves were exposed by lateral incision of the thigh and reflexion of the fascia and underlying muscle. Commercial preparations of low- and high-potency ester and amide local anesthetics (among them 2% mepivacaine) were administered using a 30-gauge needle adjacent to the nerve, but external to the epineurium. The wounds were closed and the rats allowed to recover. After 48 h, nerves were excised after anesthesia and prepared for light and electron microscopy. They were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer at a pH of 7.4. After glutaraldehyde fixation for 24 h, nerves were rinsed in buffer and postfixed for 3 h in 1% osmium tetroxide. The tissue was subsequently dehydrated in serial alcohol solutions and later in propylene oxide prior to infiltration with araldite. Blocks were sectioned for light microscopy and stained with either paraphenylenediamine or methylene blue azure II. Electron microscopy was performed on selected bocks after preparation of ultrathin sections stained with uranyl acetate and bismuth subnitrate. Sections were examined in Siemens 1001 ultramicroscope operation at 80 kV.

EVALUATION

Concentration dependence of nerve injury was tested using a subjective scoring system of 0, 1, or 2. Maximum severity was assigned a score of 2, a score of 0 indicated no injury, and moderate or equivocal injury was indicated by a score of 1. The three measures of interest were edema ("structureless space"), nerve fiber injury (degeneration and demyelinization), and lipid droplets (osmophilic inclusions in epineural, perineural, and endoneural cells).

MODIFICATION OF THE METHOD

Henn and Brattsand (1966) tested tissue irritancy after intraneural and perineural injection in rabbits. The test solution—0.25 ml intraneural and 1.0 ml perineural, respectively – was injected into/around the exposed N. ischiadicus of adult-mixed bred rabbits anesthetized with thiopental sodium. Four and eight days after the operation, tissue specimens were excised. The nerve tissues were treated by two different methods – according to the Marchi technique and according to the hematoxylin–eosin staining procedure after fixation in Bouin's solution. At all injection sites, some connective tissue proliferation was found, and after intraneural injection, some loosening-up of neurofibrils was observed. These reactions were also seen among the controls with NaCl and were therefore due to the trauma caused by the operation and injection. In no case were there pathological findings in the axons of the nerves. On the other hand, at high concentrations, some moderate degenerative effects upon the myelin sheaths were observed. At concentrations of 0.5–3% mepivacaine used clinically, no such signs were found.

Knox et al. (1961) studied nervous tissue toxicity in anesthetized rabbits. Using a sterile technique, the right and left sciatic nerves were exposed. In one series of 15 animals, 0.25 ml of lidocaine and mepivacaine in concentrations of 0.5, 1.0, 1.5, and 2% were injected directly into the right and left sciatic nerves. In a second series, three animals received 0.25 ml

mepivacaine 4% directly into both nerves. Controls received 0.25 ml normal saline. In a fourth series of five animals, 1.0 ml of test drugs was deposited around the nerve utilizing the same concentrations. All animals except the controls had a satisfactory sciatic nerve block as evidenced by hind limb paresis on recovery from the ether anesthesia. The rabbits were sacrificed on the second, fourth, and eighth day following injections, and the sciatic nerves were excised and prepared for histologic examination utilizing hematoxylin and eosin, Luxol fast blue, and Masson's trichrome staining techniques. In none of the microscopic sections of sciatic nerves which were exposed to the 0.5 through the 2.0% concentrations of drugs was there evidence of nerve damage (myelin and/or axon degeneration). No demonstrable difference was noted between nerves blocked with lidocaine and those blocked with mepivacaine. The usual reaction seen in these sections was a connective tissue proliferation which was believed to be caused by the trauma of surgery or the injection and which did not differ markedly from control. Nerve degeneration was seen in the right sciatic nerves of two of the three rabbits that received mepivacaine 4% in both sciatic nerves. This reaction was noted in the rabbits sacrificed on the fourth and eighth days.

Gentili et al. (1980) performed a light and electron microscopic, fluorescent microscopic, and horseradish peroxidase study in rats on nerve injection injury with local anesthetic agents: the sciatic nerve of pentobarbital-anesthetized adult Wistar rats was exposed in the upper thigh with the aid of an operating microscope; care was taken to avoid damage to the vascular supply. Using a 30-gauge needle, the nerve was injected at a standard location 1 cm distal to its exit from under the piriform muscle. The agents were injected either directly into the nerve fascicle or applied to the surrounding epineural tissues (extrafascicular). The nerve was examined at 1–2 h and 9–12 days after injection. Extrafascicular injections did not result in any significant nerve injury or disturbance of the blood-nerve barrier. After intrafascicular injection, the degree of injury varied significantly depending on the specific agent injected. Minimal damage was seen after the injection of 1% mepivacaine and 0.5% bupivacaine. The most severe injury with widespread axonal and myelin degeneration was seen after the injection of 2% procaine and 1% tetracaine.

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8.1.7 Irritancy After Epidural Anesthesia

PURPOSE AND RATIONALE

Activity irritancy after epidural anesthesia and tolerability of new local anesthetics after intrathecal injection have to be studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients. Myers and Sommer (1993) published a survey on methodology for spinal neurotoxicity studies. Studies in rats were reported by Blomberg and Rickstein (1988); in guinea pigs by Siems and Soehring (1952) and Åkerman et al. (1988b); rabbits by Hughes et al. (1993) and Chernyakova et al. (1994); dogs by Defalque and Stoelting (1966), Feldman and Covino (1988), Raner et al. (1994), and Kamibayachi et al. (1995); cats by Ide et al. (2001); laboratory pigs by Richer et al. (1998); and sheep by Lebeaux (1975) and Feldman et al. (1997). Kief and Bähr (1970) described epidural tolerance of local anesthetics in dogs.

PROCEDURE

Epidural tolerance of articaine with and without addition of Suprarenin was studied in beagle dogs weighing 9–12 kg. For preoperative sedation, the dogs received

0.03 ml/kg Combelen (propionylpromazine) intravenously. The fur of the lumbosacral area was shaved and the skin disinfected. A single dose of 5 ml of a 2% articaine solution was administered epidurally under sterile conditions. All dogs showed the typical symptoms of spinal anesthesia, which subsided after a few hours. The dogs were sacrificed after 1 or 3 days. The portion of the vertebral column with the site of injection in the middle was removed and placed in 8% buffered formalin. When semifixed, the vertebral arches were opened, and the spinal cord, as well as the roots of the spinal nerves with the adipose tissue of the epidural space, was removed. After embedding in gelatin and Paraplast, the serial sections from the area of injection were stained with fast red 7B, hematoxylin–eosin, and myelin sheath staining according to Olivecrona was performed. Furthermore, the PAS and iron reaction was performed in one section.

EVALUATION

The presence or absence of nerve damage and of inflammation was noted.

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8.1.8 Irritancy After Intrathecal (Spinal) Injection

PURPOSE AND RATIONALE

Transient irritancy after intrathecal (spinal) injection neurological symptoms have been observed in patients after spinal anesthesia (Hampl et al. 1995). Activity and tolerability of new local anesthetics after intrathecal injection were studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients.

Myers and Sommer (1993) published a survey on methodology for spinal neurotoxicity studies. Studies in rats were performed by Yaksh and Rudy (1976), Hylden and Wilcox (1980), Bahar et al. (1984a), Åkerman (1985), Ossipov et al. (1988), Cole et al. (1990), Wang et al. (1991), Dirksen et al. (1992), Mestre et al. (1994), Omote et al. (1995), Chanimov et al. (1997), Grouls et al. (1997), and De la Calle and Palíno (2002); in mice by Hylden and Wilcox (1980), Åkerman (1985), Åkerman et al. (1988a,b), and Langerman et al. (1994); in rabbits by Bieter et al. (1936a, b), Luduena et al. (1960), and Langerman et al. (1991); in dogs by Wagner et al. (1940), Feldman and Covino (1981), Kozody et al. (1985), and Dohi et al. (1987); in marmosets by Bahar et al. (1984b); in rhesus monkeys by Denson et al. (1981); in sheep by Lebeaux (1975) and Kyles et al. (1992).

For Detailed Description of the Pharmacological Effects, See Chap. 11 "Blood Constituents and Safety Pharmacology."

Kirihara et al. (2003) compared neurotoxicity of intrathecal and epidural lidocaine in rats.

PROCEDURE

Kirihara et al. (2003) compared neurotoxicity of intrathecal and epidural lidocaine in rats. Male Sprague Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and 1.5% halothane. A catheter of stretched polyethylene tubing PE-10 was introduced into the subarachnoid or epidural space using an aseptic technique. Catheters were passed through the L4–L5 intervertebral space and advanced 1.3 cm in the caudal direction. Rats were allowed 4 days to rest for recovery from the operation.

To measure the response to noxious heat stimulus, a tail flick test was performed. A 100-W projector lamp was focused on the distal segment of the tail approximately 5 cm from the tip. The time at which rats withdrew the tail was defined as the tail flick latency. A cutoff time of 10 s was used to avoid damage of the tail.

To measure the response of legs to noxious mechanical stimulus, a paw pressure test was applied to the dorsal surface of both hind paws using a device capable of progressively increasing the pressure at a rate of 15 g/s. The pressure at which the rat withdrew the paw from the device was defined as the paw pressure threshold, and the mean of both paws was used for analysis. A cutoff pressure of 400 g was used to prevent damage to the paws.

Motor function in the lower limbs was assessed by grading: 0 = none, 1 = partially blocked, and 2 = completely blocked.

Various concentrations of local anesthetic or saline are injected intrathecally in a volume of 20 μl or epidurally in a volume of 100 μl followed by 10 μl saline to flush the catheter. Tail flick test, paw pressure test, and motor function were performed 10, 20, 30, 60, 120, 180, and 240 min after injection and continued daily for 4 days.

After the last experiments, the rats are euthanized by injection of an overdose of pentobarbital and then perfused intracardially with a phosphate-buffered 2.0% paraformaldehyde—2.5% glutaraldehyde fixative. Methyl green solution was injected to confirm the location of the catheter after the perfusion. The spinal cord and nerve roots were dissected out and immersed in the same fixative for 4 h. Two specimens (10 mm rostral and caudal to the conus medullaris) from each rat were postfixed with cacodylate-buffered 1% osmium tetroxide dehydrated in a series of graded alcohol solutions and embedded in epoxy resin. From the embedded tissue, 1-μm transverse sections were obtained and stained with toluidine blue dyes. Sections obtained from 10 mm rostral to the conus (caudal spinal cord) were used for qualitative evaluation. Quantitative analysis of nerve injury was performed using the sections obtained from 10 mm caudal to the conus. Each fascicle present in the cross section was assigned to an injury score 0–3. The injury score for each cross section was then calculated as the average score of all fascicles present in the cross section.

EVALUATION

Data are presented as mean ± SEM. Tail flick latencies and paw pressure thresholds were converted to the percentage of maximal possible effect. The area under the time-effect curve was calculated by accumulating the effect measured at discrete time intervals using the trapezoidal integration method. The results were analyzed by ANOVA with repeated measures followed by Scheffe and Dunnett tests. The injury score for each technique and each solution was compared using two-way ANOVA followed by the Scheffe test. The frequency (i.e., the number of rats with lesions) in each group was analyzed by chi-square test.

MODIFICATION OF THE METHOD

Wakamatsu et al. (1999), Ohtake et al. (2000), and Oka et al. (2001) studied the effects of intrathecally administered local anesthetics on glutamate release and neuronal injury in rabbits. New Zealand white rabbits were anesthetized with isoflurane. With the rabbits in prone position, midline skin and subcutaneous fascia were incised between the third lumbar and the first sacral spinous process after infiltration with 0.25% bupivacaine. Muscles were dissected; the third to seventh processes, ligamentum flavum, and epidural fat were sequentially removed; and the underlying dura was exposed. Using an operating microscope, a small slit was made in the dura and arachnoid membrane at the L3–4 interlaminar space. A loop-type dialysis probe was then implanted. A PE-10 catheter for the administration of saline or the local anesthetic to be tested was implanted intrathecally through the slit made at the L6–7 interlaminar space so that the tip of the catheter was located at the level of the cauda equina. The implanted dialysis probe was perfused with artificial cerebrospinal fluid bubbled with 95% oxygen and 5% CO_2 at pH 7.2. Samples were collected

before and after administration of test substance and analyzed for glutamate.

After collecting the last sample (90 min after intrathecal administration of test substance), the catheters were removed, and all incisions were sutured. Isoflurane was discontinued, and the lungs were ventilated with 100% oxygen. Extubation of the trachea was performed when adequate spontaneous ventilation occurred. The animals were allowed to recover with infusion of Ringer's solution and antibiotic treatment.

The animals were neurologically assessed daily until 1 week after test drug administration by an observer unaware of the treatment group. Sensory function was evaluated by seeking an aversive response to pinprick stimulation with a 23-gauge needle, progressing from sacral to thoracic dermatomes. The score of the sensory function was assessed by a three-point grading scale. The hind-limb motor function was assessed by a five-point grading scale.

After completion of the neurologic function scoring at 1 week, the animals were reanesthetized, and transcardiac perfusion and fixation were performed. The spinal cord was removed and refrigerated in phosphate-buffered formalin 10% for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. The coronal sections of the spinal cord at L3, L4, and L5 levels were cut at a thickness of 8 μm and stained with hematoxylin and eosin. The degree of the spinal cord damage was assessed for the vacuolation of the dorsal funiculus with a four-point grading scale and the chromatolytic changes of the motor neuron. The neurons with chromatolytic appearance were identified by round-shaped cytoplasm with loss of Nissl substance from the central part of the cell and eccentric nuclei. The motor neurons with chromatolytic appearance were counted in two sections for each animal and averaged.

Parametric data were presented as mean ± SD. To determine differences in glutamate concentrations, a repeated measures analysis of variance was performed. The cutaneous sensation, hind-limb motor function, and morphological changes of the spinal cord were analyzed with a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney *U*-test.

Muschaweck et al. (1971) performed comparative intrathecal tolerance studies in dogs. Beagle dogs weighing 8–12 kg were anesthetized with 30 mg/kg sodium pentobarbital intravenously. The animals were intubated and submitted to artificial respiration. The fur on the neck was shaved and the skin disinfected. All further procedures were carried out under sterile conditions. The spinal canal was punctured through the foramen magnum at the atlanto-occipital joint. Successful entry of the spinal cervical canal was checked by withdrawal of cerebrospinal fluid. Five milli liter of cerebrospinal fluid was withdrawn and used as solvent for the tested local anesthetics. The same volume was injected intrathecally either as solution of local anesthetics in concentrations used in therapy or as control (saline solution). Artificial respiration was continued until spontaneous breathing resumed. Motor performance was checked during 24 h. Two days later, the animals were sacrificed under anesthesia. Dissection included the cerebellum, medulla oblongata, and sections from the cervical, thoracic, and lumbar cord, carefully observing that always the same segments were taken including the injection site. Segments were semifixed in 8% buffered formaldehyde for 2 days. When semifixed, the vertebral arches were opened and the spinal cord, as well as the roots of the spinal nerves, was removed and completely fixed. After embedding in gelatin and Paraplast, a fat red 7B and myelin sheath staining according to Olivecrona were performed on serial sections from the site of injection as well as from cervical, thoracic, and lumbar marrow. Hematoxylin–eosin staining, as well as PAS and iron reaction, was performed in one section. The presence or absence of nerve damage and of inflammation was noted.

Yaksh et al. (1995) studied the safety of chronically administered neostigmine methylsulfate in rats and dogs. Adult beagle dogs weighing 13–17 kg were adapted for 5 days to experimental protocols and placement of a nylon vest. For placement of the spinal catheter, the dogs were sedated (atropine 0.04 mg/kg and xylazine "Rompun" 1–2 mg/kg i.m.) given an i.m. injection of penicillin G and procaine and brought an anesthetic depth by mask administration of halothane (3–5%), and then the trachea was intubated. The dog was maintained under spontaneous ventilation with 1–2% halothane and 50% N_2/50% O_2. Surgical areas on the back of the neck and head were shaved and prepared with alcohol and a povidone iodine scrub, and the dog was placed in a stereotaxic head holder.

After draping and using sterile technique, the cisterna magna was exposed, and a small incision (10–2 mm) was made. The intrathecal catheter (polyethylene rubbing PE-50 stretched by 30%, making the nominal diameter 0.6 mm) was inserted and passed caudally at a distance of 40 cm, to a level corresponding approximately to the L3–L4 segment. Presence of the catheter in the intrathecal space was confirmed by free withdrawal of cerebrospinal fluid. A small stainless steel screw was placed in the skull and the catheter fixed to the screw. The catheter was tunneled subcutaneously and caudally to exit on the upper left back at the level of the scapula. The incision was closed by sutures, the halothane turned off, and the animal allowed to recover. An analgesic was administered for postoperative pain medication. At this time, the catheter was connected to the infusion pump placed into a vest side pocket, and an infusion of sterile saline (2 ml/day) was started.

For a 28-day infusion study, dogs were randomly assigned to receive saline or neostigmine (4 mg/4 ml). After 28 days of infusion, the dogs were sacrificed. After induction of a deep anesthesia, the animal was manually ventilated to maintain adequate oxygenation. A percutaneous puncture of the cisterna magna was performed and cerebrospinal fluid withdrawn for analysis. The chest was opened and a large-bore cannula placed in the aortic arch through which was perfused saline followed be 10% formalin. After fixation, the dura was exposed by an extensive laminectomy of the spinal canal and the lower brainstem, being careful to leave the catheter and the dura undisturbed. Dye was injected through the catheter to determine its integrity, visualize the position of the intrathecal catheter, and determine the spread of dye around the catheter; the spinal cord was removed in four blocks (cervical, thoracic, caudal, and rostral from the catheter tip), taking care to keep the dura intact, and placed in formalin. After fixation, tissue blocks were embedded in paraffin and then decalcified overnight, embedded in paraffin, sectioned at a thickness of 6–7 μm, and stained with hematoxylin and eosin. Particular attention was given to the presence or absence of fibrosis and other reactions around the catheter, dural thickening or other reaction; inflammation in the epidural space, leptomeninges/subarachnoid space, or spinal cord parenchyma, microglial nodules, demyelinization, or gliosis. The degree of chronic and/or acute inflammation was graded as normal, mild, moderate, or severe.

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8.1.9 Studies on Porphyrogenicity

PURPOSE AND RATIONALE

Acute porphyrogenicity hereditary porphyrias are disorders of heme synthesis in which overproduction of heme precursors is often accompanied by severe clinical manifestations. Most of the time, these diseases remain clinically latent and only occasionally result in acute abdominal and neuropsychiatric symptoms. Occurrence of the symptoms often follows exposure to drugs, such as barbiturates, sulfonamides, estrogens, and some local anesthetics (Blanloeil et al. 1989).

De Verneuil et al. (1983), Deybach et al. (1987), and Blanloeil et al. (1989) studied according to the method of Anderson (1978) the propensity of various drugs to induce hepatic porphyria. A situation analogous to the latent stage of human hepatic porphyria could be produced in chick embryos by injecting an inhibitor of ferrochelatase (the last enzyme in the heme biosynthesis pathway). The simultaneous injection of a porphyrinogenic drug (e.g., barbiturate) results in a strong increase of the porphyrin levels in the chick embryo. This method is used for drug screening: drugs that are found porphyrinogenic in this test were considered to be potentially dangerous to patients with hepatic porphyria.

PROCEDURE

Fertilized 16-day-old chick embryos are kept in an incubator at 37°C with 70% humidity. Drugs are dissolved in a small volume (0.1–0.3 ml) of 0.15 M NaCl. According to Anderson (1978), a small dose of DDC (1,4-dihydro-3,5-dicarbethoxycollidine) is added that leads to the formation of N-methylprotoporphyrin, which is the inhibitor of ferrochelatase, the last enzyme in heme biosynthesis. Sterile injections are made when the eggs are 18 days old. After 24 h, the embryos are killed by decapitation and the livers removed, separated from the gall bladder, and rinsed with saline prior to homogenization with 3 vol of 0.25 M sucrose/0.02 M Tris buffer, pH 7.4. At least six embryo livers are pooled for each determination.

For determination of porphyrins, the livers are extracted from the whole homogenate with 1 N perchloric acid:methanol (1:1 v/v). The type and the concentrations of porphyrins are studied with a spectrofluorimeter (Grandchamp et al. 1980). The type of accumulated porphyrins is confirmed by

high-pressure liquid gas chromatography (de Verneuil et al. 1978).

For enzyme assays, the homogenates are centrifuged at 800 g for 15 min, and the supernatant is sonicated three times for 15 s. Activity of δ-aminolevulinate synthase (ALA-S) is measured following the radiochemical method of Strand et al. (1972) with [^{14}C]succinic acid and a succinyl-CoA-generating system. Enzyme activity is expressed as pmoles δ-aminolevulinic acid per 30 min per mg of protein. Ferrochelatase activity is measured using a modified radiochemical method (Bonkowsky et al. 1975; Deybach et al. 1981).

To measure cytochrome P-450, 10 ml of the 800 g supernatant is centrifuged at 12,000 g for 15 min; 5 ml of the supernatant is then centrifuged at 105,000 g for 30 min; after washing twice, microsomes (pellet) are resuspended in phosphate buffer and the protein concentration adjusted to 2 mg/ml. The level of cytochrome P-450 is measured in the microsomal fraction using the method of Omura and Sato (1964).

EVALUATION

The tests allow judgment of the propensity of local anesthetics to induce symptoms of hepatic porphyria. The local anesthetics lidocaine, bupivacaine, etidocaine, mepivacaine, prilocaine, and procaine belong to this group, but procaine, butacaine, oxybuprocaine, proxymethacaine, and tetracaine had no (or very slight) porphyrinogenic effect.

MODIFICATION OF THE METHOD

Schütz and Fuchs (1985a, b) studied the porphyrinogenic potential of the local anesthetic articaine (Ultracain). Rats and dogs were given 25–30 intravenous and intramuscular injections of ultracaine without a vasoconstrictor in sublethal or maximum tolerated doses. No signs of any hepatotoxic effect were observed. Rabbits found to have porphyrinuria following administration of allyl-isopropyl-acetyl-carbamide (Apronal) were injected, the local anesthetic intramuscularly on five successive days in a dosage (50 mg/kg) which caused cramps without resulting in urinary porphyrin secretion or hepatic damage.

Bayar and Sümer (1995) investigated the effect of some local anesthetics on methemoglobin levels and erythrocyte enzymes.

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8.2 Tolerance of Neuromuscular Blocking Agents

8.2.1 General Considerations

Neuromuscular tolerance of neuromuscular blocking agents transmission is mediated by nicotinic acetylcholine receptors, for which various subtypes are described (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

Neuromuscular blocking agents are distinguished by whether or not they cause depolarization of the motor end plate. They are classified either as competitive (stabilizing) agents, of which **d**-tubocurarine is the classical example, or as depolarizing, desensitizing agents such as succinylcholine.

For safety evaluation, the absence of cardiovascular side effects is important (Vizi et al. 2003).

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8.2.2 Evaluation of Autonomic Margins of Safety

PURPOSE AND RATIONALE

Savarese (1979) determined not only the potencies of metocurine and **d**-tubocurarine but also the autonomic margins of safety in anesthetized cats.

PROCEDURE

Adult cats of either sex are anesthetized with α-chloralose, 80 mg/kg, and pentobarbital 7 mg/kg, given intraperitoneally. Cannulas are placed in the left femoral vein and artery for drug injection and recording blood pressure and heart rate. The lungs are mechanically ventilated through a tracheostomy and a small animal ventilator set to deliver 15 ml/kg tidal volume and 20 breaths/min.

The right vagus nerve and the right sympathetic trunk are exposed and divided in the neck. The distal ends are placed on the same shielded platinum wire electrode to permit preganglionic stimulation of both nerve trunks. The left sympathetic trunk is also dissected along its postganglionic portion at the base of the skull and cut distal to the superior cervical ganglion to permit postganglionic stimulation through another electrode. Trains of square wave pulses (20 Hz for 10 s) are delivered at supramaximal voltage every 4 min simultaneously to all three autonomic nerve trunks. The resulting bradycardia and hypotension (the vagal response) are measured. Contractions of both nictitating membranes, one (the right) elicited pregangionically and the other (the left) elicited postganglionically, are recorded. The maximal vagal response (i.e., cardiac arrest for 10 s) is achieved by stimulation of the right vagus nerve or of both vagus nerves.

Twitches of the right tibialis anterior muscle are elicited at 0.15 Hz via the peroneal branch of the right sciatic nerve, to which square-wave shocks of 0.2 ms are applied at supramaximal voltage. Twitch recording is done via a transducer. All nerves and tendons are kept moist in small pools of mineral oil or in cotton pledges soaked in mineral oil. Tibialis anterior and esophageal muscle temperatures are monitored and kept between 35°C and 38°C by heat lamps.

Simultaneous recordings of heart rate, arterial pressure, pre- and postganglionic-elicited contractions of the nictitating membrane, and twitches of the tibialis anterior muscle are made on a polygraph. Cumulative dose–response curves for inhibition of neuromuscular, vagal (parasympathetic), and sympathetic functions are determined simultaneously for each animal. The mechanism of vagal inhibition is localized at parasympathetic ganglia or cardiac muscarinic receptors by determining whether the bradycardic response to methacholine (20 μg/kg) is blocked as well as the neurally elicited bradycardia.

A single-bolus dose of the neuromuscular relaxants producing the delayed depressor response plus tachycardia (Paton 1957) is determined in each animal. This response being pathognomonic for histamine release is defined as sudden hypotension to less than 80% of the control arterial pressure within 2 min of relaxant injection and with tachycardia to more than 25% above the baseline value.

Test drugs are given intravenously.

EVALUATION

Data analysis is done by the method of Litchfield and Wilcoxon. Mean dose–response curves are plotted on

log-probit paper. Best fit to straight lines on these scales is determined by computerized regression. The cumulative ED_{50} values for vagal and sympathetic inhibition and the cumulative ED_{95} values for neuromuscular blockade are determined from the lines, and 95% confidence limits are calculated. Differences in potency are considered significant when $P < 0.05$.

The occurrence of histamine release is also treated as an all-or-none response to permit log-probit plotting. The delayed depressor response plus tachycardia is judged to have or have not occurred after each single-bolus injection of the drugs. The percentage of animals responding at each dose level is then determined and the data handled by the Litchfield–Wilcoxon method.

The autonomic margins of safety of the test drugs are calculated as the ratios of cumulative doses producing 50% block (ED_{50}) of vagal (parasympathetic) and sympathetic transmission and the ED_{50} for histamine release, each divided by the ED_{50} of neuromuscular blockade.

MODIFICATION OF THE METHOD

Clutton et al. (1992) studied the autonomic and cardiovascular effects of neuromuscular blockade antagonism in the dog. Neuromuscular blockade was antagonized with various anticholinesterase–antimuscarinic drug combinations including atropine, neostigmine, and glycopyrrolate.

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Safety of Intravenous and Inhalation Anesthetics

9

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9.1 Determination of Safety of Intravenous Anesthetics

9.1.1 General Considerations

PURPOSE AND RATIONALE

The determination of safety of intravenous anesthetics first agents used as intravenous anesthetics were *barbiturates*. Barbiturates with a duration of action appropriate to the requirements of surgery became available with the introduction of hexobarbital and thiopental (Volwiler and Tabern 1930; Miller et al. 1936). The studies with barbiturates were extended (Butler and Bush 1942; Christensen and Lee 1973). Intravenous anesthetics from other chemical groups were developed, such as *acetamidoeugenol* (Estil, Domenjoz 1959), steroid derivatives (Presuren = *hydroxydione sodium*, Laubach et al. 1955), *alfaxolone* (CT1341, Child et al. 1971), *propanidid* (Epontol, Goldenthal 1971), *ketamine* (CI-581, Chen et al. 1966; Reich and Silvay 1989), *etomidate* (Janssen et al. 1975), *propofol* (ICI 35868, Glenn 1980), and *midazolam* (Pieri 1983; Reilly and Nimmo 1987).

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9.1.2 Tests for Safety of Intravenous Anesthetics

PURPOSE AND RATIONALE

Besides determination of the ratio between anesthetic and lethal dose, intravenous anesthetics have to be tested for their influence on the cardiovascular and pulmonary system. Borkowski et al. (1990) described a method to compare intravenous anesthetics in rabbits.

PROCEDURE

Adult New Zealand white rabbits with a mean weight of 4.5 kg are used. To provide access for direct blood pressure measurement and arterial blood samples, an 18-gauge catheter is implanted into the left carotid artery under halothane anesthesia. Following a minimum 24-h recovery period, the rabbit is placed in a sling and a pneumograph is fitted around the rabbit's caudal thorax at the level of the 10th to 12th rib to monitor respiratory rate and pattern. From the arterial catheter, blood is withdrawn for blood gas analysis. Then the catheter is connected to a blood pressure transducer. A 10-min acclimatization period is allowed before control measurements are recorded. Each rabbit serves as its own control in that cardiopulmonary parameters and responses to noxious stimuli are determined before anesthesia is induced. The right marginal ear vein is catheterized with a 22-gauge catheter, which is secured with adhesive tape, flushed with physiologic sterile saline, and used for the administration of the anesthetic agents.

One third of the dose of the anesthetic to be tested is injected manually over a 1-min period. When the rabbit is relaxed, it is removed from the sling and is placed in left lateral recumbence on a heating blanket. The degree of muscle tension and reaction to noxious stimuli are determined while the rabbit is in the sling and at 15-min intervals following anesthesia. The assessments performed include those of jaw tone, leg muscle tone, palpebral reflex, corneal reflex, ear pinch reflex, and pedal withdrawal reflex. Jaw tone is evaluated subjectively by pulling the lower jaw open by an index finger. Leg muscle tone is evaluated by flexion and extension of the right rear leg according to subjective scores. The corneal reflex is tested by placing a moistened cotton swab on the cornea. The palpebral reflex is tested by touching the medial canthus with a dry cotton swab. Assessment of the ear pinch reflex is performed by applying a compression force with an alligator clip. The pedal withdrawal reflex is determined by applying the same clip on the right rear fifth digit at the distal phalanx.

Cardiopulmonary parameters and rectal body temperature are determined while the rabbit is in the sling and also at 15-min intervals following induction of anesthesia with the rabbit in lateral recumbency. Heart rate, mean arterial blood pressure, respiratory rate, and respiratory pattern are calculated from tracings from the physiological recorder. Arterial blood pH, partial pressure of oxygen (PaO_2), and partial pressure of carbon dioxide ($PaCO_2$) are determined from arterial blood samples.

EVALUATION

The heart rate, mean arterial blood pressure, respiratory rate, pH, PaO_2, and $PaCO_2$ are analyzed using a two-factor analysis on repeated measures. The control values are treated as covariate to allow standardization of the inherent variation between rabbits.

The single *t*-test for paired differences is used to compare control values to data obtained during the later testing intervals. The standard error of the mean (SEM) is calculated for each variable at each time interval. Data for muscle tone and responses to noxious stimuli are calculated as frequency percentages. The Fisher's exact test is used to compare between treatments. For all of the statistical analyses, a p value of less than 0.05 is considered significant.

MODIFICATIONS OF THE METHOD

Details of anesthesia in the rabbit were also described by Murdock (1969).

Peeters et al. (1988) performed a comparative study of four methods for general anesthesia in rabbits.

Glenn (1980) examined the anesthetic activity of propofol (ICI 35868) in mice, rats, rabbits, cats, pigs, and monkeys, including cardiovascular and respiratory parameters and EEG studies.

Korkmaz and Wahlström (1997) developed the electroencephalographic (EEG) threshold test in rats to determine the central nervous sensitivity to several depressant drugs, mainly intravenous anesthetic drugs. The test drugs were administered by continuous intravenous infusion until a defined EEG criterion indicating deep anesthesia was reached. The criterion was a burst suppression which lasted 1 s or more, the "silent second" (SS). The dose of the drug needed to induce the SS, the threshold dose, was the dependent variable. In the intact animal, it is influenced by the potency of the drug and the dose administration rate of the infusion. With the method, it is possible under in vivo conditions to monitor continuously the electrical changes in the CNS.

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9.2 Determination of Safety of Inhalation Anesthetics

9.2.1 General Considerations

PURPOSE AND RATIONALE

The determination of safety of inhalation anesthetics efficacy and safety of new inhalation anesthetics has to be evaluated in pharmacological experiments. Robbins (1946) defined the anesthetic AD_{50} as the concentration of anesthetic at which 50% of mice failed to right themselves for 15 s when placed in a rotating bottle with a known concentration of anesthetic. The concentration of the anesthetic that caused apnea in 50% of the mice in 10 min was defined as the LD_{50}, and the ratio LD_{50}/AD_{50} as an index of safety.

Wolfson et al. (1972) recommended brain anesthetic concentration for construction of anesthetic indices.

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9.2.2 Safety Margin of Inhalation Anesthetics

PURPOSE AND RATIONALE

To assess the safety margin of an inhalation anesthetic, not only should the ED_{50} values but also the maximally effective dose and the dose with a minimal danger of fatal outcome be determined. In particular, *cardiovascular parameters* are observed (Kissin et al. 1983).

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are placed into a clear chamber with the tail protruding from a special opening. An anesthetic-oxygen nonhumidified mixture is directed into the chamber at a rate of 4 l/min. The inhalation anesthetics, e.g., halothane or isoflurane, are vaporized in Draeger vaporizers, and the level in the chamber is monitored

with a gas analyzer which is calibrated with a mass spectrometer. Rectal temperature is monitored and maintained at 37°C with a heating pad. Each rat is exposed to only one predetermined concentration of anesthetic for 30 min, at which time the presence or absence of the end point of anesthesia is determined. For the lethal endpoint, rats are tracheotomized and ventilated at 60 strokes/min through an endotracheal catheter. Tidal volume is adjusted to maintain Pa_{CO_2} at 40 ± 5 mmHg.

Endpoints of anesthesia used:

1. Loss of righting reflex. The test is regarded as positive if the animal fails to right itself with all 4 ft on the floor within 15 s after being placed in a side position.
2. Prevention of purposeful movements response to a noxious stimulus. The animals are stimulated for 60 s by placement of a 1-kg weight on the middle of the tail. Only the purposeful movement of the head or legs is considered to be a response.
3. Prevention of the heart rate increase to a noxious stimulus (ECG signals). An increase in heart rate of greater than 1% is regarded as a positive response.
4. The endpoint for the lethal effect is 7 mmHg in the femoral artery with artificial respiration.

With each of the anesthetics, four series of experiments are performed: to determine the righting reflex, purposeful movement response, heart rate response, and lethal effect. The concentrations of the test compounds and the standard are spaced equally between the above-mentioned doses.

After determination of the heart rate effect and the lethal effect, the rats are sacrificed for determinations of brain tissue concentrations. The whole brain is removed, and tissue anesthetic concentration is determined by gas chromatography.

EVALUATION

For calculation of the dose-effect curves, the probit method of statistical analysis is used.

For the assessment of anesthetic safety, not only the therapeutic ratio (LD_{50}/ED_{50}) but also the standard safety margin

$$SSM = (LD_5 - ED_{95})/ED_{95} \times 100$$

is used. This represents the percentage by which the ED_{95} has to be increased before LD_5 is reached.

CRITICAL ASSESSMENT OF THE METHOD

The standard safety margin has definitive advantages over therapeutic ratio. In contrast to the LD_{50}/ED_{50} index, the standard safety margin is influenced not only by the distance between central points of the anesthetic and lethal dose-effect curves but also by the slope of these curves.

MODIFICATIONS OF THE METHOD

A similar concept, based on response to tail clamping, respiratory arrest, and cardiovascular failure in the *rat*, was published as an anesthetic index by Wolfson et al. (1973).

Another attempt to determine anesthetic requirements in rats was published by White et al. (1974).

Kissin et al. (1984) studied the morphine-halothane interaction in rats.

Fukuda et al. (1996) investigated the effects of sevoflurane and isoflurane on bupivacaine-induced arrhythmias and seizures in rats.

Kanaya et al. (1998) compared myocardial depression by sevoflurane, isoflurane, or halothane in *cultured neonatal rat ventricular myocytes*. Changes in beating rate and amplitude during exposure to the anesthetics were measured.

Chaves et al. (2003) used noninvasive electrocardiography in *mice* to study the effects of intravenous and inhalation anesthetics and of age.

Krantz et al. (1941, 1953) described an anesthetic index between surgical anesthesia (cornea and wink reflexes abolished) and respiratory failure in *dogs*.

Van Poznak and Artusio (1960a, b) determined the anesthetic properties of fluorinated compounds in dogs using a face mask for the induction of anesthesia and a cuffed endotracheal tube later on. ECG (lead II) and EEG were monitored.

Steffey and Howland (1978) determined the potency of enflurane in dogs in comparison with halothane and isoflurane.

Johnson et al. (1998) compared isoflurane with sevoflurane for anesthesia induction and recovery in adult dogs.

Salmempera et al. (1992) studied in dogs the potency of remifentanil, a short-acting opioid analgesic, which is used as anesthetic adjunct by variable-rate infusion. Enflurane minimal alveolar concentration was measured by the tail-clamp method in dogs before and after sequential infusion of various doses of

remifentanil. The plasma concentration causing a 50% reduction of enflurane minimal alveolar concentration was determined.

Kataoka et al. (1994) studied the negative inotropic effects of sevoflurane, isoflurane, enflurane, and halothane in canine blood-perfused papillary muscles.

Hirano et al. (1995) compared the coronary hemodynamics during isoflurane and sevoflurane anesthesia in dogs.

Mutoh et al. (1997) compared the cardiopulmonary effects of sevoflurane with those of halothane, enflurane, and isoflurane in dogs.

Hashimoto et al. (1994) examined the effects of sevoflurane and halothane on the effective refractory period and ventricular activation in a canine myocardial infarction model.

The effects of desflurane, sevoflurane, and halothane on postinfarction spontaneous dysrhythmias in dogs were examined by Novalija et al. (1998).

Cardiopulmonary effects in *cats* were studied for desflurane by McMurphy and Hodgson (1996) and for sevoflurane by Hisaka et al. (1997).

Saeki et al. (1996) determined the effects of sevoflurane, enflurane, and isoflurane on baroreceptor-sympathetic reflex in *rabbits*.

Hanagata et al. (1995) found that isoflurane and sevoflurane produce a dose-dependent reduction in the shivering threshold in rabbits.

Antognini and Eisele (1993) determined anesthetic potency and cardiopulmonary effects of enflurane, halothane, and isoflurane in *goats*.

The effects of multiple administrations of sevoflurane to *cynomolgus monkeys* were evaluated by Soma et al. (1995).

The effect of inhalation anesthetics on the *respiratory system* was investigated in several studies:

Mazzeo et al. (1996) compared the relaxing effects of desflurane and halothane at various *MAC*s on isolated proximal and distal airways of dogs precontracted with acetylcholine.

Hashimoto et al. (1996) compared the bronchodilating effect of sevoflurane, enflurane, and halothane in dogs using a superfine fiber-optic bronchoscope. The dogs were anesthetized with pentobarbital, paralyzed with pancuronium, and the lungs were mechanically ventilated. The endotracheal tube had an additional lumen to insert the superfine fiber-optic bronchoscope (outer diameter 2.2 mm) which was located between a second and third bronchial bifurcation to monitor continuously the bronchial cross-sectional area of third or fourth generation bronchi. Bronchoconstriction was produced by histamine injection and infusion. The bronchial cross-sectional area was printed out by a video printer at the end of expiration and was calculated on a computer using an image program after various *MAC*s of the different inhalation anesthetics.

Mitsuhata et al. (1994) induced systemic anaphylaxis in dogs sensitized to *Ascaris suum* by intravenous injection of the antigen and measured pulmonary resistance and dynamic pulmonary compliance. Sevoflurane was as effective as isoflurane in attenuating bronchoconstriction associated with anaphylaxis in dogs.

Cervin and Lindberg (1998) examined the short-term effects of halothane, isoflurane, and desflurane on mucociliary activity in the rabbit maxillary sinus in vivo.

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9.2.3 Determination of Minimal Alveolar Anesthetic Concentration (*MAC*)

PURPOSE AND RATIONALE

The determination of minimal alveolar anesthetic concentration (MAC) term "minimum alveolar anesthetic concentration" (*MAC*) was coined by Merkel and Eger (1963) as an index to compare various anesthetic agents.

The use of *MAC* which represents the partial anesthetic pressure in the brain has gained wide acceptance (Eger et al. 1965; Quasha et al. 1980).

For *man*, Saidman and Eger (1964) defined *MAC* as the point at which 50% of the patients moved in response to a surgical incision.

A method for determining minimum alveolar concentration of anesthetic in the *rat* was published by Waizer et al. (1973). Kashimoto et al. (1997) determined the minimum alveolar concentration of sevoflurane in rats. Eger et al. (1999) studied minimum alveolar anesthetic concentration of fluorinated alkanols in rats and discussed the relevance to theories of narcosis. Eger et al. (2003) studied additive minimum alveolar concentration effects of halothane and isoflurane in rats.

Issues in the design and interpretation of minimum alveolar anesthetic concentration studies were discussed by Sonner (2002).

PROCEDURE

Minimum alveolar anesthetic concentrations are determined in Sprague Dawley rats weighing 300–450 g. Each rat is placed in an individual gas-tight plastic cylinder closed at both ends by rubber stoppers. The stoppers are pierced with holes for various purposes. A rectal temperature probe (temperature maintained between 36°C and 38.5°C) and the rat's tail are drawn separately through holes in the rubber stopper closing the distal end of the cylinder. Delivered gases at an average inflow rate of 1 L/min to each rat enter through ports at the head (proximal) end of the cylinder and exit at the tail (distal end), a flow to minimize rebreathing (inspired $CO_2 < 10$ mmHg). Exiting gases are scavenged.

The anesthetics are introduced from conventional vaporizers. For the determination of *MAC*, an initial concentration is used that permits movement of the rats in response to noxious stimulation. A tail clamp is applied for 1 min or until the animal moves, and the anesthetic partial pressure is measured by gas chromatography. If the animal moves, the partial pressure

is increased by 0.2% or 0.3% atmospheres. After equilibration for 30 min, the tail clamp is applied again and the anesthetic partial pressure measured by gas chromatography. This procedure is repeated until the partial pressures bracketing movement-nonmovement are determined for each rat.

EVALUATION

MAC is defined as the average of the partial pressures that just prevented movement in response to clamping of the tail. Differences between anesthetics are accepted at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Fang et al. (1997) found that maturation decreases ethanol minimum alveolar anesthetic concentration more than desflurane *MAC* in rats.

Gong et al. (1998) assessed the effect of rat strain on susceptibility to anesthesia and convulsions produced by inhaled compounds in five different rat strains. Strain minimally influenced anesthetic and convulsant requirements of inhaled compounds in *rats*.

Doquier et al. (2003) studied the minimum alveolar anesthetic concentration of volatile anesthetics in rats as tools to assess antinociception in animals.

Davis et al.'s (1975) determination of the minimal alveolar concentration of halothane in the white New Zealand *rabbit* was published.

Determination of an anesthetic index (Apnea/*MAC*) in experiments in dogs has been proposed by Regan and Eger (1967).

Murphy and Hug (1982), Hall et al. (1987) used the reduction of enflurane *MAC* values in dogs as parameter for the anesthetic potency of fentanyl or sufentanil, respectively.

Seifen et al. (1987) used *MAC* values for comparison of cardiac effects of enflurane, isoflurane, and halothane in the dog *heart-lung preparation*.

Ide et al. (1998) used airway occlusion in *cats* as a noxious respiratory stimulus that induces a visceral sensation of choking for determination of minimum alveolar anesthetic concentrations during halothane, isoflurane, and sevoflurane anesthesia. These values were compared with *MAC* values for somatic noxious stimuli such as toe pinch or tetanic stimulus. The authors recommended this method as a new concept for *MAC* determination.

Eger et al. (1988) determined minimum alveolar concentration of fluorinated anesthetics in *pigs*.

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Drug-Addiction and Drug-Dependency 10

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10.1 Drug Addiction and Drug Dependency

10.1.1 General Considerations

Drug abuse is a complex phenomenon, and many factors (e.g., availability, cost) contribute to whether a particular drug will be abused by a particular individual. Nevertheless, many drugs that are abused have common neurobiological and behavioral effects. Consequently, some of the properties of drugs that contribute to abuse can be examined systematically in animals using well-established and validated behavioral procedures. A major strength of this area of research is that the effects of drugs in these procedures (i.e., in nonhuman species) are highly predictive of the effects of the same drugs in humans; thus, behavioral assessments are used both to study the underlying biological and behavioral phenomena associated with drug abuse (e.g., drug reinforcement, physical dependence) and to assess whether new chemical entities have properties in animals that would indicate a likelihood of abuse in humans. Preclinical abuse and dependence liability studies typically comprise the following approaches and procedures:

- Physical dependence
- Tolerance
- Drug discrimination
- Self-administration
- Conditioned place preference

No single procedure or any set of procedures can exactly predict whether a drug is likely to be abused. However, when considered within the context of other known properties of the drug (e.g., receptor binding, pharmacokinetic profile), results of behavioral studies

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can be very useful in estimating the likelihood that new chemical entities will be abused, largely by comparing (in standardized assays) those new entities to reference compounds and known drugs of abuse.

10.1.2 Physical Dependence Studies

PURPOSE AND RATIONALE

Withdrawal phenomena, either after abrupt cessation of chronic treatment or after administration of a pharmacologic antagonist (e.g., naltrexone), can be observed in a variety of nonhuman species. Importantly, the withdrawal that emerges in some nonhumans topographically resembles important features of withdrawal in humans. On this basis, tests for drug dependence and withdrawal have been developed for monkeys (Seevers 1936; Seevers and Deneau 1963; Aceto 1990; Woods et al. 1993), dogs (Martin et al. 1974, 1976), rats (Buckett 1964; Cowan et al. 1988), and mice (Way et al. 1969; VonVoigtlander and Lewis 1983). Two general approaches are used to evaluate physical dependence potential: primary physical dependence and single-dose substitution. In the former, a test substance is administered repeatedly over days, and the assessment of dependence (i.e., by the emergence of withdrawal) occurs either after discontinuation of drug treatment or by administration of a pharmacologic antagonist. Precipitated withdrawal studies are warranted only when the mechanism or site of action of the test substance is known and when an appropriate pharmacologic antagonist is available. In a single-dose substitution study, a reference substance (e.g., morphine) is administered repeatedly over a sufficient number of days to produce dependence; after discontinuation of treatment with the reference substance, and when reliable withdrawal signs have emerged, the test substance is assessed for its ability to attenuate withdrawal signs. In this type of study, the test substance can be administered just once to assess its acute withdrawal-reversing effects or can be administered repeated over days (i.e., replace the reference substance) and subsequently discontinued followed by assessment of withdrawal signs.

A well-established in vitro procedure has also been used to test for opioid dependence (i.e., antagonist-precipitated withdrawal) in opioid-treated guinea pig ileum (Villarreal et al. 1977; Rodríguez et al. 1978; Collier et al. 1981; Cruz et al. 1991).

PROCEDURES

10.1.2.1 Opioid Withdrawal Responses in the Guinea Pig Ileum Made Dependent In Vitro

A 40-cm-long segment of the small intestine of male guinea pigs weighing 600–900 g is removed and placed in a low-magnesium Krebs solution. The terminal section of the guinea pig ileum is used after discarding the portion of 10 cm closest to the ileocecal junction. The ileum is cut in eight 3-cm-long segments. The intestinal content is gently removed with the aid of a glass rod. To produce opioid dependence, segments are incubated in 500-ml Erlenmeyer flasks containing 480 nM morphine in 250 ml Krebs solution saturated with a 95% O_2/5% CO_2 gas mixture at a temperature ranging between 4°C and 6°C for 1–48 h. One hour before completion of the incubation time, the segments are removed, placed in glass chambers with 50 ml Krebs solution bubbled with 95% O_2/5% CO_2 gas mixture at 36°C, and mounted on a vertical electrode with one edge fixed to the chamber plug and the opposite fixed to an isometrical force transducer (Grass FT 03) connected to a polygraph for recording the contractile activity of the longitudinal muscle. The ilea are set up with an initial tension of 1 g and left for a period of 30 min for stabilization. Thereafter, all segments are electrically stimulated with supramaximal rectangular pulses (10–40 V) of 0.5-ms duration at a frequency of 0.1 H.

Five minutes before naloxone administration, the electrical stimulation is suspended. The response to naloxone is recorded by administration of up to 100 nM. The response to the antagonist is recorded for 20 min, and thereafter the electrical stimulation is reinitiated and maintained for 10 min.

Thirty-five minutes after naloxone administration, various doses of nicotine are administered to provide a positive control. For comparison, a concentration-response curve for nicotine (1, 1.78, 3.2, 5.6, 10, 17.8, 32, and 56 μM) is obtained in untreated ilea. Moreover, the concentration-response curve for nicotine is obtained in ilea that are treated as follows: (1) exposed to 10 nM naloxone for 20 min, (2) exposed to 480 nM of morphine for 1 h, or (3) pretreated for 10 min with 3 or 10 nM of naloxone and exposed to 480 nM of morphine for 1 h. The response to nicotine is attenuated after pretreatment with morphine, and this attenuation is dose dependently antagonized by naloxone.

A correlation between the response to supramaximal electrical stimulation and the withdrawal response (contraction) precipitated with 100 nM naloxone as well as a correlation between withdrawal and nicotine response after long-term exposure (12–48 h) with 480 nM morphine is used to determine whether physical dependence developed and, therefore, whether the naloxone-induced contraction indicates withdrawal.

10.1.2.2 Test for Physical Dependence in Rats

Male albino rats receive either morphine or saline i.p. twice daily. The starting dose of morphine is 20 mg/kg and is increased by 40 mg/kg increments daily until, by day 11, the dose is 400 mg/kg. Maintenance at 400 mg/kg is continued through day 20. The test compound is similarly administered to groups of ten rats each, typically in ascending doses and a maximally tolerated dose. The daily increments have to be adjusted to a maximum level that is not lethal for the duration of the experiment.

Primary physical dependence capacity is measured on days 11 and 17 when all animals receive an injection of 10 mg/kg of naltrexone or naloxone i.p. in the morning. Signs of withdrawal are recorded during a 30- to 60-min period. Rats are scored for the presence or absence of withdrawal signs (e.g., diarrhea, wet-dog-type shaking) using standardized scoring.

A single-dose substitution study substitutes either a single dose or multiple doses (from day 20 through day 23) of the test substance in morphine-dependent rats; scoring for suppression of withdrawal occurs on days 20–23 and after discontinuation of the test substance.

10.1.2.3 Test for Physical Dependence in Monkeys

Groups of 3–4 rhesus monkeys (3–6 kg body weight) receive morphine four times daily (s.c. or i.m.) beginning with a dose of 1.0 mg/kg. Progressively, the unit dose is increased to a final dose of 3.2 mg/kg/6 h. The test substance is similarly administered to groups of 3–4 monkeys. For the test compound, the daily increments in drug administration are adjusted to a maximally tolerated (nontoxic) dose and frequency of injection. Both groups of monkeys are then maintained at their appropriate dose levels for a minimum of 112 days. On days 35, 60, and 91, 1 mg/kg of naltrexone or naloxone is administered (s.c. or i.m.) in the morning. On days 50 and 112, all doses are omitted for 24 h. Signs of withdrawal are recorded during a 30- to 60-min period using standardized scoring (e.g., Katz 1986; Becker et al. 2008; Brandt and France 1998).

CRITICAL ASSESSMENT OF THE METHOD

The emergence of withdrawal signs after discontinuation of drug treatment is dependent of the duration of action of the treatment compound. Thus, after discontinuation of morphine treatment, withdrawal reliably emerges within 12–24 h. For drugs with an unusually long duration of action (e.g., buprenorphine), observations for withdrawal signs need to occur over longer periods of time (e.g., several days); for drugs with an exceptionally long duration of action, the gradual and prolonged offset of drug action might preclude emergence of significant withdrawal, despite development of dependence. Opioid antagonists will precipitate withdrawal in animals treated with opioid agonists. If a test substance has actions at non-opioid receptors, a negative result with naltrexone or naloxone in a precipitated withdrawal study might not provide useful information regarding dependence potential. Thus, both precipitated withdrawal and treatment discontinuation-induced withdrawal need to be studied for test compounds.

Rhesus monkeys have been used extensively for assessing physical dependence potential of opioid receptor agonists. An excellent correlation between humans and rhesus monkeys has been shown regarding the physical dependence liability of opioids, although there are some compounds for which the relative potency between humans and monkeys is not what is predicted from other data.

Nonhuman species can also be used to assess physical dependence potential of other classes of drugs, including sedative/hypnotics such as benzodiazepines and barbiturates. Physical dependence potential alone cannot be assumed to predict abuse liability because some drugs that are not abused (e.g., kappa opioid receptor agonists) can produce marked physical dependence (Gmerek et al. 1987) and discontinuation of some widely used therapeutic drugs (selective serotonin reuptake inhibitors) can result in a discontinuation syndrome that does not appear to promote drug taking (Black et al. 2000; Hosenbocus and Chahal 2011).

MODIFICATIONS OF THE METHODS

Mouse jumping as a simple screening method to estimate the physical dependence capacity of opioid agonists has been recommended by Saelens et al. (1971). Mice receive seven i.p. injections over 2 days. The test compound is given at doses increasing in multiples of two until a maximally tolerated dose is reached. Two hours after the last injection, the animals receive an i.p. injection of 100 mg/kg naloxone and are placed individually into glass cylinders. The number of jumps is recorded during 10 min.

Rothwell et al. (2011) used acoustic startle reflex and conditioned place aversion to examine long-lasting changes in behavior caused by a single injection of naloxone at different times after a single injection of morphine.

Opioid-receptor agonist-induced dependence can be studied by measuring withdrawal in the guinea pig ileum made dependent in vitro (Cruz et al. 1991).

Kest et al. (2002) compared naloxone-precipitated withdrawal jumping in several strains of mice after acute or multiple injections of morphine or after chronic infusion of morphine with osmotic minipumps.

Becker et al. (2010) used Pavlovian conditioning to demonstrate the ability of environmental stimuli that are paired with the administration of naloxone to elicit withdrawal in morphine-dependent rats.

Yoshimura et al. (1993) studied physical dependence on morphine induced in dogs via the use of osmotic minipumps. Naloxone-precipitated withdrawal signs were recorded such as hyperactivity, biting, digging, tremors, nausea, hyperthermia, increased wakefulness, and by EEG activation in the amygdala and hippocampus, followed by a dissociation of the EEG in the cortex (fast wave) from that in the limbic (slow wave) system, increased heart rate and raised blood pressure. Withdrawal signs were more severe in animals with osmotic minipumps than in those receiving the same dose by syringe injections.

Pierce and Raper (1995) studied the effects of laboratory handling procedures on naloxone-precipitated withdrawal behavior in morphine-dependent rats, and Gellert and Holtzman (1978) used access to drug in drinking solutions to study morphine dependence and withdrawal in rats.

Pierce et al. (1996) used slow release emulsion formulations of methadone to induce dependence in rats. Withdrawal was induced following i.p. challenge with either naloxone or saline, and dependence was assessed in terms of the presence or absence of characteristic withdrawal signs.

Antagonist-precipitated and discontinuation-induced withdrawal in morphine-dependent rhesus monkeys was studied by Becker et al. (2008) using several behavioral procedures as well as telemetry. Changes in heart rate and body temperature persisted for much longer (several weeks) than other directly observable indices of withdrawal or discriminative stimulus effects.

Korkmaz and Wahlström (1999) used EEG threshold and sensitivity to hexobarbital to compare withdrawal in rats treated for different durations with benzodiazepines such as diazepam and lorazepam.

Gallaher et al. (1986) used directly observable changes in behavior to characterize withdrawal in mice that consumed diazepam for 53 days (as much as 1,000 mg/kg/day) in laboratory chow.

McMahon et al. (2007) compared directly observable behavioral effects, rate of operant responding, discriminative stimulus effects, and serum drug concentration to characterize withdrawal after discontinuation of chronic treatment with diazepam in rhesus monkeys.

Stewart and McMahon (2010) compared directly observable behavioral effects and discriminative stimulus effects to withdrawal-like behavior produced by administration of the cannabinoid receptor antagonist rimonabant in rhesus monkeys treated chronically with the cannabinoid receptor agonist delta-9-tetrahydrocannabinol.

Weerts et al. (2005) demonstrated dependence to gamma hydroxybutyrate, and Goodwin et al. (2006) demonstrated dependence to gamma-butyrolactone in baboons using directly observable behavioral effects after administration of an antagonist and after discontinuation of chronic drug treatment.

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10.1.3 Tolerance Studies

PURPOSE AND RATIONALE

Repeated treatment with some drugs can decrease sensitivity to the effects of the same drugs (tolerance) and to the effects of pharmacologically related drugs (cross-tolerance). The development of tolerance can limit the effectiveness of drugs (e.g., analgesics), thereby necessitating an increase in the dose for recovery of the desired effect. The radiant heat or the hot plate method for testing antinociceptive activity of opioid receptor agonists in mice is adapted to measure drug-induced changes in the sensitivity to a noxious stimulus.

PROCEDURE

Male mice (10–12 per condition) with an initial weight of 18–20 g are used. They are placed in restraining cages. A noxious stimulus is produced by an intense light beam directed to the proximal part of the tail. The subject can respond to this stimulus by flicking its tail. The reaction time, the interval between stimulus onset and response, is measured automatically with commercially available equipment or manually with a stopwatch. A maximum time of exposure to the stimulus (e.g., 12-s cutoff time) prevents tissue damage. Prior to drug administration, two control measures of reaction time are obtained for each animal. After administration of the drug, the test is repeated 15, 30, and 60 min after s.c. injection or 30, 60, and 120 min after oral administration. In this way, time of peak activity can be determined. Mice showing a reaction time of the average control value plus two times the standard deviation in the control experiment are regarded as positive. Complete dose–response curves are determined, and ED_{50} values are calculated. Subsequently, the animals are treated for 5 days once every day with a dose which is four times higher than the ED_{50} in the first experiment. On the following day, dose–response curves are determined using at least three doses and the ED_{50} is calculated again. Frequency and duration of drug administration should be adjusted to insure adequate exposure for assessing tolerance. Cross-tolerance can be assessed in the same animals shown to be tolerant to one drug by determining a dose–response curve for a second drug.

EVALUATION

Reduced effectiveness of a fixed dose and/or the need for larger doses to obtain a constant response indicates the development of tolerance. ED_{50} values obtained before and after repeated daily treatment are compared to assess the magnitude of tolerance. Similarly, comparison of ED_{50} values for one drug, obtained in untreated animals and in animals treated with (and shown to be tolerant to) a second drug, is used to determine cross-tolerance.

CRITICAL ASSESSMENT OF THE TEST

Tolerance is observed with a variety of drugs including opioid receptor agonists, barbiturates, benzodiazepines, and ethanol. The measurement of antinociception after single and repeated administration, therefore, has to be regarded as a primary test. Moreover, a decrease in the potency of a drug after daily drug treatment, while providing evidence for tolerance, does not give insight to the mechanism by which tolerance has developed (i.e., pharmacodynamic, pharmacokinetic, behavioral). Demonstration that the antinociceptive effects of a new drug do not decrease after repeated daily treatment with high doses indicates that it is not necessary to escalate dose in order to maintain effectiveness and represents the first step for establishing the absence of tolerance liability. For drugs that do not have antinociceptive actions, other tests need to be employed using a similar dosing strategy for assessing tolerance and cross-tolerance.

MODIFICATIONS OF THE METHOD

Other authors (e.g., Glassman 1971) injected the dose which induced a full antinociceptive effect in mice twice daily for a period of 21 days and evaluated the stepwise decay of effectiveness. After 21 days, the effect of 10 mg/kg morphine or 30 mg/kg meperidine i.p. decreased to approximately 50% of the value of the first day.

Boisse et al. (1986, 1990) demonstrated tolerance and dependence to both short-acting (midazolam) and long-acting (chlordiazepoxide) benzodiazepines in rats.

Langerman et al. (1995) evaluated the acute tolerance to continuous morphine infusion up to 8 h in the

rat with various doses using the hot plate and the tail flick assay. Tolerance was observed with the hot plate assay but not with the tail flick assay suggesting tolerance development at a supraspinal site.

Smith et al. (2003) used twice-daily injections of morphine and implantation of morphine-containing pellets to study mechanisms of opioid tolerance in mice.

Riba et al. (2002) showed that the role of mu opioid receptors in modifying the antinociceptive effects of delta opioid receptor agonists changes during morphine tolerance.

Schwandt et al. (2008) report rapid tolerance to the motor impairing effects of ethanol in adolescent rhesus monkeys.

Eppilito and Gerak (2010) treated rats daily with the neuroactive steroid pregnenolone and showed tolerance to some, but not all, effects on operant responding for food.

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10.1.4 Tests for Abuse Liability

10.1.4.1 General Considerations

Drug abuse often occurs in the absence of physical dependence. The term "psychological dependence" is often used to describe abuse-related phenomena that are not specifically related to physical dependence (Deneau 1964), and laboratory procedures have been developed in animals, not physically dependent on drugs, which are predictive of abuse-related effects in humans. For example, drug discrimination procedures are frequently used to complement other assays of abuse liability; discrimination procedures have the advantage of having a high degree of pharmacologic selectivity which can be used to identify mechanism of action (e.g., receptor type) or to compare mechanism of action between a reference substance and a test substance. Importantly, drug discrimination procedures are predictive of the subjective effects of drugs in humans (Holtzman 1983, 1990; Brady et al. 1987; Colpaert 1987; Overton 1987; Hoffmeister 1988). Self-administration procedures are used to study the reinforcing effects of drugs and are the procedures used most often for predicting abuse liability of new chemical entities (Deneau et al. 1969; Hoffmeister 1979; Littmann et al. 1979; Woolverton and Schuster 1983; Bozarth 1987; Meisch and Carroll 1987; Weeks and Collins 1987; Yokel 1987; Woolverton and Nader 1990). More recently, and in a more limited context, conditioned place preference procedures have been used to examine drug effects that might be predictive of abuse. Finally, based on the early observations of Olds and colleagues (Olds et al. 1956; Olds 1979) on intracranial self-stimulation, procedures have also been developed for studying drug-induced changes in brain-stimulation reward (Kornetsky and Bain 1990), although this methodology has not been used systematically with a sufficiently wide range of drugs for it to be included in a standard abuse liability assessment.

10.1.4.2 Drug Discrimination Studies

PURPOSE AND RATIONALE

Many laboratories use two-choice discrimination procedures to investigate the mechanism or site of action

of test substances by examining compounds in animals trained to discriminate a reference substance and known drug of abuse (Shannon and Holtzman 1976, 1986; Holtzman 1983, 1990; Brady et al. 1987; Colpaert 1987; Overton 1987; Hoffmeister 1988; Carboni et al. 1989). Because of the pharmacologic selectivity of this procedure, test substances might need to be assessed in different groups of animals trained to discriminate different reference substances (e.g., cocaine or heroin). Less common are studies in which animals are trained to discriminate a test substance (presumably with a mechanism[s] of action that is not fully known) and reference substances are examined for their ability to produce discriminative stimulus effects like the test substance.

PROCEDURE

Rats are trained to press one of two choice levers either to receive a food pellet or to avoid/escape electric foot shock which is delivered intermittently beginning 5 s after the start of the trial. The occurrence of a trial is signaled by the illumination of a light in the operant chamber. In some procedures, a third (observing) lever is mounted in the wall of the chamber opposite the two-choice levers and must be pressed before the choice response is made. This contingency prevents the rat from persevering on a single response lever; thus, the choice response in each trial is relatively independent of the consequences of choice responses in the preceding trials of the session. The rats are tested in 20-trial sessions. Animals are trained to discriminate a prototype of the drug of interest. Morphine and fentanyl have served well as training drugs for exploring the discriminative effects of prototypic mu opioid receptor agonists; however, many drugs from a variety of pharmacologic classes have been used as training stimuli in drug discrimination studies (see Glennon and Young 2011). Training often occurs more rapidly when the dose of the training drug is the largest dose that does not disrupt behavior. For discrimination training, the animal is placed in the operant chamber and trained to perform the required response, initially under a schedule of continuous reinforcement where a single response on either lever delivers a food pellet or postpones/terminates electric shock. As performance improves, the response requirement is increased progressively across days (e.g., to a maximum of 10 [fixed-ratio 10]), and discrimination training commences whereby responding on just one of the levers is reinforced in each session. In two-choice procedures, the left choice lever and the right choice lever are designated for drug and vehicle training sessions, respectively, for half of the animals in a group; the lever designation is reversed for the other half of the animals. Acquisition of the discrimination is a function of the drug, training dose, and the number of training sessions. Training continues until the subject reaches predetermined performance criteria, which typically could be the following: at least 80% of the total session responses on the injection (drug or vehicle) appropriate lever and less than one fixed-ratio value (e.g., 10) of responses on the injection-inappropriate lever prior to delivery of the first reinforcer (i.e., food pellet delivery or first postponement/termination of shock) for six consecutive training sessions. A morphine discrimination can be established in rats, according to these criteria, in 6–12 weeks. Once stable discrimination performance is achieved; tests of generalization to test substances can be interposed among the training sessions. During test sessions, the reinforcer is available after completion of the response requirement on either lever. Complete dose–response curves for the training drug and the test drug are obtained. In cases where the test drug does not produce responding on the training drug-appropriate lever, the test drug should be evaluated up to doses that decrease rates of lever pressing or until other behavioral effects are observed, in order to insure that the substance is evaluated up to behaviorally active doses. Drugs from a wide variety of classes have been used as training stimuli in these types of procedures and in a variety of species.

EVALUATION

Results of the stimulus-generalization test usually are evaluated with the quantitative or graded method, whereby the amount of responding on the training drug-associated lever is expressed as a percentage of the total number of responses during a test (i.e., responding on the drug-appropriate lever plus responding on the vehicle-appropriate lever). This percentage is compared with the percentage of drug-appropriate responses normally engendered by the training dose of the training drug (reference standard). The discriminative stimulus effects of the test drug substitute for those of the training drug if the maximal percentages of drug-appropriate responding are not significantly different from each other. When stimulus control of behavior transfers from one drug to another,

it can be inferred that the test drug produced discriminative stimulus effects that are similar to those of the training drug. Advantages of this procedure are that it is pharmacologically very selective and that the discriminative stimulus effects of drugs are related to and predictive of subjective effects in humans. When appropriate pharmacological tools (e.g., antagonists acting at the same receptor as the training drug) are available, the mechanism(s) mediating the discriminative stimulus effects of a drug can be confirmed by combining drugs (agonists with antagonists).

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures display a high degree of pharmacologic selectivity. Test drugs that dose dependently occasion responding on the drug-appropriate lever likely share a mechanism of action with the training drug; drugs that do not occasion responding on the drug-appropriate lever likely are pharmacologically dissimilar to the training drug (in terms of site of action and/or in terms of efficacy) and will typically cause responding predominantly if not exclusively on the lever that is appropriate for the drug vehicle, up to behaviorally active doses. The pharmacologic selectivity of these procedures permits differentiation not only among compounds acting on different receptors or neurochemical systems (e.g., dopamine receptors vs. opioid receptors) but also among compounds acting on different subtypes of receptors within the same receptor class (e.g., mu vs. kappa opioid receptor agonists). Because of the importance of pharmacokinetic factors to the overall abuse liability of drugs, and because drug discrimination procedures are relatively insensitive to pharmacokinetic factors (as compared to self-administration procedures), positive results from a drug discrimination study are not in themselves sufficient to predict abuse liability. Along with other measures of drug action, results of drug discrimination studies are used to predict the likelihood of new compounds having abuse liability. Typically, self-administration data are used along with drug discrimination data, since these two assays are sensitive to different, though related aspects of drug activity. A negative effect with a test compound in a drug discrimination procedure indicates that the test substance, at the doses and pretreatment times studied, does not share discriminative stimulus effects with the training drug; it does not indicate that the test substance is devoid of discriminative stimulus effects or that the test substance is not likely to be abused. Thus, the high pharmacological selectivity of drug discrimination procedures is both an asset and a limitation.

MODIFICATIONS OF THE METHOD

Drug discrimination studies are performed in a variety of species including squirrel monkeys, rhesus monkeys, pigeons, gerbils, and mice (Hein et al. 1981; Herling and Woods 1981; Bertalmio and Woods 1987; Bertalmio et al. 1982; Dykstra et al. 1987, 1988; France and Woods 1993; France et al. 1994, 1995; Jarbe and Swedberg 1998; Shelton et al. 2004; Stolerman et al. 2004). Operant responding can be maintained with different reinforcers, including food, liquids, and aversive stimuli (e.g., electric shock). While the percentage of the total responses made on the drug-appropriate lever (averaged among subjects) is commonly used to express and analyze drug effects, some investigators express and analyze data in terms of the percentage of animals emitting some predetermined minimum (e.g., 90%) percentage of responses on the drug-appropriate lever.

The pharmacologic selectivity of drug discrimination procedures is particularly evident with opioid receptor agonists that bind selectively to different receptor subtypes. For example, monkeys trained to discriminate injections of a mu opioid receptor agonist (codeine, etorphine, or alfentanil) generalize to other mu opioid receptor agonists and not to non-opioid drugs, not to opioid receptor antagonists, and not to opioid receptor agonists acting at other (e.g., kappa) opioid receptors. Conversely, monkeys trained to discriminate a kappa opioid receptor agonist such as ethylketocyclazocine or U-50,488 generalize to other kappa receptor agonists and not to mu receptor agonists, antagonists, or to non-opioids (Woods et al. 1993; France et al. 1994).

Meert et al. (1989) used drug discrimination studies to characterize risperidone as an antagonist of the discriminative stimulus effects of LSD.

Meert and Janssen (1989) and Meert et al. (1990) showed differences between ritanserin and chlordiazepoxide in drug discrimination procedures.

Discrimination procedures have also been used to examine stimulus conditions that are believed to reflect drug withdrawal. Some of those procedures involve Pavlovian conditioning of aversive stimuli (Davis et al. 2009) whereas others involve operant procedures in which pharmacological antagonists are the training

stimuli in animals treated chronically with an agonist (Becker et al. 2008; Stewart and McMahon 2010).

Delta-9-tetrahydrocannabinol discrimination in rats was proposed as model for cannabis intoxication in humans (Balster and Prescott 1990).

The drug discrimination method has also been applied to study anxiolytic drugs using pentylenetetrazol at subconvulsive doses (Sherman and Lal 1979, 1980; Sherman et al. 1979; Lal and Sherman 1980).

The conditioned taste aversion procedure has been described as a more rapid alternative to two-lever operant procedures in drug discrimination research (Garcia et al. 1955; van Heest et al. 1992).

Others have studied combinations of drugs in animals trained to discriminate one or more drugs alone or in combination (McMillan et al. 2009; Stolerman et al. 1999).

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10.1.4.3 Self-Administration Studies

PURPOSE AND RATIONALE

Drug self-administration is studied under a variety of conditions that can involve free or limited access. Some procedures (e.g., two-bottle choice for alcohol drinking in rodents) provide conditions that promote voluntary drug intake without particular attention to patterns of drug taking whereas other procedures (e.g., operant self-administration) permit detailed examination of specific aspects of drug taking. Operant procedures involving i.v. drug administration are the most widely used procedures for assessing abuse liability, and these procedures have a high degree of predictive validity for drugs that are abused by humans. The goal of self-administration procedures is often to compare self-administration of a test substance to self-administration of a standard reference substance and known drug of abuse (e.g., heroin). Alternatively, other procedures test whether drug naïve animals will initiate and maintain self-administration of a test substance.

PROCEDURE

Self-administration procedures in rodents commonly use the i.v. route of drug administration as this route maximizes the likelihood of detecting positive reinforcing effects because of the rapid onset of drug action after i.v. administration. Unlike drug discrimination and conditioned place preference procedures, where onset and duration of drug action are not critical factors so long as drugs are studied at times (and doses) that have activity, pharmacokinetics are critically important for self-administration studies. Even drugs that otherwise are very effective, positive reinforcers can be less effective or ineffective when their administration is delayed after a response. Self-administration procedures also are available for oral, intragastric, parenteral, and inhalation routes of administration.

Male Sprague–Dawley rats weighing 250–300 g are used for these studies. The apparatus consists of commercially available operant chambers equipped with levers, lights, a food hopper, and a mechanism for i.v. drug delivery (e.g., syringe pump).

Food-restricted rats are first trained to press levers for food prior to catheter implantation; the useful period of the catheter is extended when rats are trained to press levers for food before surgery. In daily sessions, a single response on either lever delivers a food pellet. After a single session in which 50 pellets are received under the continuous reinforcement schedule, one of the levers is designated (randomly or systematically balanced across subjects in a group) the active lever for the remainder of the study. The response requirement is increased over days, so long as rats receive the maximum of 50 pellets in a session, to a maximum of 5 (fixed-ratio [FR] 5). Once responding is reliable under the FR 5 schedule (e.g., 50 pellets delivered per session for three consecutive sessions), food training is suspended and the rats receive a chronic indwelling catheter.

Surgery is conducted under isoflurane anesthesia with the catheter (3 French) implanted in the jugular or femoral vein. The catheter is tunneled s.c., exteriorized in the midscapular region, and connected to an access port that is mounted in a jacket. For daily 90-min sessions, a Huber point needle connected to a syringe pump by sterile tubing delivers drug or vehicle to the access port and catheter. The port and catheter are filled with heparinized saline after each session. The beginning of the self-administration session is signaled by illumination of lights over the active lever; five responses on the active lever deliver drug or vehicle with each injection followed by a 3-min timeout when the chamber is dark and lever presses have no programmed consequence.

Stable self-administration responding is established with a reference compound (e.g., 0.32 mg/kg/injection, cocaine, i.v.), defined by three consecutive sessions when the number of injections received per session is greater than 20 and the number of injections in each session does not vary by more than $\pm 20\%$ of the mean number of injections for those sessions. Next, saline is substituted for the reference compound in order to extinguish responding and, thereby, to confirm the positive reinforcing effects of the reference compound under these conditions. Extinction is defined as three consecutive sessions when the number of saline injections received per session is less than eight. Once these criteria are satisfied, a dose of test substance is substituted to see if it maintains self-administration responding. The test substance is studied for a minimum of five and a maximum of ten sessions or until stable responding is observed, as defined by three consecutive sessions when the number of injections received per session does not vary by more than $\pm 20\%$ of the mean number of injections for those sessions. Following the test substance, saline is

available for self-administration for a minimum of five sessions and until the number of saline injections received is less than eight for three consecutive sessions. Finally, a retest with the reference substance (e.g., cocaine) confirms the sensitivity of the assay (and the subject) to positive reinforcing effects of a known drug of abuse. Different doses of a test substance are studied in different groups (n = 8/group) of rats that are initially trained to self-administer a reference substance.

EVALUATION

Self-administration of a test substance is compared to self-administration of vehicle and self-administration of the reference substance. Data are expressed either as the number of injections (mean ± SEM) received per session or as the response rate on the active lever. A range of doses of the test substance must be examined in order to insure that sufficient exposure occurred to test whether that substance has positive reinforcing effects.

CRITICAL ASSESSMENT OF THE METHOD

Self-administration procedures are, generally, not pharmacologically specific insofar as animals with a history of self-administering a drug from one pharmacological class (e.g., cocaine-like stimulant) will readily self-administer a drug from a different pharmacological class (e.g., heroin-like opioid), although there are examples where specific drug and behavioral history can facilitate subsequent drug self-administration (Collins and Woods 2009). While the predicative validity of self-administration studies in nonhumans is quite high for abuse liability in humans, it is not unanimous insofar as some drugs that are abused by humans (e.g., lysergic acid dimethylamide) are not readily self-administered by nonhumans. Conversely, some drugs that are self-administered by nonhumans do not appear to have high-abuse liability in humans (e.g., modafinil). Nevertheless, i.v. self-administration procedures remain the "gold standard" in preclinical studies for assessing and predicting abuse liability.

MODIFICATIONS OF THE METHOD

In addition to fixed-ratio schedules, a variety of different schedules of reinforcement have been used to study drug reinforcement including second-order schedules (Howell et al. 2007), multiple schedules (Ginsburg and Lamb 2006), and progressive ratio schedules (Carroll et al. 2011).

Winsauer et al. (2000) examined the effects of self-administered cocaine on acquisition and performance of response sequences for food using a multiple schedule in monkeys.

Henry and Howell (2009) studied reinstatement of responding by non-contingent i.v. cocaine injections in monkeys with a history of i.v. cocaine self-administration under a second-order schedule.

Wang and Woolverton (2007) used progressive ratio schedules to compare reinforcing effects of the isomers of MDMA and of methamphetamine in monkeys.

Modification by amphetamine of the reinforcing effects of cocaine under a progressive ratio schedule was studied in rats (Chiodo et al. 2008) and rhesus monkeys (Czoty et al. 2010).

Responding that has been extinguished (by responding in the absence of drug or the absence of drug and drug-paired stimuli) can be reinstated by presenting various stimuli, including drugs, non-drug stimuli that were paired with contingent drug administration, or by stress. Such reinstatement procedures are used to examine, in the preclinical laboratory (Bossert et al. 2007; Holtz et al. 2011), some of the factors that might contribute to reinitiation of drug taking in abstinent individuals.

Extending the period of drug access in self-administration procedures increases drug intake more than the proportional increase in session length (Ahmed et al. 2000) and can modify sensitivity to drugs (Morgan and Roberts 2004) as well as increase the likelihood of dependence developing (O'Dell et al. 2007).

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10.1.4.4 Conditioned Place Preference Studies

PURPOSE AND RATIONALE

Conditioned place preference procedures have been used to examine behavioral actions that are thought to be related to positive reinforcing effects as measured by other procedures, such as self-administration (van der Kooy 1987; Hoffman 1998; Tzschenke 1998; Self and Stein 1992). Particular environmental stimuli are paired with the presence or absence of a presumed reinforcer (e.g., drug or food), and later, in the absence of that reinforcer, animals are tested for their preference for either environment.

PROCEDURE

To induce place preference with food, a food-restricted animal is exposed to an experimental chamber that consists of two compartments (which differ in floor texture and/or wall color) and that are separated by a removable barrier. In some iterations of this procedure, the two compartments are joined by a small tunnel or a third (neutral) compartment. On alternate days, the animal is confined to one or the other compartment, with food available in only one of the compartments. Thus, food is selectively paired with one of the distinctive environments. After several (e.g., four in each compartment for a total of eight) conditioning sessions, the animal is placed in the same chamber without the barrier in place (for procedures that use a third [neutral] compartment, the animal is placed in that compartment and otherwise in the middle of the chamber). In the absence of the reinforcer (e.g., food), animals demonstrate a relative increase in the amount of time spent in the environment that was paired with the reinforcer (e.g., food) as compared to the compartment that was not paired with the reinforcer. Place conditioning with drugs is conceptually similar and involves the differential pairing of drug effect with one compartment and the absence of drug effect (vehicle) with the other. Drugs can be administered by various different routes (Amalric et al. 1987, Bals-Kubik et al. 1990; Iwamoto 1988; Shippenberg and Herz 1987), and usually animals are placed in the chamber immediately after drug administration for a 40-min conditioning session.

Male Sprague–Dawley rats weighing 250–300 g are typically used for these studies. Drugs are usually administered i.p. or s.c. When drugs are to be administered intracerebroventricularly, rats are anesthetized with 60 mg/kg i.p. sodium hexobarbital and 23-gauge guide cannulae aimed at the lateral ventricle ($AP = -0.9$ mm, $L = +1.5$ mm, $DV = 3.5$ mm) (Paxinos and Watson 1982) are stereotaxically implanted; conditioning commences 1 week later.

The apparatus consists of $30 \times 60 \times 30$-cm Plexiglas boxes. For conditioning sessions, each box is divided into two equal-sized compartments by means of a removable sliding wall. One compartment is white with a textured floor, the other black with a smooth floor. For testing, the central wall is raised 12 cm above the floor to allow passage from one compartment to the other.

Conditioning sessions are conducted once a day for 8 days and consist of administering drug or its vehicle

on alternate days. The rats are immediately confined to one compartment of the box following drug injection and to the other compartment following vehicle injection. Conditioning sessions last 40 min although, for drugs with delayed onset or very short duration of action, the temporal conditions need to be adjusted to insure that conditioning occurs at a time of biological activity. Test sessions are carried out 1 day after the last training session and in the absence of drug. The rats are placed in a neutral position (either in the center or in the neutral compartment) of the test box and allowed free access to both sides of the box for 15 min. A video camera with integrated stopwatch is used for data recording. Alternatively, photocells mounted along the sides of each compartment can be used to electronically monitor the location of the subject in the apparatus. The time spent in each compartment is assessed by visual analysis of the recorded videotape or by data collected through photocell beam breaks.

For intracerebroventricular injections, a 30-gauge injection needle is attached to a microsyringe via polyethylene tubing. The drug solutions are administered over a 60-s period, and the injection needles are left in place for an additional 30 s to ensure complete delivery of the solution. For antagonism tests, groups of rats receive an intracerebroventricularly injection of the antagonist (naltrexone or naloxone) or vehicle 10 min before the microinjection of the conditioning drug. At the end of the experiments, the rats are anesthetized and sacrificed by decapitation. The brains are removed and sectioned in a cryostat to verify the location of the cannulae. Alternatively, antagonists can be administered systemically.

EVALUATION

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place and are expressed as means ± SEM. In cases where animals show a bias toward one compartment prior to conditioning, drug conditioning can be established with the non-preferred compartment, thereby increasing the confidence that preference for that compartment is specifically related to drug administration. A range of doses should be studied since the dose–response curve for conditioned place preference can be biphasic such that smaller doses produce preference whereas larger doses have no effect or produce an aversion.

CRITICAL ASSESSMENT OF THE METHOD

Conditioned place preference procedures are not pharmacologically selective in the manner that drug discrimination studies are insofar as drugs from several different classes (e.g., opioids, ethanol, and stimulants) can generate positive results. Generally, there is a strong positive correlation between drugs that can be used to establish conditioned place preference and those that are positive reinforcers by other measures (e.g., i.v. self-administration); however, one of the most effective reinforcers in self-administration studies, that is also widely abused by humans, does not unanimously generate strong conditioned place preference in nonhumans—cocaine. Thus, results from conditioned place preference studies should be used in concert with results from other measures of reinforcing effects (e.g., self-administration) in order to determine the likelihood that a drug exerts a profile of behavioral effects that would indicate its abuse. For the purpose of opioids, in general, mu opioid receptor agonists are effective for establishing place preference whereas kappa receptor agonists are not. In fact, kappa receptor agonists can generate place aversion (e.g., Sante et al. 2000).

MODIFICATIONS OF THE METHOD

In order to distinguish place preference and place aversion, place-conditioning behavior can be expressed by a difference in the time spent in the preferred and the non-preferred sides in the postconditioning and preconditioning tests, respectively. Positive values indicate preference and negative values aversion (Kitaichi et al. 1996). For non-biased procedures, where animals do not show an inherent preference for either compartment, results are presented simply as a difference score (i.e., time spent in the drug-paired compartment minus time spent in the vehicle-paired compartment).

In addition to place preference, others (Mucha and Herz 1985; Broadbent et al. 2002) used taste preference conditioning.

Foltin and Evans (1997) established place preference for cocaine in rhesus monkeys, and Wang et al. (2011) established a preference with morphine in monkeys.

Cunningham (Bormann and Cunningham 1998; Gabriel et al. 2004) and others (Sevak et al. 2007, 2008a, b) use the same chamber for training and testing with the exception that only floor texture varies

according to treatment condition. Thus, drug and vehicle are paired with different floor textures, and during test sessions, the time spent on each section of a floor comprising the two different textures (half of the floor with each) is used as an index of preference or aversion. This procedure has the advantage that the size of the test chamber is not different from the size of the training chamber.

Perks and Clifton (1997) used sucrose solution to generate a place preference which was subsequently devalued using a LiCl taste aversion procedure.

Brockwell et al. (1996) described a computerized system for the simultaneous monitoring of place conditioning and locomotor activity in rats consisting of four independent conditioning boxes, each equipped with six pairs of photosensors connected to an Experiment Controller, an electronic board containing a microprocessor, a programmable timer, and 16 K of RAM used to store both instructions and data.

Steinpreis et al. (1996) investigated place preference in Sprague–Dawley rats treated with graded i.p. doses of methadone. Place preference for methadone peaked at 4 mg/kg, and aversion was produced at 10 mg/kg.

Using the conditioned place preference paradigm, Mamoon et al. (1995) assessed the rewarding properties of butorphanol in comparison to morphine after unilateral microinjections into the ventral tegmental area of male Lewis rats.

Gaiardi et al. (1997) assessed rewarding and aversive effects of buprenorphine by place preference and taste aversion conditioning. After s.c. administration of doses ranging from 0.025 to 0.1 mg/kg, buprenorphine caused a significant increase in the amount of time spent on the drug-paired compartment but no significant decrease of saccharin consumption. Rewarding and aversive effects did not occur within a similar dose range.

Contarino et al. (1997) found no tolerance to the rewarding properties of morphine, after repeated i.p. injections of morphine, in prolonged conditioned place preference trials.

Tsuji et al. (1996) studied the effect of microinjections of GABA receptor agonists and antagonists into the ventral tegmental area of Sprague Dawley rats on morphine-induced place preference.

Sufka (1994) recommended the conditioned place preference paradigm as a novel approach for assessing effects of opioids in chronic pain induced in rats by unilateral injections of Freund's adjuvant into the hind paw.

Conditioned place avoidance by naloxone was attenuated by clonidine (Kosten 1994).

In addition to morphine and other mu opioid receptor agonists, other drugs with known or putative abuse liability were tested in the place-conditioning paradigm including the following: cocaine (Lepore et al. 1995; Suzuki and Misawa 1995; Calcagnetti et al. 1996; Martin-Iverson and Reimer 1996; Martin-Iverson et al. 1997), caffeine (Brockwell et al. 1991; Brockwell and Beninger 1996), cannabinoids (Lepore et al. 1995; Sañudo-Peña et al. 1997), LSD (Parker 1996), methamphetamine (Suzuki and Misawa 1995), amphetamine (Hoffman and Donovan 1995; Turenne et al. 1996), methylphenidate (Gatley et al. 1996) and fenfluramine (Davies and Parker 1993), 7-OH-DPAT (Khroyan et al. 1995; Chaperon and Thiébot 1996), gamma-hydroxybutyric acid (Martellotta et al. 1997), propofol (Pain et al. 1997), alcohol (Kennedy et al. 2011; Voorhees and Cunningham 2011; Zarrindast et al. 2010), methylenedioxymethamphetamine (Daza-Losada et al. 2011), and NMDA receptor antagonists (Steinpreis et al. 1995; Papp et al. 1996).

Furthermore, 5-HT_3 receptor antagonists (Acquas et al. 1990), 5-HT_3 receptor agonists (Higgins et al. 1993), dopamine release inhibitors (Schechter and Meehan 1994), dopamine D1 receptor antagonists (Acquas and Di Chiara 1994), dopamine D3 receptor agonists (Khroyan et al. 1997), and antiemetic agents (Frisch et al. 1995) were studied in the place-conditioning paradigm.

Suzuki et al. (1991, 1993) and del Poso et al. (1996) studied opioid-induced place preference in mice, and Bechtholt et al. (2004) studied the effects of handling on conditioned place aversion and conditioned place preference by ethanol in mice.

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11.1 In Vitro Tests

11.1.1 Blood Coagulation Tests

PURPOSE AND RATIONALE

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated conversion of fibrinogen to fibrin, which is one major component of a thrombus. The coagulation cascade can be initiated either by the "exogenous pathway," the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa complex, or by the "endogenous pathway," so-called contact activation leading via factors XII, XI, and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X, which induces the formation of the prothrombinase complex consisting of factor Xa, factor Va, and Ca^{2+} on a phospholipid's surface. The latter leads to the activation of thrombin, which in turn cleaves fibrinogen to fibrin. The three coagulation tests (PT, APTT, and TT) allow differentiating between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) ex vivo.

PROCEDURE

Male Sprague–Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a midline incision, and 1.8 ml blood is collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behring Werke, Marburg, Germany). The sample is immediately agitated and centrifuged in a plastic tube at 1,500 g for 10 min. Plasma is transferred to another plastic tube, and the coagulation tests for the determination of thrombin time (TT), thromboplastin time (PT), and activated partial thromboplastin time (APTT) are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below), and the time to clot formation is determined in the coagulometer (=coagulation time). For detailed laboratory diagnosis of bleeding disorders and assessment of blood coagulation, see Palmer (1984) and Nilsson (1987).

11.1.1.1 Prothrombin Time (PT)

An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37°C. Then, 0.2 ml of human thromboplastin (Thromborel®, Behring Werke, Marburg) is added, and the coagulometer (Schnittger+Gross coagulometer, Amelung, Brake) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

11.1.1.2 Activated Partial Thromboplastin Time (APTT)

To 0.1 ml of citrated plasma, 0.1 ml of human placenta lipid extract (Pathrombin®, Behring Werke, Marburg) is added, and the mixture is incubated for 2 min at 37°C. The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started, and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

11.1.1.3 Thrombin Time (TT)

To 0.1 ml of citrated plasma, 0.1 ml of diethyl barbiturate-citrate buffer pH 7.6 (Behring Werke, Marburg) is added, and the mixture is incubated for 1 min at 37°C. Then, 0.1 ml of bovine test thrombin (30 IU/ml, Behring Werke, Marburg) is added, and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

EVALUATION

Mean values of TT, PT, and PTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's *t*-test.

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11.1.2 Thrombelastography

PURPOSE AND RATIONALE

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph (Haemoscope Corp., Skokie, Illinois, USA) is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction. The blood samples are transferred to cuvettes and maintained at 37°C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermo paper. The modern thrombelastograph transfers the analogous recording to a digital signal that is evaluated by a computer program.

PROCEDURE

Thrombelastography can be performed in either whole blood or in citrated platelet-rich or platelet-poor plasma after recalcification. Blood samples are obtained from Beagle dogs, weighing 12–20 kg, from rabbits, weighing 1.7–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the compound by intravenous, subcutaneous, or by oral administration. Ten or 20-min postdosing (i.v., s.c. administration) or 60, 90, or 180-min postdosing (oral administration) blood is collected. The blood samples are mixed with 3.8% trisodium citrate solution (one-part citrate solution to nine-part blood) as anticoagulant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the recalcified whole blood is transferred to the prewarmed cup of the thrombelastograph. After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

EVALUATION

The following measurements are the standard variables of TEG:

- *Reaction time* (*r*): the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
- *Clot formation time (k)*: the difference from the 1-mm *r* to 20-mm amplitude. *k* represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot caused by fibrin build up and cross-linking.
- *Alpha angle (α)*: angle formed by the slope of the TEG tracing from the *r* to *k* value. It denotes speed at which solid clot forms.
- *Maximum amplitude (MA)*: greatest amplitude on the TEG trace. *MA* represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
- *Clot strength (G in dynes per square centimeter)*: defined by $G = (5{,}000\ MA)/(96–MA)$. In a tissue-factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
- *Lysis 30, lysis 60 (Ly30, Ly60)*: reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis.

Effect of various stimuli on platelet/fibrin clot dynamics as shown by Mousa et al. (2000)

TEG parameters	TF (25 ng)	LPS (0.63 ug)	Xa (0.25 nM)	Thrombin (0.3 mU)
	Mean±SEM			
R (minutes)	29.7±2.3	23.4±1.4	15.6±2.9	3.4±0.6
K (minutes)	5.8±1.0	7.6±0.9	4.8±0.5	5.5±0.8c
α (angle)	45.0±2.6	47.8±3.2	61.5±2.1	57.8±2.9
MA (mm)	58.2±1.7	50.0±2.0	65.0±0.8	50.1±2.4

Citrated human whole blood plus 2.5 mM calcium. Data represent mean for n = 6±SEM

MODIFICATIONS OF THE METHOD

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by thrombelastography in vitro using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and thrombelastography to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described a short-time, endotoxin-induced rabbit model of hypercoagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters including thrombelastography.

Khurana et al. (1997) introduced tissue-factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II). Additionally, Mousa et al. (2005) utilized the TEG in phase II clinical trial in monitoring the efficacy of oral platelet GPIIb/IIIa antagonist on platelet/fibrin clot dynamics.

CRITICAL ASSESSMENT OF THE METHOD

Zuckerman et al. (1981) compared thrombelastography with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count, and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies; it contains additional information on the hemostatic process. This is due to the following: (1) the fact that most laboratory measurements end with the formation of the first fibrin strands while TEG measures the coagulation process on whole blood from initiation of clotting to the final stages of clot lysis and retraction and (2) the possibility of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

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11.1.3 Chandler Loop

PURPOSE AND RATIONALE

The Chandler loop technique allows producing in vitro thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to human thrombi formed in vivo (Robbie et al. 1997) with platelet-rich upstream sections ("white heads") that are relatively resistant to t-PA-mediated thrombolysis in contrast to the red blood-cell-rich downstream parts ("red tails") (Stringer et al. 1994).

PROCEDURE

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm tubing). The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23°, and rotated at 17 rpm. When the developing thrombus inside the tube becomes large enough to occlude the lumen; the blood column becomes static and moves around in the direction of rotation of the tube.

EVALUATION

Time to thrombus occlusion establishes a definite end point in this system.

MODIFICATIONS OF THE METHOD

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2 C8) on the t-PA-induced lysis of Chandler thrombi in vitro.

They used citrated blood and supplemented it with 5.8 μM [^{125}I]–labeled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (prevalue). The head and the tail were then subjected to thrombolysis by adding 300 μl phosphate-buffered saline containing plasminogen (2 μM) and t-PA (0.9 nM). During the observation time of 240 min aliquots of 10 μl were taken at 30, 60, 120, 180, and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the prevalue was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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11.1.4 Platelet Aggregation and Disaggregation in Platelet-Rich Plasma or Washed Platelets (Born Method)

PURPOSE AND RATIONALE

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of nonactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V-complex at high shear rates and binding of collagen to two receptors, integrin α2β1 and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa-ligand binding sites. These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation, platelets are activated by the addition of one of the following agonists to platelet-rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A2) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, and PAF. The formation of platelet aggregates with stirring leads to changes in optical density that are monitored photometrically, usually for 4 min. The test was developed originally by Born (1962a, b) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation in vitro or ex vivo. For in vitro studies, human PRP is preferred.

PROCEDURE

11.1.4.1 Materials and Solutions

Anticoagulating substances:	
Hirudin (Sigma)	200 μg/ml
Trisodium citrate	0.11 M
Acid-citrate-dextrose (ACD) solution:	
Citric acid	38 mM
Sodium citrate	75 mM
Glucose	124 mM
Platelet aggregating substances (final concentrations in the test):	
ADP: for reversible or biphasic aggregation	0.1–5 μM
ADP: for irreversible aggregation (Sigma)	3–10 μM
Sodium arachidonate (Biodata)	0.3–1 mM
Calcium ionophore A 23187 (Calbiochem)	10 μM
Collagen (Hormonchemie)	3 μg/ml
PAF-acether (C 16-PAF, Bachem)	0.1 μM
Thrombin (Sigma)	0.02–0.05 IU/ml
TRAP (SFLLRNP, Bachem)	1–10 μM
U 46619 (ICN)	1–10 μM
Ristocetin	0.1–1 mg/ml
GPRP (fibrin antipolymerant, Bachem)	0.5 mM
Four-channel aggregometer (PAP 4, BioData)	

The test is carried out either ex vivo or in vitro

For ex vivo assays, mice, rats, or guinea pigs from either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal, or intravenous administration. At the end of the absorption time, blood is collected by caval venipuncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardiopuncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For in vitro assays, human blood is collected from the antecubital vein of adult volunteers who had not received any medication for the last 2 weeks.

11.1.4.2 Preparation of PRP, PPP, and WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 170 g for 15 min to obtain platelet-rich plasma (PRP). The PRP supernatant is carefully removed, and the rest is further centrifuged at 1,500 g for 10 min to obtain platelet poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3×10^8/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5 by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430 g. The pellet is resuspended in the original volume with Tyrode solution (mM: NaCl 120, KCl 2.6, $NaHCO_3$ 12, NaH_2PO_4 0.39, HEPES 10, glucose 5.5, albumin 0.35%) and set to platelet count of 3×10^8/ml. Studies should be completed within 3 h after blood withdrawal.

For ex vivo assays, duplicate samples of 320 µl PRP from drug-treated and vehicle control subjects (for rabbits: control samples before drug administration) are inserted into the aggregometer at 37°C under continuous magnetic stirring at 1,000 rpm. After the addition of 40 µl physiological saline and 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For in vitro assays, 40 µl of the test solution are added to samples of 320 µl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37°C for 2 min under continuous magnetic stirring at 1,000 rpm. After the addition of 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, glycine-proline-aspartate-proline (GPRP) is added in order to avoid fibrin formation. In order to measure disaggregation, experimental compounds are added to stimulated PRP at 70% or 100% of control aggregation, and monitoring is performed for further 10 min. Deaggregation is measured by the decrease of light transmission (see Haskel and Abendschein 1989).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation (0% = transmission of PRP, 100% = transmission of PPP).

For in vitro assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls.
   Statistical significance is evaluated by means of the unpaired Student's *t*-test.
2. IC_{50} values are determined from the nonlinear curve fitting of concentration-effect relationships. IC_{50} is defined as the concentration of test drug for half maximal inhibition of aggregation.
3. Percent disaggregation is determined at 10 min after addition of compound; IC_{50} is calculated from the concentration-effect relationship.

For ex vivo assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits: control values before drug administration).
   Statistical significance is evaluated by means of the Student's *t*-test (paired for rabbits, unpaired for others).
2. *ED*50 values are determined from the dose–response curves. *ED*50 is defined as the dose of drug leading to 50% inhibition of aggregation in the animals.

CRITICAL ASSESSMENT OF THE METHOD

The assay, introduced by Born (1962a, b), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of

a functional parameter in intact human platelets. However, processing of platelets during the preparation of PRP, washed or filtered platelets from whole blood, results in platelet activation and separation of large-size platelets.

MODIFICATIONS OF THE METHOD

Several authors described modifications of the assay procedure. Breddin (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratantoni and Poindexter (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96-well microtiter plate method with conventional aggregometry showed similar dose–response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neill (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor.

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11.1.5 Platelet Aggregation After Gel Filtration

PURPOSE AND RATIONALE

Triggering of platelet activation by low concentrations of ADP, epinephrine, or serotonin—so-called weak platelet agonists—in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet aggregation induced by thrombin, collagen, or prostaglandin endoperoxide—so-called strong agonists—is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX–V.

PROCEDURE

11.1.5.1 Materials and Solutions

Acid-citrate-dextrose (ACD) solution:	
Citric acid	0.8%
Sodium citrate	2.2%
Glucose	2.45%
Hirudin	0.6 U/ml
Tyrode solution:	
NaCl	137 mM
KCl	2.7 mM
$MgCl_2$	5.5 mM
NaH_2PO_4	3.0 mM
HEPES	3.5 mM
Glucose	5.5 mM

(*continued*)

Albumin	0.2%
Hirudin	0.06 U/ml
Apyrase	40 μg/ml
pH	7.2
ADP	10 μM
Thrombin	0.02–0.05 U/ml
$CaCl_2$	0.5 mM
Fibrinogen (American Diagnostca)	1 mg/ml
von Willebrand factor	10 μg/ml
Sepharose CL 2B (Pharmacia)	
Acrylic glass column (Reichelt Chemietechnik, 3-cm inner diameter, 18-cm length)	
Aggregometer (PAP 4, Biodata)	

11.1.5.2 Preparation of Gel-Filtered Platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marguerie et al. (1979).

Blood is drawn from healthy adult volunteers who had no medication for the last 2 weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD solution and centrifuged for 10 min at 120 g. The platelet-rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD solution and centrifuged at 285 g for 20 min. The resulting pellet is resuspended in Tyrode buffer (approximately 500 μl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8/ml. Gel-filtered platelets (GFP) are kept at room temperature for 1 h until the test is started.

11.1.5.3 Experimental Course

For the aggregation studies, GFP in Tyrode buffer is incubated with $CaCl_2$ (final concentration 0.5 mM) with or without fibrinogen (final concentration 1 mg/ml) in polystyrene tubes. After 1 min, 20 μl of the test compound or the vehicle (controls) are added, and the samples are incubated for another 2 min. After the addition of 20 μl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37°C (1,000 rpm) in the aggregometer. Samples with added $CaCl_2$ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregation response of GFP to 10 μM ADP shows intact platelets (with only minor preactivation with gel filtration).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor GFP; in the case of an antiaggregating effect, the test is performed with 4–6 GFPs.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student's *t*-test.

The percent inhibition of platelet aggregation in the dosage groups is calculated relative to the vehicle controls. IC_{50} values (50% inhibition of aggregation) are determined from the concentration-effect curves.

For detailed methodology and evaluation of different agents, see Marguerie et al. (1979, 1980) and Markell et al. (1993).

References and Further Reading

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11.1.6 Platelet Aggregation in Whole Blood

PURPOSE AND RATIONALE

The method uses a whole blood platelet counter that counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anticoagulated human whole blood samples by the addition of the aggregating agent's arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC_{50} values are calculated in dosage groups. The effect of compounds on other blood cells that secondarily can

influence platelet aggregation is included in this test system. The method has been described by Lumley and Humphrey (1981) and Cardinal and Flower (1980).

PROCEDURE

11.1.6.1 Materials and Solutions

Anticoagulant: sodium citrate to induce platelet aggregation	3.8%
Sodium arachidonate (Bio/Data)	3.6×10^{-4} M
Collagen (Hormonchemie)	10 µg/ml
Serono Hematology System 9000 or Sysmex Microcell counter F 800	

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers who had not received medication for the last 2 weeks. Nine milliliters of venous blood is anticoagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30 min until the start of the test.

For the aggregation studies, 10 µl test substance or vehicle (control) are added to 480 µl citrated blood. Samples in closed tubes are preincubated for 5 min in a 37°C water shaker bath at 75 strokes/min. Ten microliters aggregating agent are added and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ("initial platelet count," "10-min-platelet count") in a hematology cell counter. The following samples for the determination of the platelet count are prepared in duplicate:

- Control aggregation = spontaneous aggregation (without aggregating agent): 480 µl blood + 20 µl vehicle. Blood samples with >20% spontaneous aggregation are not used to test for induced aggregation.
- Maximal aggregation: 480 µl blood + 10 µl vehicle + 10 µl aggregating agent.
  Values represent the maximal induced aggregation rate of the blood sample.
- Test substance aggregation: 480 µl blood + 10 µl test substance + 10 µl aggregating agent.

EVALUATION

1. From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\%\ \text{maximal aggregation} = 100 - \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

[*This value for maximal aggregation is taken as* 100%].

2. From the samples for test substance–induced aggregation, the percentage of aggregation in dosage groups is calculated according to the following formula:

$$\%\ \text{aggregation} = \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

3. IC_{50} values (50% inhibition of aggregation) are determined from the dose–response curves (log concentration test substance versus % inhibition of aggregation).

References and Further Reading

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11.1.7 Platelet Micro- and Macroaggregation Using Laser Scattering

Purpose and Rationale

- A new highly sensitive method to study platelet aggregation based on the measurement of mean radius or particle size makes it possible to record kinetics of formation of micro- and macroaggregates in real time.
- Sensitivity in measurements of spontaneous aggregation is higher than in routine light transmittance.

A new platelet aggregometer (AG-10; Kowa, Japan) that uses a laser-light-scattering beam was introduced (Tohgi et al. 1996). Platelet aggregates, the size of which can be measured as total voltage of light-scatter intensity at 1.0-s intervals for a 10-min period, can be divided into three ranges: small aggregates (diameter 9–25 µm), medium (26–50 µm), and large (>50 µm). Using laser-scatter aggregation, it was found that young smokers had an increased number of

small platelet aggregates, which cannot be detected with a conventional aggregometer based on the turbidometric method (Matsuo et al. 1997). This device detects platelet aggregation in the small-aggregate size range by the addition of unfractionated heparin (UFH), and the aggregates are disaggregable in incubation with protamine sulfate. When platelet aggregation induced by UFH at a final concentration of 0.5 U/ml was observed in 36 normal subjects with no history of heparin exposure, 13 had a positive response in excess of 0.5 V of light intensity in the small-aggregate size range. In chronic hemodialysis patients in whom heparin had been used regularly for many years, a positive response with heparin-induced aggregates was noted in 37 of 59 patients, which was increased compared with that of normal subjects. The light intensity in the small-aggregate size range was enhanced during heparinized dialysis. In patients with a positive heparin response, the intensity of aggregates after heparin was significantly increased compared with that in nonresponders to heparin. The findings of enhanced platelet aggregation during heparin infusion could be directly obtained without the addition of ADP or TRAP using laser aggregometry (Xiao and Théroux 1998).

11.1.7.1 Limitations

This technique cannot be applied in whole blood yet but can be used with PRP, washed platelet, or GFP.

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11.1.8 Fibrinogen Receptor Binding

PURPOSE AND RATIONALE

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I-fibrinogen (30–50 nM) is incubated with increasing concentrations of a nonlabeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug. Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I-fibrinogen binding at the GPIIb/IIIa receptor.

PROCEDURE

11.1.8.1 Preparation of Gel-Filtered Platelets

From a healthy volunteer, 200 ml blood is collected. An aliquot of 8.4 ml blood is mixed with 1.4 ml ACD buffer in polystyrol tubes and centrifuged at 1,000 rpm for 15 min. The resulting platelet-rich plasma (PRP) is collected, and an aliquot is taken for platelet counting. Ten milliliters of PRP is mixed with 1 ml ACD buffer (ACD-PRP, pH∼6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at 1,600 rpm for 20 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 µl Tyrode buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to the Sepharose-packed column that has been eluted with approximately 100 ml degassed Tyrode buffer B (2 ml/min). The column is closed and eluted with degassed Tyrode buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4×10^8 platelets/ml with Tyrode buffer B and kept at room temperature until the start of the test.

11.1.8.2 Experimental Course

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt). The total volume of each incubation sample is 500 µl. The concentration of ^{125}I-fibrinogen is constant for all samples (10 µg/500 µl).

11.1.8.3 Competition Experiments

The competition reaction is characterized by one buffer value (bidistilled water) and various concentrations of nonlabeled fibrinogen or test compound.

- 100 µl ^{125}I-fibrinogen
- 100 µl nonlabeled fibrinogen or test drug (various concentrations, 10^{-10}–10^{-3} M)
- 50 µl ADP

Nonspecific binding: The nonspecific binding of ^{125}I-fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of nonlabeled fibrinogen. The binding reaction is started by adding 250 µl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 µl aliquot of the incubation sample is transferred to a Microtainer tube containing 400 µl glucose solution. The tubes are centrifuged at 11,750 rpm for 2 min to separate ^{125}I-fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radioligand. The supernatant is carefully decanted and is allowed to run off for approximately 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3%.

11.1.8.4 Materials and Solutions

Solutions for platelet preparation		
Stock solution I	Citrate	0.8%
	Sodium citrate	2.2%
Stock solution II	NaCl	120 mM
	KCl	2.8 mM
	NaH_2PO_4	10.0 mM
	HEPES	10.0 mM
ACD buffer	Stock solution	
	+ Glucose	2.45%
	+ Hirudin	0.06 U/ml
Tyrode buffer A	Stock solution II	
	+ $NaHCO_3$	12.0 mM
Tyrode buffer B	Stock solution II	
	+ $NaHCO_3$	12 mM
	+ Glucose	5.5 mM
	+ Bovine albumin	0.35%
Tyrode buffers A and B are degassed by aspiration for approximately 1 h after setting the pH to 7.2		
Tyrode buffer C	Tyrode buffer B	
	(degassed)	
	+ Apyrase	40 µg/ml
	+ Hirudin	0.06 U/ml
Chromatography column	Acryl glass column (200×170 mm, 30-mm diameter), closed with three perlon filters, pore sizes 63, 90, and 230 µm, and gauze 50 µm filled with degassed Sepharose CL2B suspension (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode buffer A (2 ml/min)	
Incubation buffer		
Stock solution	NaCl	120 mM
	KCl	2.6 mM
	NaH_2PO_4	0.3 mM
	HEPES	10.0 mM
	$CaCl_2$	0.5 mM
Incubation buffer, pH 7.2	Stock solution	
	+ $NaHCO_3$	12 mM
	+ Glucose	5.5 mM
	+ Human albumin	0.35%
Glucose solution (in incubation buffer)		
Radioligand	^{125}I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 µCi/mg fibrinogen) (Amersham), 1 mg radiolabeled fibrinogen is dissolved in 10 ml incubation buffer	
Nonlabeled fibrinogen (mw 340,000, grade L, Sigma; in bidistilled water)		10^{-10}–10^{-3} M
ADP (in incubation buffer)		10 µM
Gamma counter (1282 Compugamma CS, LKB)		

EVALUATION

The quantity of the specific ^{125}I-fibrinogen binding results from the difference between the total and the nonspecific binding.

Platelet glycoprotein IIb-IIIa receptor binding is given as fmol ^{125}I-fibrinogen/10^8 platelets or ^{125}I-fibrinogen molecules bound per platelet.

The dissociation constant (Ki) of the test drug is determined from the competition experiment of ^{125}I-fibrinogen versus nonlabeled drug by a computer-supported analysis of the binding data:

$$K = \frac{K_D{}^{125}\mathrm{I} \times IC_{50}}{K_D{}^{125}\mathrm{I} + [^{125}\mathrm{I}]},$$

where IC_{50} = concentration of the test drug, which displaces 50% of the specifically glycoprotein IIb-IIIa receptor bound ^{125}I-fibrinogen in the competition experiment.

[^{125}I] = concentration of ^{125}I-fibrinogen in the competition experiment.

KD^{125}I = dissociation constant of ^{125}I-fibrinogen, determined from the saturation experiment.

The K_i value of the test drug is the concentration, at which 50% of the fibrinogen receptors are occupied by the test drug.

For detailed methodology and evaluations of various mechanisms and agents, see the following selected references: Bennett and Vilaire (1979); Marguerie et al. (1979, 1980); and Mendelsohn et al. (1990).

References and Further Reading

Bennett JS, Vilaire G (1979) Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393–1401

Kornecki E, Niewiarowski S, Morinelli TA, Kloczewiak M (1981) Effects of chymotrypsin and adenosine diphosphate on the exposure of fibrinogen receptors on normal human and Glanzmann's thrombasthenic platelets. J Biol Chem 256:5696–5701

Marguerie GA, Edgington TS, Plow EF (1980) Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. J Biol Chem 255:154–161

Marguerie GA, Plow EF, Edgington TS (1979) Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254:5357–5363

Mendelsohn ME, O'Neill S, George D, Loscalz o J (1990) Inhibition of fibrinogen binding to human platelets by *S*-nitroso-*N*-acetylcysteine. J Biol Chem 265:19028–19034

11.1.9 Euglobulin Clot Lysis Time

PURPOSE AND RATIONALE

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood according to Gallimore et al. (1971). The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator, and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e.g., plasminogen activators). Thus, compounds can be detected that stimulate the release of tissue-type plasminogen activator from the vessel wall.

PROCEDURE

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37°C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intraperitoneally. Twenty-five minutes later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

11.1.9.1 Plasma Preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a midline excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8% sodium citrate solution. The sample is thoroughly mixed, transferred to a plastic tube, and immediately immersed in ice. Plasma is prepared by centrifugation at 2,000 g for 10 min at 2°C.

11.1.9.2 Euglobulin Preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1% acetic acid. The diluted plasma is kept on ice for 10 min, and the precipitated euglobulin fraction is collected by centrifugation at 2,000 g for 10 min at 2°C. The supernatant is discharged, and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

11.1.9.3 Euglobulin Lysis Assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behring Werke) (25 U/ml) are added. The tubes are transferred to a water bath at 37°C. The time interval between the addition of thrombin and the complete lysis of the clots is measured.

EVALUATION

The lysis time [min] is determined. ELT is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage groups as compared to controls.

Statistical evaluation is performed by means of the Student's *t*-test.

References and Further Reading

Gallimore MJ, Tyler HM, Shaw JTB (1971) The measurement of fibrinolysis in the rat. Thromb Diath Haem 26:295–310

11.1.10 Flow Behavior of Erythrocytes

PURPOSE AND RATIONALE

The deformation of erythrocytes is an important rheological phenomenon in blood circulation according to Teitel (1977). It allows the passage of normal red cells

through capillaries with diameters smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to two basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

- Addition of calcium ions (increase in erythrocyte rigidity)
- Addition of lactic acid (decrease in pH value)
- Addition of 350–400 mmol NaCl (hyperosmolarity)
- Storing the sample for at least 4 h (cellular aging, depletion of ADP)

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

PROCEDURE

11.1.10.1 Apparatus

Erythrocyte filtrometer MF 4 (Fa. Myrenne, 52159 Roetgen, Germany)

Membrane filter (Nuclepore Corp.) pore diameter: 5–10 μm, pore density: 4×10^5 pores/cm^2

11.1.10.2 Ex Vivo

Blood is collected from Beagle dogs, weighing 12–20 kg; from rabbits, weighing 1.2–2.5 kg; or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

11.1.10.3 In Vitro

Following addition of the test compound, blood is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA, (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at 3,000 rpm for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25% human albumin, and the hematocrit value is fixed at 10%. The red blood cells are altered by one or several of the stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the filtrometer, and the initial flow rate is determined. The filtration curve is plotted automatically.

EVALUATION

The cumulative volume of the filtered suspension is recorded per time unit (10 min).

The slope of the curve is determined at different time intervals.

The initial flow rate (10% of the cell suspension having passed the filter) is recorded.

11.1.10.4 Statistics

Data of each set are first tested for normal distribution using the Kolmogorov-Smirnov test. The normal distribution hypothesis is eliminated if the data having a significance level of 5% are not normally distributed. In case that both data sets to be compared are normally distributed, the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5%. The *t*-test for paired and nonpaired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the *U*-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; *U*-test = Wilcoxon–Mann–Whitney or Mann–Whitney test, respectively).

References and Further Reading

Teitel P (1977) Basic principles of the "Filterability test" (FT) and analysis of erythrocyte flow behavior. Blood Cells 3:55–70

11.1.11 Filterability of Erythrocytes

PURPOSE AND RATIONALE

The single erythrocyte rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a synthetic membrane are determined according to Kiesewetter et al. (1982a), Roggenkamp et al. (1983), and Seiffge and Behr (1986). The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is

produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore, the current is interrupted. The test is used to detect compounds that improve filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified either by one or by a combination of the following stress factors:

- Addition of calcium ions (increase in erythrocyte rigidity)
- Addition of lactic acid (decrease in pH value)
- Addition of 350–400 mmol NaCl (hyperosmolarity)
- Storing the sample for at least 4 h (cellular aging, depletion of ADP)

PROCEDURE

11.1.11.1 Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany)

Data

Driving pressure: $dp = 70$ Pa (dog, rabbit, rat), $dp = 100$ Pa (man)

Wall shear stress: $\tau = 3$ Pa

Single pore membrane: length: 30 μm, Diameter: 3.5 μm (rat), 4.0 μm (rabbit, dog), 4.5 μm (man)

11.1.11.2 Ex Vivo

Blood is collected from Beagle dogs, weighing 12–20 kg; from rabbits, weighing 1.2–2.5 kg; from Wistar rats, weighing 150–300 g; or from man. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

11.1.11.3 In Vitro

Following addition of the test compound, the blood samples are incubated at 37°C for 5 or 30 min. The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at 3,000 rpm for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in filtrated HEPES-buffer containing 0.25% human albumin, and the hematocrit value is fixed to <1%. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device, and the passage time of a population of 250 erythrocytes (tm) is determined. Cells remaining in the pore for more than 100 ms (tm >100 ms) lead to a rheological occlusion. Untreated red blood cell suspensions serve as control.

EVALUATION

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes are determined.

11.1.11.4 Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

References and Further Reading

Kiesewetter H, Dauer M, Teitel P, Schmid-Schoenbein H, Trapp R (1982) The single erythrocyte rigidometer (SER) as a reference for RBC deformability. Biorheology 19:737–753

Roggenkamp HG, Jung F, Kiesewetter H (1983) Ein Gerät zur elektrischen Messung der Verformbarkeit von Erythrocyten. A device for the electrical measurement of the deformability of red blood cells. Biomed Techn 28:100–104

Seiffge D, Behr S (1986) Passage time of red blood cells in the SER; their distribution and influences of various extrinsic and intrinsic factors. Clin Hemorheol 6:151–164

11.1.12 Erythrocyte Aggregation

PURPOSE AND RATIONALE

The aggregation of red blood cells into rouleaux and from rouleaux into three-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood especially in disturbed microcirculation. In the following procedure, an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/plate configuration) is transilluminated by light of a defined wavelength. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation according to Kiesewetter et al. (1982b) and Schmid-Schoenbein et al. (1973).

PROCEDURE

11.1.12.1 Apparatus

Selective erythrocyte rigidometer (Fa. Myrenne, 52159 Roetgen, Germany)

11.1.12.2 Ex Vivo

Blood is collected from Beagle dogs, weighing 12–20 kg; from rabbits, weighing 1.2–2.5 kg; or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

11.1.12.3 In Vitro

Following addition of the test compound, the blood sample is incubated at 37°C for 5 or 30 min. Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood of 40% hematocrit. A sample of 40 µl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis, and the extent of erythrocyte aggregation is determined photometrically.

EVALUATION

11.1.12.4 Statistics

The statistical significance is evaluated according to the procedure for flow behavior of erythrocytes described above.

References and Further Reading

Kiesewetter H, Radtke H, Schneider R, Mußler K, Scheffler A, Schmid-Schönbein H (1982) Das Mini-Erythrozyten-Aggregometer: Ein neues Gerät zur schnellen Quantifizierung des Ausmaßes der Erythrocytenaggregation. Mini Erythrocyte Aggregometer: a new apparatus for rapid quantification of the extent of erythrocyte aggregation. Biomed Tech 27:209–213

Schmid-Schoenbein H, Gosen J, Heinich L, Klose HJ, Volger E (1973) A counter-rotating "Rheoscope chamber" for the study of the microrheology of blood cell aggregation by microscopic observation and microphotometry. Microvasc Res 6:366–376

11.1.13 Determination of Plasma Viscosity

PURPOSE AND RATIONALE

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice according to Harkness (1971). In this test, a defined volume of plasma is transferred into a capillary viscometer, and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either ex vivo or in vitro.

PROCEDURE

11.1.13.1 Ex Vivo

Beagle dogs weighing 12–20 kg, rabbits weighing 2.0–3.0 kg, or Wistar rats weighing 150–300 kg of either sex are used as test animals. Likewise, the test procedure can be performed in man. The test subjects receive the test compound by oral, subcutaneous, or intravenous administration 15, 60, 90, or 180 min before the withdrawal of blood.

11.1.13.2 In Vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ml blood K^+−EDTA or heparin sodium (5 IU/ml blood) and centrifuged at 3,000 rpm for 5 min. The supernatant (plasma) is removed, and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr. LTD, England) provided with a glass capillary of 0.5-mm inside diameter. The temperature during measurement is 37°C. The flow time t, required for the plasma to flow through the capillary, is measured. Untreated plasma serves as control.

EVALUATION

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho,$$

where

η = viscosity of plasma

K = calibration constant of viscometer

t = flow time of 0.9 ml plasma
ρ = density of plasma.

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's t-test.

References and Further Reading

Harkness J (1971) The viscosity of human blood plasma; its measurement in health and disease. Biorheology 8:171–179

11.2 In Vitro Models of Thrombosis

PURPOSE AND RATIONALE

There is abundant evidence suggesting that platelets play a pivotal role in the pathogenesis of arterial thrombotic disorders, including unstable angina (UA), myocardial infarction (MI), and stroke. The underlying pathophysiological mechanism of these processes has been recognized as the disruption or erosion of a vulnerable atherosclerotic plaque leading to local platelet adhesion and subsequent formation of partially or completely occlusive platelet thrombi.

The specific platelet surface receptors that support these initial adhesive interactions are determined by the local fluid dynamic conditions of the vasculature and the extracellular matrix constituents exposed at the sites of vascular injury. Konstantopoulos et al. (1998) and Alevriadou et al. (1993) demonstrated that under high shear conditions, the adhesion of platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is primarily mediated by the platelet glycoprotein (GP)Ib/IX/V complex. This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa. In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels as shown by Turitto (1982), may be important. Shear-induced platelet aggregation is dependent upon the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation that also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.

A variety of methods have been utilized to assess the ex vivo and/or in vitro efficacy of platelet antagonists, including photometric aggregometry, whole blood electrical aggregometry, and particle counter methods as described in the above segments. In photometric aggregometry, a sample is placed in a stirred cuvette in the optical light path between a light source and a light detector. Aggregate formation is monitored by a decrease in turbidity, and the extent of aggregation is measured as percent of maximal light transmission. The major disadvantage of this technique is that it cannot be applied in whole blood since the presence of erythrocytes interferes with the optical responses. Furthermore, it is insensitive to the formation of small aggregates. Particle counters are used to quantitate the size and the number of particles suspended in an electrolyte solution by monitoring the electrical current between two electrodes immersed in the solution. Aggregation in this system is quantitated by counting the platelets before and after stimulation and is usually expressed as a percentage of the initial count as shown by Jen and McIntire (1984). However, the disadvantage of this technique is that it cannot distinguish platelets and platelet aggregates from other blood cells of the same size. Thus, one is limited to counting only a fraction of single platelets, as well as aggregates that are much larger than erythrocytes and leukocytes. The technique of electrical aggregometry allows the detection of platelet aggregates as they attach to electrodes immersed in a stirred cuvette of whole blood or platelet suspensions. Such an attachment results in a decrease in conductance between the two electrodes that can be quantitated in units of electrical resistance. However, a disadvantage of this method is that it is not sensitive in the detection of small aggregates as demonstrated by Sweeney et al. (1989).

This segment discusses two complementary in vitro flow models of thrombosis that can be used to accurately quantify platelet aggregation in anticoagulated whole blood specimens and evaluate the inhibitory efficacy of platelet antagonists. (1) A viscometric-flow cytometric assay to measure direct shear-induced platelet aggregation in the bulk phase as demonstrated by Konstantopoulos et al. (1995) and (2) a parallel-plate

perfusion chamber coupled with a computerized videomicroscopy system to quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood flowing over an immobilized substrate (e.g., collagen I) as shown by Konstantopoulos et al. (1995) and Mousa et al. (2002). Furthermore, Mousa et al. (2002) demonstrated a third in vitro flow assay in which surface-anchored platelets are preincubated with a GPIIb/IIIa antagonist, and unbound drug is washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling us to distinguish agents with markedly distinct affinities and receptor-bound lifetimes.

PROCEDURES

11.2.1 Isolation of Human Platelets

The steps described in the next subheading outline the procedure for isolation and purification of platelets from whole blood obtained by venipuncture from human volunteers. Obtain blood sample by venipuncture from an antecubital vein into polypropylene syringes containing either sodium citrate (0.38% final concentration) or heparin (10 U/ml final concentration). Centrifuge anticoagulated whole blood at 160 g for 15 min to prepare platelet-rich plasma (PRP).

11.2.2 Isolation of Washed Platelets

PRP specimens are subjected to a further centrifugation (1,100 g for 15 min) in the presence of 2 μM PGE_1 (Evangelista et al. 1996).

1. The platelet pellet is resuspended in HEPES-Tyrode buffer containing 5 mM EGTA and 2 μM PGE_1.
2. Platelets are then washed via centrifugation (1,100 g for 10 min), resuspended at 2×10^8/ml in HEPES-Tyrode buffer, and kept at room temperature for no longer than 4 h before use in aggregation/adhesion assays.

11.2.3 Materials

1. Anticoagulant solution (sodium citrate, porcine heparin, PPACK, etc.)
2. Fluorescently labeled platelet-specific antibody
3. Dulbecco phosphate-buffered saline (D-PBS) (with and without Ca^{2+}/Mg^{2+})
4. Formaldehyde
5. Type I collagen, from bovine Achilles tendon
6. 0.5 mol/L glacial acetic acid in water
7. Glass coverslips (24×50 mm; Corning; Corning, NY)
8. Silicone sheeting (gasket) (0.005-in or 0.010-in thickness; Specialty Manufacturing Inc; Saginaw, MI)
9. Quinacrine dihydrochloride
10. Prostaglandin E_1 (PGE_1) and EGTA
11. Thrombin
12. Bovine serum albumin
13. HEPES-Tyrode buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM K_2PO_4, 0.8 mM $MgCl_2{\bullet}6H_2O$, 1 mM $CaCl_2$, 10 mM HEPES, 5.6 mM dextrose)
14. 3-Aminopropyltriethoxysilane (APES)
15. Acetone
16. 70% nitric acid in water
17. THP-1 monocytic cells
18. Platelet antagonists such as abciximab

EVALUATIONS

The methods described below outline three dynamic adhesion/aggregation assays used to assess the in vitro and/or ex vivo efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure shear-induced platelet-platelet aggregation in the bulk phase and (2) a perfusion chamber coupled with a computerized videomicroscopy system to visualize in real time and quantify (a) the adhesion and subsequent aggregation of platelets flowing over an immobilized substrate (e.g., extracellular matrix protein) and (b) free-flowing monocytic cell adhesion to immobilized platelets.

References and Further Reading

Alevriadou BR, Moake JL, Turner NA, Ruggeri ZM, Folie BJ, Phillips MD, Schreiber AB, Hrinda ME, McIntire LV (1993) Real-time analysis of shear dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. Blood 81:1263–1276

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conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta2 integrin CD11b/CD18. Blood 88:4183–4194
Jen CJ, McIntire LV (1984) Characteristics of shear-induced aggregation in whole blood. J Lab Clin Med 103:115–124
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Konstantopoulos K, Kukreti S, McIntire LV (1998) Biomechanics of cell interactions in shear fields. Advanced Drug Delivery Reviews 33:141–164
Mousa SA, Abulencia JP, McCarty OJ, Turner NA, Konstantopoulos K (2002) Comparative efficacy between glycoprotein IIb/IIIa antagonists roxifiban and orbofiban in inhibiting platelet responses in flow models of thrombosis. J Cardiovasc Pharmacol 39: 552–560
Sweeney JD, Labuzzetta JW, Michielson CE, Fitzpatrick JE (1989) Whole blood aggregation using impedance and particle counter methods. Am J Clin Pathol 92:794–797
Turitto VT (1982) Blood viscosity, mass transport, and thrombogenesis. Prog Hemost Thromb 6:139–177

11.2.4 Cone-and-Plate Viscometry Under Shear Flow Cytometry

PURPOSE AND RATIONALE

The cone-and-plate viscometer is an in vitro flow model used to investigate the effects of bulk fluid shear stress on suspended cells. Anticoagulated whole blood specimens (or isolated cell suspensions) are placed between the two platens (both of stainless steel) of the viscometer. Rotation of the upper conical platen causes a well-defined and uniform shearing stress to be applied to the entire fluid medium as described by Konstantopoulos et al. (1998). The shear rate (γ) in this system can be readily calculated from the cone angle and the speed of the cone using the formula:

$$\gamma = \left(\frac{2\pi\omega}{60\theta_{cp}}\right)$$

where γ is the shear rate in sec^{-1}, ω is the cone rotational rate in revolutions per minute (rev/min), and θ_{cp} is the cone angle in radians. The latter is typically in the range of 0.3–1.0. The shear stress, τ, is proportional to shear rate, γ, as shown by: $\tau = \mu \bullet \gamma$, where μ is the viscosity of the cell suspension (the viscosity of anticoagulated whole blood is ~0.04 cp at 37°C). This type of rotational viscometer is capable of generating shear stresses from ~2 dyn/cm^2 (venous level) to greater than 200 dyn/cm^2 (stenotic arteries).

PROCEDURES

Single platelets and platelet aggregates generated upon shear exposure of blood specimens are differentiated from other blood cells on the basis of their characteristic forward scatter and fluorescence (by the use of fluorophore-conjugated platelet-specific antibodies) profiles by flow cytometry as described by Konstantopoulos et al. (1995). This technique requires no washing or centrifugation steps that may induce art factual platelet activation and allows the study of platelet function in the presence of other blood elements. Konstantopoulos et al. (1995) described the procedure used to quantify platelet aggregation induced by shear stress as follows: *Incubate anticoagulated whole blood with platelet antagonist or vehicle (control) at 37 ℃ for 10 min*.

1. Place a blood specimen (typically ~500 μl) on the stationary platen of a cone-and-plate viscometer maintained at 37°C.
2. Take a small aliquot (~3 μl) from the presheared blood sample, fix it with 1% formaldehyde in D-PBS (~30 μl), and process it as outlined in steps 6–8.
3. Expose the blood specimen, in the presence or absence of a platelet antagonist, to well-defined shear levels (typically 4,000 s^{-1} to induce significant platelet aggregation in the absence of a platelet antagonist) for prescribed periods of time (typically 30–60 s).
4. Take a small aliquot (~3 μl) from the sheared blood specimen and immediately fix it with 1% formaldehyde in D-PBS (~30 μl).
5. Incubate the fixed blood samples with a saturating concentration of a fluorescently labeled platelet-specific antibody, such as anti-GPIb(6D1)-FITC, for 30 min in the dark.
6. Dilute specimens with 2 ml of 1% formaldehyde and analyze them by flow cytometry.
7. Flow cytometric analysis is used to distinguish platelets from other blood cells on the basis of their characteristic forward scatter and fluorescence profiles, as shown in Fig. 11.1. Data acquisition is then carried out on each sample for a set period (usually 100 s), thereby allowing equal volumes for both the presheared and sheared specimens to

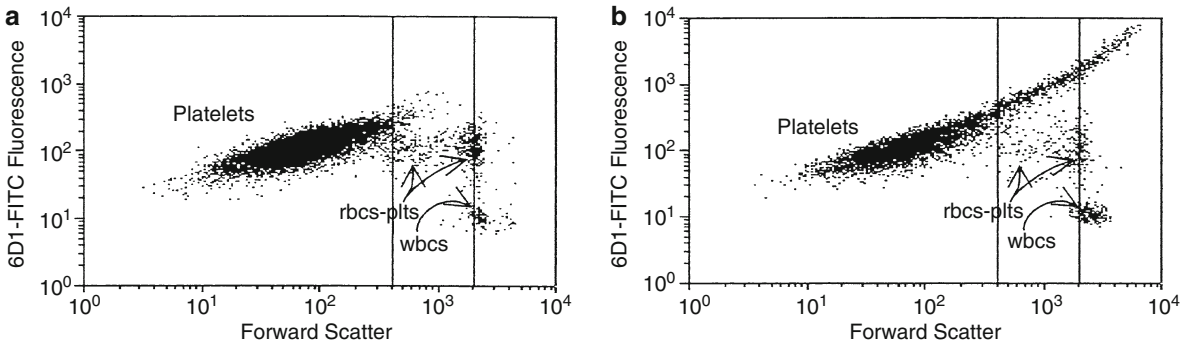

Fig. 11.1 Quantification of shear-induced platelet aggregation by flow cytometry. Panel a corresponds to an unsheared blood specimen. Panel b corresponds to a blood specimen that has been subjected to a pathologically high level of shear stress for 30 s. As can be seen in the figure, there are three distinct cell populations. The upper population consists of platelets and platelet aggregates. The "rbcs-plts" population corresponds to platelets associated with erythrocytes and leukocytes. The "wbcs" population consists of some leukocytes that have elevated levels of FITC autofluorescence. The left vertical line separates single platelets ($\leq$4.5 μm in diameter) from platelet aggregates, whereas the right vertical line separates "small" from "large" platelet aggregates. The latter were defined to be larger than 10 μm in equivalent sphere diameter

be achieved. As a result, the percent platelet aggregation can be determined by the disappearance of single platelets into the platelet aggregate region using the formula:

% Platelet aggregation = (1−Ns/Nc×100), where Ns represents the single platelet population of the sheared specimen and Nc represents the single platelet population of the presheared specimen. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be readily determined.

References and Further Reading

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Konstantopoulos K, Kukreti S, McIntire LV (1998) Biomechanics of cell interactions in shear fields. Adv Drug Delivery Rev 33:141–164

11.2.5 Platelet Adhesion and Aggregation Under Dynamic Shear

PURPOSE AND RATIONALE

The steps described and outlined an in vitro flow model of platelet thrombus formation, which can be used to evaluate the ex vivo and/or in vitro efficacy of platelet antagonists. Thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and vWF, with subsequent platelet activation and aggregation. Konstantopoulos et al. (1995) described the use of a parallel-plate flow chamber that provides a controlled and well-defined flow environment based on the chamber geometry and the flow rate through the chamber. The wall shear stress, τ_w, assuming a Newtonian and incompressible fluid, can be calculated using the formula:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, and w is the channel width. A flow chamber typically consists of a transparent polycarbonate block, a gasket whose thickness determines the channel depth, and a glass coverslip coated with an extracellular matrix protein such as type I fibrillar collagen. The apparatus is held together by vacuum. Shear stress is generated by flowing fluid (e.g., anticoagulated whole blood or isolated cell suspensions) through the chamber over the immobilized substrate under controlled kinematic conditions using a syringe pump. Mousa et al. (2002) combined the parallel-plate flow chamber with a computerized epifluorescence videomicroscopy system, enabling us to visualize in real time and separately

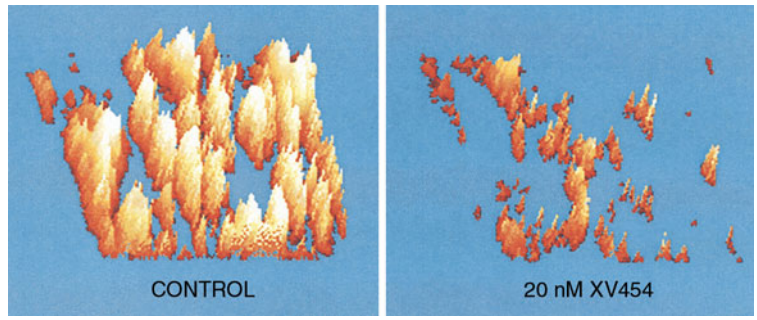

Fig. 11.2 Three-dimensional computer-generated representation of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused in the absence (control) or presence of a GPIIb/IIIa antagonist (XV454) at 37°C for 1 min at 1,500 s^{-1}

quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood (or isolated platelet suspensions) flowing over an immobilized substrate.

PROCEDURES

Preparation of collagen-coated surfaces (Folie et al. 1988)

1. Dissolve 500 mg collagen type I from bovine Achilles tendon into 200 ml of 0.5 mol/L acetic acid in water, pH 2.8.
2. Homogenize for 3 h.
3. Centrifuge the homogenate at 200 g for 10 min, collect supernatant, and measure collagen concentration by a modified Lowry analysis.
4. Coat glass coverslips with 200 µl of fibrillar collagen I suspension on all but first 10 mm of the slide length (coated area = 12.7×23) and place in a humid environment at 37°C for 45 min.
5. Rinse excess collagen with 10 ml of D-PBS maintained at 37°C before assembly into the flow chamber.

Platelet Perfusion Studies

1. Add the fluorescent dye quinacrine dihydrochloride to anticoagulated whole blood samples at a final concentration of 10 µM immediately after blood collection.
2. Prior to the perfusion experiment, incubate blood with either a platelet antagonist or vehicle (control) at 37°C for 10 min.

Perfuse anticoagulated whole blood through the flow chamber for 1 min at wall shear rates ranging from 100 s^{-1} (typical of venous circulation) to 1,500 s^{-1} (mimicking partially constricted arteries) for prescribed periods of time (e.g., 1 min). Platelet-substrate interactions are monitored in real time using an inverted microscope equipped with an epifluorescent illumination attachment and silicon-intensified target video camera and recorded on videotape. The microscope stage and flow chamber are maintained at 37°C by an incubator heating module and incubator enclosure during the experiment.

3. Videotaped images are digitized and computer analyzed at 5, 15, and 60 s for each perfusion experiment. The number of adherent individual platelets in the microscopic field of view during the initial 15 s of flow is determined by image processing and used as the measurement of platelet adhesion that initiates platelet thrombus formation. The number of platelets in each individual thrombus is calculated as the total thrombus intensity (area×fluorescence intensity) divided by the average intensity of single platelets determined in the 5-s images. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be determined (Fig. 11.2). Along these lines, any potential inhibitory effects of a platelet antagonist on platelet adhesion can be readily assessed.

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11.2.6 Cell Adhesion to Immobilized Platelets: Parallel-Plate Flow Chamber

PURPOSE AND RATIONALE

In this assay, immobilized platelets are pretreated with a GPIIb/IIIa antagonist, and any unbound drug is washed away before the perfusion of monocytic THP-1 cells. Agents with slow platelet off-rates such as XV454 ($t_{1/2}$ of dissociation = 110 min; K_d = 1 nM) and abciximab ($t_{1/2}$ of dissociation = 40 min; K_d = 9.0 nM) that are distributed predominantly as receptor-bound entities with little unbound in the plasma can effectively block these heterotypic interactions as shown by Abulencia et al. (2001) and by Mousa et al. (2002). In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation = 0.2 min; K_d >110 nM), whose antiplatelet efficacy depend on the plasma concentration of the active drug, do not exhibit any inhibitory effects as described by Mousa et al. (2002).

PROCEDURES

Preparation of 3-aminopropyltriethoxysilane Treated Glass Slides

1. Soak glass coverslips overnight in 70% nitric acid.
2. Wash coverslips with tap water for 4 h.
3. Dry coverslips by washing once with acetone followed by immersion in a 4% solution of APES in acetone for 2 min.
4. Repeat step 3 followed by a final rinse of the glass coverslips with acetone.
5. Wash coverslips three times with water and allow them to dry overnight.

Immobilization of Platelets on 3-aminopropyltriethoxysilane Treated Glass Slides

1. Layer washed platelets or PRP (2×10^8 cells/ml) on the surface of a coverslip at ~30 μl/cm^2.
2. Allow platelets to bind to APES-treated cover slip in a humid environment at 37°C for 30 min.

Monocytic THP-1 Cell-Platelet Adhesion Assay

1. Assemble the platelet-coated cover slip on a parallel-plate flow chamber that is then mounted on the stage of an inverted microscope equipped with a CCD camera connected to a VCR and TV monitor.
2. Perfuse the antiplatelet antagonist at the desirable concentration or vehicle (control) over surface-bound platelets and incubate for 10 min. The extent of platelet activation can be further modulated by the presence of chemical agonists such as thrombin (0.02–2 U/ml) during the 10-min incubation. The microscope stage and flow chamber are maintained at 37°C by an incubator heating module and incubator enclosure during the experiment.
3. In some experiments, unbound platelet antagonist is removed by a brief washing step (4 min) prior to the perfusion of the cells of interest over the platelet layer. In others, the desirable concentration of the platelet antagonist is continuously maintained in the perfusion buffer during the entire course of the experiment.
4. Perfuse cells (e.g., THP-1 monocytic cells, leukocytes, tumor cells, protein-coated beads, etc.) over surface-bound platelets, either in the presence or absence of a platelet antagonist (see above), at the desirable flow rate for prescribed periods of time. THP-1 cell binding to immobilized platelets is monitored in real time and recorded on videotape.
5. Determine the extent of THP-1 cell tethering, rolling, and stationary adhesion to immobilized platelets as well as the average velocity of rolling THP-1 cells. By comparing the corresponding extents of THP-1 cell tethering, rolling, and stationary adhesion to immobilized platelets in the presence and absence of a platelet antagonist (Fig. 11.3), its antiplatelet efficacy can be determined.

EVALUATIONS

1. Low-speed centrifugation results in the separation of platelets (top layer) from larger and denser cells such as leukocytes and erythrocytes (bottom layer). To minimize leukocyte contamination in PRP specimens, slowly aspirate the uppermost 2/3 of the platelet layer. Furthermore, certain rare platelet disorders, such as Bernard-Soulier Syndrome (BSS), are characterized by larger than normal platelets that must therefore be isolated by allowing whole blood to gravity separate for 2-h post venipuncture.
2. The mechanical force most relevant to platelet-mediated thrombosis is shear stress. The normal time-averaged levels of venous and arterial shear stresses range between 1–5 and 6–40 dyn/cm^2, respectively. However, fluid shear stress may reach levels well over 200 dyn/cm^2 in small arteries and arterioles partially obstructed by atherosclerosis or vascular spasm. The cone-and-plate viscometer and

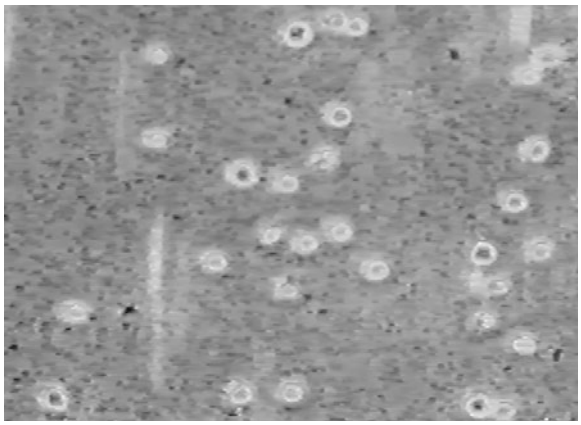

Fig. 11.3 Phase-contrast photomicrograph of THP-1 cells (phase bright objects) attached to a layer of thrombin-treated platelets (phase dark objects) after THP-1 cell perfusion for 3 min at a shear stress level of 1.5 dyn/cm^2

parallel-plate flow chamber are two of the most common devices used to simulate fluid mechanical shearing stress conditions in blood vessels.

3. Due to the large concentration of platelets and erythrocytes in whole blood, small aliquots (~3 μl) of presheared and postsheared specimens must be obtained and processed prior to the flow cytometric analysis. This will minimize an artifact produced as a platelet and an erythrocyte pass through the light beam of a flow cytometer at the same time.
4. The "rbcs-plts" population represents 3–5% of the displayed cells. A small fraction (~5%) of this population seems to be leukocyte-platelet aggregates as evidenced by the use of an anti-CD45 monoclonal antibody. The remaining events correspond to erythrocytes associated with platelets. However, it appears that the majority of the latter population is an artifact generated by the simultaneous passage of a platelet and an erythrocyte through the beam of a flow cytometer. This concept is corroborated by the fact that further dilution of presheared and sheared blood specimens and/or reduction of the sample flow rate during the flow cytometric analysis results in a dramatic relative decrease of the "rbcs-plts" population.
5. The collagen density remaining on glass coverslips after D-PBS rinsing can be measured by the difference in weight of 20 clean uncoated slides versus 20 collagen-treated slides.
6. Experiments are optimally monitored ~100–200 μm downstream from the collagen/glass interface using a 60×FLUOR objective and 1× projection lens, which gives a 3.2×10^4 μm^2 field of view. A field of view closer to the interface may lead to nonreproducible results due to variations in the collagen layering in that region. In contrast, positions farther downstream are avoided in order to minimize the effects of upstream platelet adhesion and subsequent aggregation on both the fluid dynamic environment as well as bulk platelet concentration.
7. The digitization of a background image (at the onset of perfusion prior to platelet adhesion to the collagen I surface) and its subtraction from a subsequent image acquired 5 s after an initial platelet adhesion event allows the determination of the fluorescence intensity emitted by a single platelet. The intensity level of each single platelet is measured as a mean gray level between 0 (black) and 255 (white) through the use of an image processing software (e.g., OPTIMAS; Agris-Schoen Vision Systems, Alexandria, VA) and is multiplied by its corresponding area (total number of pixels covered by each single platelet). The aforementioned products are then averaged for all single platelet events detected at the 5-s time point, thus enabling us to calculate the average intensity of single platelets.
8. A single field of view (10×0.55 mm^2) is monitored during the 3-min period of the experiment, and at the end, five additional fields of view (0.55 mm^2) are monitored for 15 s each. The following parameters can be quantified: (a) the number of total interacting cells per mm^2 during the entire 3-min perfusion experiment, (b) the number of stationary interacting cells per mm^2 after 3 min of shear flow, (c) the percentage of total interacting cells that are stationary after 3 min of shear flow, and (d) the average rolling velocity (μm/s) of interacting cells. The number of interacting cells per mm^2 is determined manually by reviewing the videotapes. Stationary interacting cells per mm^2 are considered as those that move <1-cell radius within 10 s at the end of the 3-min attachment assay. To quantify their number, images can be digitized from a videotape recorder using an imaging software package

(e.g., OPTIMAS). Rolling velocities can be computed as the distance traveled by the centroid of the rolling THP-1 cell divided by the time interval using image processing.

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11.3 In Vivo or Ex Vivo Models

PURPOSE AND RATIONALE

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of blood flow, changes in the properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two, or all three factors of the Virchow triad. Therefore, they differ with respect to the prothrombotic challenge—either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, laser light), insertion of foreign surface, or injection of a prothrombotic factor—with respect to the vessel type and with respect to the animal species.

Roughly, two types of models can be differentiated (Didisheim 1972): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor resulting in fibrin-rich "red" venous type thrombi and (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis resulting in platelet rich "white" mural thrombi. But the differentiation is not strict because platelets and coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in humans namely, atherosclerosis or thrombophilias are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. Despite these limitations, animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadley et al. (2000). Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires preclinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that the second- and third-generation antithrombotic drugs are devoid of in vitro anticoagulant effects, yet in vivo, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit in vitro anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models utilizing species such as rats, rabbits, dogs, pigs, and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat, and guinea pig have also been utilized. Species variations are an important consideration in selecting a model and interpreting the results as these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thrombosis have been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models have been well established and can be used for the development of antithrombotic drugs. It is also possible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of in vitro potency, the endogenous effect of antithrombotic drugs can also be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases, and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models in particular have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor antibodies relies largely on these models. These models are, however, of pivotal value in the development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human usage.

PROCEDURES

11.3.1 Animal Models of Thrombosis

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiological) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

1. *Stasis-Thrombosis Model*. Since its introduction by Wessler et al. (1959), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents. This model has also been adapted for use in rats (Meuleman et al. 1991). In the *stasis thrombosis model*, a hypercoagulable state is mimicked by administration of one of a number of thrombogenic challenges including human serum (Carrie et al. 1994), thromboplastin (Walenga et al. 1986), activated prothrombin complex concentrates (Vlasuk et al. 1991), factor Xa (Millet et al. 1994), and recombinant relipidated tissue factor (Callas et al. 1995). This administration serves to produce a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a thrombus.
2. *Models Based On Vessel Wall Damage*. The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a nonthrombogenic surface over which the blood flows. Disruption of the endothelium not only limits the beneficial effects enumerated above but also exposes subendothelial tissue factor and collagen that serve to activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means (clamping, catheter), chemical means (FITC, Rose Bengal, ferrous chloride), thermal injury, or electrolytic injury.

EVALUATIONS

Each setting in the design of an animal model can answer specific question in relation to certain thrombotic disorders in human. However, the ultimate model of human thrombosis is in human.

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11.3.2 Stenosis- and Mechanical-Injury-Induced Coronary Thrombosis (Folts Model)

PURPOSE AND RATIONALE

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction, or sudden death. Treatment with angioplasty, thrombolysis, or bypass grafts can expose new thrombogenic surfaces, and rethrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFRs) in stenosed canine coronary arteries. Uchida described a similar model in 1975. The model includes various aspects of unstable angina pectoris, i.e., critical stenosis, vascular damage, and downstream vasospasm induced by vasoconstrictors released or generated by platelets. The cyclic variations in coronary blood flow are a result of acute platelet thrombi that may occlude the vessel but that either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts et al. 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases, but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin, but the effect can be reversed by increases in catecholamines and shear forces (Folts and Rowe 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

11.3.2.1 Coronary Thrombosis Induced by Stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. These alterations in coronary blood flow, called cyclic flow reductions (CFR), are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder upon the damaged part of the vessel. In dogs, the stenosis is critical, i.e., the reactive hyperemic response to a 10-s occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i.e., there is a partial reactive hyperemia left (Just et al. 1991; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis are not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF) according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

11.3.2.2 Coronary Thrombosis Induced by Electrical Stimulation

In this preparation, coronary thrombosis is induced by delivery of low amperage electrical current to the intimal surface of the artery according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

PROCEDURE

11.3.2.3 Coronary Thrombosis Induced by Stenosis

Protocol 1: Critical Stenosis

Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approximately 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flow probe, the vessel is squeezed with a 2-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2–1.8 mm (depending on the size of the LCX), is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site. Only dogs with regularly repeated cyclic flow reductions (CFRs) of similar intensity within a pretreatment phase of 60 min are used in these experiments. The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2–4×60 min and compared to pretreatment values. Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical Stenosis

Male castrated pigs (German landrace, weighing (20–40 kg)) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.), and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericard is opened and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flow probe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flow probe, the vessel is squeezed with a 1-mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces cyclic flow reductions. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at reduction of blood flow by shortly lifting the vessel with forceps. Only pigs with regularly repeated CFRs of similar intensity within a pretreatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2×60 min and compared to pretreatment values.

Protocol 3: Stenosis+Epinephrine Infusion

If protocol 1 does not lead to CFRs, additionally epinephrine (0.2 μg/kg/min) is infused into a peripheral vein for 2×60 min (60 min before and 60 min following drug administration). CFRs are registered and compared in the 60-min postdrug phase to the 60-min predrug phase.

Protocol 4: Stenosis+PAF Infusion

The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus

formation (subcritical stenosis). For the induction of CFRs, in addition, PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into one cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty minutes later, concomitantly, the test substance is administered and a second PAF infusion is started for 30 min.

CFRs are registered and compared in the drug treated, second PAF phase, to the pre-drug, first PAF phase.

11.3.2.4 Coronary Thrombosis Induced by Electrical Stimulation

Protocol 5

The LCX is punctuated distal to the flow probe with a chrome-vanadium-steel electrode (3-mm length, 1-mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon-coated wire to a 9-V battery, a potentiometer, and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracic muscle layer to complete the electrical circuit. The intima is stimulated with 150 μA for 6 h. During this time, gradually, an occluding thrombosis is formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) is determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols, the following preparations and measurements are performed:

- To measure peripheral arterial blood pressure (BP) [mm Hg], the right femoral artery is cannulated and connected to a Statham pressure transducer.
- Left ventricular pressure (LVP) (mm Hg) is determined by inserting a microtip catheter via the carotid artery retrogradely.
- Left ventricular end diastolic pressure (LVEDP) (mm Hg) is evaluated through sensitive amplification of the LVP.
- Contractility (LV *dp*/*dt* max) (mm Hg/s) is determined from the initial slope of the LVP curve.
- Heart rate (min^{-1}) is determined from the pulsatile blood pressure curve.
- The ECG is recorded in lead II.
- Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
- Blood hematocrit values (37–40%) and number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.
- Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
- Template buckle mucosal bleeding time is carried out using the Simplate® device.

At the end of the test, animals are sacrificed by an overdose of pentobarbital sodium (Fig. 11.4).

EVALUATION

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic) [mm Hg] is determined.

Protocol 1–4

The following parameters are determined to quantify stenosis-induced coronary thrombosis:

- Frequency of cyclic flow reductions = cycle number per time
- Magnitude of cyclic flow reductions = cycle area (mm^2) (total area of all CVRs per time measured by planimetry)

Percent change in cycle number and cycle area after drug treatment is calculated compared to pretreatment controls.

Statistical significance is assessed by the paired Student's *t*-test.

Protocol 5

The following parameters are determined to quantify electrically induced coronary thrombosis:

- Occlusion time (min) = time to zero blood flow
- Thrombus size (mg) = wet weight of the thrombus immediately after removal, percent change in mean values for occlusion time, and thrombus size in drug-treated groups are compared to the control group.

Statistical significance is assessed by the nonpaired Student's *t*-test.

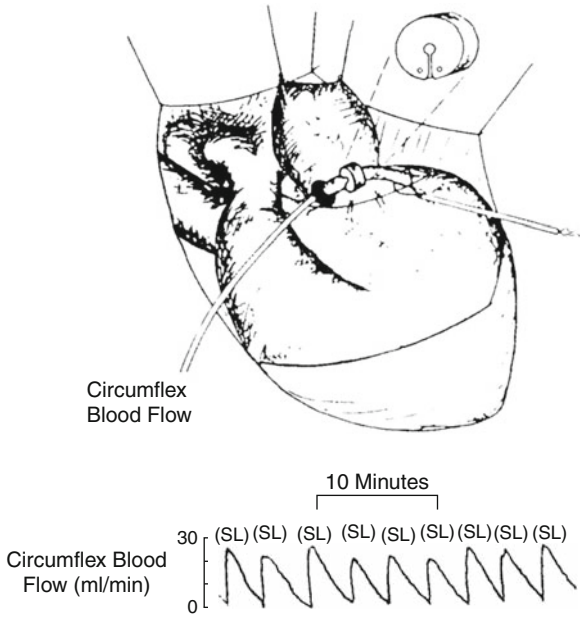

Fig. 11.4 Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes measure blood flow. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2–3 mm, the thrombus must he mechanically shaken loose to restore blood. For detailed application of the Folts model, see Folts (1991); Folts and Rowe (1974, 1988); and Folts et al. (1976, 1982)

Standard data

		% Decrease in CFVs			
Standard	Dose	Number	Area	Protocol	Species
Acetylsalicylic acid	1 mg/kg, i.v.	56	80	1	dog
	20 mg/kg, i.d.	87	95	1	dog
	30 mg/kg, i.v.	56	77	2	pig

CRITICAL ASSESSMENT

Both, the stenosis (Folts) and the electrical (Romson/Lucchesi) models of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e.g., thromboxane, prostacyclin, cyclooxigenase, serotonin, NO donors, and other vasodilators. The effect of an NO donor could be reversed by the NO scavenger oxyhemoglobin indication that indeed NO was responsible for the antithrombotic action (Just and Schönafinger 1991). Recent mechanisms of antithrombotic drug action that have been studied in either of the two coronary thrombosis models are the oral GPIIb/IIIa antagonist DMP 728 (Mousa et al. 1996), the LMWH enoxaparin (Leadley et al. 1998) that inhibited CFRs in contrast to unfractionated heparin, the thrombin-inhibitors PEG-hirudin (Ruebsamen and Kirchengast 1998) and melagatran (Mehta et al. 1998), an anti-P-selectin antibody (Ikeda et al. 1999), and an activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive, e.g., aspirin has no effect, and with some drugs higher dose levels are required; but in principle, most drug mechanisms act in both models if at all.

MODIFICATIONS OF THE METHOD

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50% increase in flow velocity, and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion. Warltier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis and the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size. Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts thrombosis has also been applied to carotid arteries in monkeys. Coller et al. (1989) induced CFRs in carotid arteries of anesthetized cynomologus monkeys and showed abolition by the GPIIb/IIIa antibody abciximab.

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11.3.3 Stenosis- and Mechanical-Injury-Induced Arterial and Venous Thrombosis: Harbauer Model

PURPOSE AND RATIONALE

Harbauer (1984) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thrombosis is produced in rabbits by stenosis of the carotid artery and the jugular vein with

simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an in vivo model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

PROCEDURE

Male Chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, intravenous, or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approximately 30 min, p.o. approximately 60 min, i.v. variable).

Sixty-five minutes before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun®) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein.

A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube. One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion.

Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow (ml/min) is measured continuously.

After reaching steady state (approximately 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching), and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approximately 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. Then, a small plastic constricting cylinder with 1.2-mm diameter and 2-mm length is placed around the site of the endothelial damage. Registration of parameters is terminated after 30 min. In addition, the template bleeding time is measured at various time intervals before and after drug treatment (depending on the route of administration) in the shaved inner ear using the Simplate® device. Care is taken to select parts of the skin without larger vessels.

EVALUATION

Percent thrombus formation (= thrombosis incidence) is judged by determination of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls.

Thrombosis incidence is always 100% in vehicle controls.

Statistical significance is assessed by means of the Fisher exact test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition, and mean values in dosed groups are compared to controls by means of the unpaired Student's *t*-test.

Mean values of occlusion times [min] in dosage and control groups are calculated and compared by means of the *t*-test.

The maximal change in systolic and diastolic blood pressure during the time period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mm Hg and of diastolic blood pressure by 20 mm Hg is quoted as a strong reduction in blood pressure.

CRITICAL ASSESSMENT OF THE METHOD

Two main factors of arterial thrombosis in men are essential in this model: high-grade stenosis and vessel wall damage. In the absence of either, no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels, thrombus formation is equally dependent on platelet function as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model (see 11.4.2) since carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: (1) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, NO donors (SNP, molsidomine) but not aspirin, and thromboxane

synthase inhibitors; (2) anticoagulants like hirudin, high-dose heparin, and warfarin; and (3) streptokinase/t-PA (Bevilacqua et al. 1991; Just 1986 and unpublished). In contrast, drugs that only lower blood pressure such as hydralazine, clonidine, and prazosin have no effect on thrombus formation in this model.

MODIFICATIONS OF THE METHOD

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH2Cl; iloprost; and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected, and the clot was removed for determination of dry weight.

Lyle et al. (1995) searched for an animal model mimicking the thrombotic reocclusion and restenosis occurring in several cases after successful coronary angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ^{111}indium-labeled platelet deposition and thrombosis were assessed 4 h after balloon injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

11.3.3.1 Thrombosis Induced by Supercooling

Meng (1975), Meng and Seuter (1977), and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized and the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove that was cooled to −15°C. The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed, and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was −12°C for a period of 5 min, and the compressing weight was 500 g. The wound was closed, and the animal was allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 h, the animals received heparin and were reanesthetized. The lesioned carotid artery was removed and thrombus wet weight was immediately measured.

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11.3.4 Electrical-Induced Thrombosis

PURPOSE AND RATIONALE

The use of electrical current to induce thrombosis in hamster and dog has been described in the early 1950s by Lutz et al. (1951) and Sawyer and Pate (1953a, b).

In general, two different approaches exist. One method produces electrical damage by means of two externally applied hook-like electrodes (Hladovec 1973; Philp et al. 1978). The other method uses a needle electrode which is advanced through the walls of the blood vessels and positioned in their lumen, the second electrode is placed into a subcutaneous site completing the circuit (Salazar 1961; Romson et al. 1980b; Benedict et al. 1986).

PROCEDURE

Anesthetized rats weighing 200–300 g are intubated, and a femoral artery is cannulated for administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm. A pair of rigid stainless-steel wire hook-like electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flow meter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
- Patency of the blood vessel over 30 min

CRITICAL ASSESSMENT OF THE METHOD

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed, and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. He concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

MODIFICATIONS OF THE METHOD

The technique described by Salazar (1961) uses a stainless steel electrode that is inserted into a coronary artery in the dog and that delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control, which complicates the method. The technique was modified by Romson et al. (1980b). They placed the electrode directly into the coronary artery of open-chest anesthetized dogs (Fig. 11.5).

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to both the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25% without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel-cadmium battery in series with a 250,000-Ω variable resistor. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by application of a 150-μA continuous pulse anodal direct current to the intimal surface of the vessel for a maximum duration of 3 h or for 30 min beyond the time of complete vessel occlusion as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50%, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 h after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996b). A 10-min

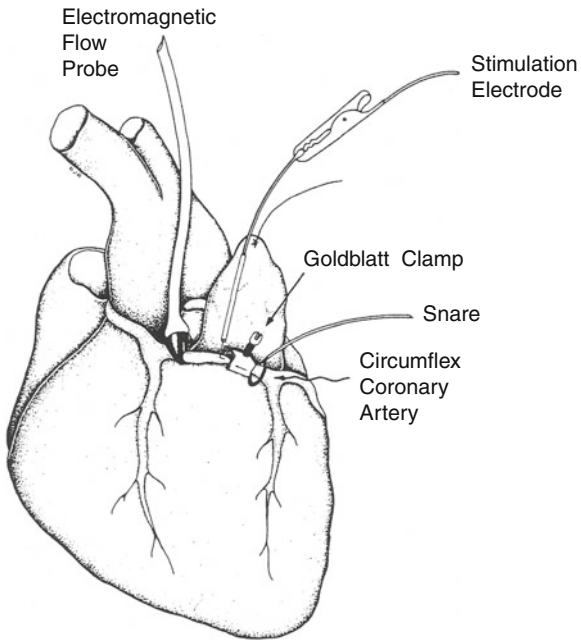

Fig. 11.5 Model of coronary artery thrombosis in the dog. Electrical injury to the intimal surface of the artery leads to occlusive thrombosis formation. The thrombosis is formed in the presence of a flow-limiting stenosis induced by a Goldblatt clamp. Upon spontaneous occlusion, heparin is administered, and the clot is aged for 1 h before initiating the t-PA infusion

anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle treated ferrets within 41 ± 3 min with an average weight of 8 ± 1 mg. Thrombus weight was reduced by aspirin or a thromboxan receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

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11.3.5 $FeCl_3$-Induced Thrombosis

PURPOSE AND RATIONALE

A variety of chemical agents has been used to induce thrombosis in animals. Topical $FeCl_3$ was described by Reimann-Hunziger (1944) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evaluation of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

PROCEDURE

Rats weighing between 250 and 300 g are anesthetized with Inactin (100 mg/kg), and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for

administration of test agents. The right carotid artery is isolated, and an ultrasonic Doppler flow probe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm "M" (American Can Co., Greenwich, CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2×5 mm), saturated with 25% $FeCl_3$ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between $FeCl_3$ application and the time at which blood flow decreases under 0.3 ml/min
- Thrombus weight after blotting the thrombus on filter paper

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11.4 Thrombin-Induced Clot Formation in Canine Coronary Artery

PURPOSE AND RATIONALE

A canine model of thrombin-induced clot formation was developed by Gold et al. (1984) in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique described by Collen et al. (1983) who used radioactively labeled fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular veins clots. The vessel was intentionally de-endothelialized by external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later, and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 h, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

PROCEDURES

In the initial study described by Gold et al. (1984), recombinant t-PA was characterized for its ability to lyse 2-h-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 μg/kg/min i.v. and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a_2-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (1988) demonstrated that SK elicited dose-dependent thrombolysis in this model. Subsequently, Gold et al. (1986, 1988) modified the model to study not only reperfusion but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45% of patients (Goldberg et al. 1985). Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

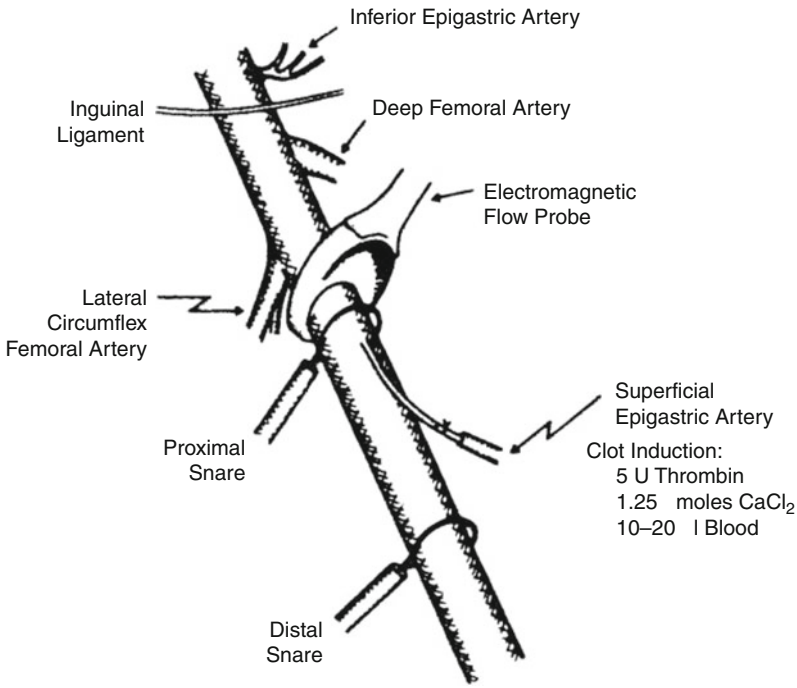

Fig. 11.6 Rabbit model of femoral arterial thrombosis. A clot is introduced into an isolated segment of femoral artery by injection of thrombin, $CaC1_2$, and whole blood. After aging for 1 h, t-PA is infused. Reperfusion is assessed by restoration of blood flow

11.4.1 Thrombin-Induced Rabbit Femoral Artery Thrombosis

Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch (Shebuski et al. 1988).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (FABF). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, $CaCl_2$ (1.25 μmoles), and a volume of blood sufficient to distend the artery. After 30 min, the snares are released, and FABF is monitored for 30 mm to confirm total obstruction of flow by the thrombus. See Fig. 11.6 above.

EVALUATIONS

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent reocclusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (1983) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion (Yasuda et al. 1988). These actions in vivo were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

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11.4.2 Laser-Induced Thrombosis

Purpose and Rationale

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam–lysed erythrocytes due to the fact that erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary, aggregation stimulus following the release reaction induced by the platelets themselves.

PROCEDURE

11.4.2.1 Apparatus

4 W Argon laser (Spectra Physics, Darmstadt, FRG) wavelength, 5,145 nm; energy below the objective, 15 mW; duration of exposure, 1/30 or 1/15 s

- Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
- Video camera (Sony, Trinicon tube)
- Recorder (Sony, U-matic 3/4")
- Videoanalyzer and correlator to determine blood flow velocity

11.4.2.2 In Vivo Experiment

Male Sprague–Dawley or spontaneously hypertensive stroke prone Wistar or Lewis rats with adjuvant induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anesthetized by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60, or 90 min postdosing. Investigations are performed in arterioles or venules of 13 $\pm$1 μm in diameter of the fat-free ileocecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37°C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 s. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of 13 μm or an area of at least 25 μm^2 are evaluated. All measuring procedures are photographed by a video system.

Standard compounds

- Acetylsalicylic acid (10 mg/kg, per os)
- Pentoxifylline (10 mg/kg, per os)

For detailed description and evaluation of various agents and mechanisms, see the following references: Arfors et al. (1968); Herrmann (1983); Seiffge and Kremer (1984, 1986); Seiffge and Weithmann (1987); and Weichert et al. (1983).

EVALUATION

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated. Results are shown graphically.

For statistical evaluation, the χ^2-test is used.

References and Further Reading

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11.4.3 Photochemical-Induced Thrombosis

PURPOSE AND RATIONALE

In 1977, Rosenblum and El-Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the photochemical reaction. He postulated that by exciting the intravascularly administrated fluorescein, singlet oxygen damages endothelial cells, which subsequently leads to platelet adhesion and aggregation.

PROCEDURE

Studies are performed in mesenteric arteries of 15–30-μm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate–dextran 70 (FITC-dextran, Sigma, 10%, 0.3 ml), the FITC-dextran in arterioles is exposed to ultraviolet light (wavelength of excitation 490 nm, wavelength of emission 510 nm).

EVALUATION

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to other thrombosis induction methods, photochemically induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets; however, the primary target of the photochemical insult is the endothelial cells by means of an oxygen radical damage.

MODIFICATIONS OF THE METHOD

Matsuno et al. (1991) reported a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose Bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wavelength: 540 nm). Blood flow is monitored by a pulsed Doppler flow meter. Occlusion is achieved after approximately 5–6 min. Pretreatment with heparin dose dependently prolongs the time required to interrupt the blood flow. The model also enables to study thrombolytic mechanisms, which had been evaluated with t-PA. A comparative data for hirudin in various models was carried out by Just et al. (1991).

References and Further Reading

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11.4.4 Foreign-Surface-Induced Thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

11.4.4.1 Wire-Coil-Induced Thrombosis

PURPOSE AND RATIONALE

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in aorta of dogs and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991; Mellot et al. 1993; Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min followed by a steady state between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 h following implantation of the wire coil. Thrombosis incidence in untreated control animals in this model is 100%. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an in vivo model of venous thrombosis in rats.

PROCEDURE

Male Sprague–Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous, or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by intraperitoneal injection of 1.3 g/kg urethane. Through a midline incision, the caudal caval vein is exposed and a stainless-steel wire coil (a dental pate carrier, Zipperer® size 40(st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire toward the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured, and the animal is placed on its back on a heating pad (37°C). The wound is reopened after 2 h, the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9% saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2% Na_2CO_3 in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry. See Fig. 11.7 below.

11.4.4.2 Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution, a polyethylene catheter is inserted in the jugular vein. One and a half hours after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 h. The wire coil is then removed, and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

EVALUATION

Thrombosis incidence (=number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content (mg) of the thrombotic material in dosage groups and vehicle controls is determined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's *t*-test.

References and Further Reading

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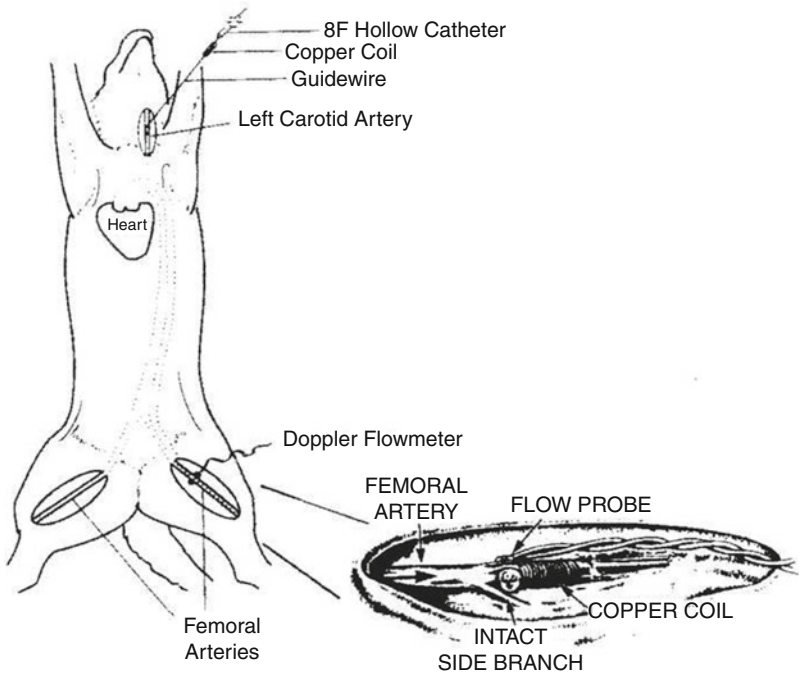

Fig. 11.7 Schematic diagram of the canine femoral artery copper coil model of thrombolysis. A thrombogenic copper coil is advanced to either femoral artery via the left carotid artery. By virtue of the favorable anatomical angles of attachment, a hollow polyurethane catheter advanced down the left carotid artery nearly always enters the descending aorta, and with further advancement, into either femoral artery without fluoroscopic guidance. A flexible, Teflon-coated guide wire is then inserted through the hollow catheter and the latter is removed. A copper coil is then slipped over the guide wire and advanced to the femoral artery (see inset). Femoral artery flow velocity is measured directly and continuously with a Doppler flow probe placed just proximal to the thrombogenic coil and distal to a prominent side branch, which is left patent to dissipate any dead space between the coil and the next proximal side branch. Femoral artery blood flow declines progressively to total occlusion over the next 10–12 mm after coil insertion

11.4.4.3 Eversion-Graft-Induced Thrombosis

PURPOSE AND RATIONALE

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted, and then reimplanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly leading to complete occlusion of the vessel. This model mimics a deep arterial injury since the adventitial surface is a nonendothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

PROCEDURE

In anesthetized New Zealand white rabbits, the right A. carotid is exposed. After double ligation, a 3-mm segment of the artery is excised, everted, and immersed in prewarmed (37°C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double occluder (2-cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9–0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flow meter (Model T106, Transonic, Ithaca NY, USA). The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right V. jugulars. Ten minutes after substance administration,

the vessel clamps are released, and the blood flow is monitored by the flow meter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration), 10, 60, and 120 min after substance administration.

EVALUATION

- Time until occlusion (time after restoring of vessel blood flow until occlusion of the vessel indicated by a flow less than 3.0 ml/min)
- Patency (time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds)

11.4.4.4 Statistical Analysis

Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p<0.05$) are calculated by the nonparametric Kruskal-Wallis test.

CRITICAL ASSESSMENT OF THE METHOD

The eversion graft is very thrombogenic, although technically difficult and time-consuming. The deep occlusive thrombi can be prevented only by intra-arterial administered thrombolytic or aggressive antithrombotic treatments such as recombinant hirudin at high dosages or PEG-hirudin. The adventitial surface is a nonendothelial tissue containing tissue factor and collagen. Thus, both the coagulation system and blood platelets are activated.

MODIFICATIONS OF THE METHOD

Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial-obstructed left-circumflexed coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal nonendothelial surface, produces total thrombotic occlusion within 5 min.

References and Further Reading

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11.4.4.5 Arteriovenous Shunt Thrombosis

PURPOSE AND RATIONALE

A method for the direct observation of extracorporal thrombus formation has been introduced by Rowntree and Shionoya (1927). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. The AV shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs and cats (Best et al. 1938), and nonhuman primates (Yokoyama et al. 1995).

PROCEDURE

Rats are anesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2-cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps that are occluding the AV shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCr-Ni thermocouple that is fixed distal to the glass capillary. If blood is flowing, the temperature rises from room temperature to body temperature. In contrast, decreases in temperature indicate the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown by Best et al. (1938) that the thrombi formed in the AV shunt are to a greater part white

arterial thrombi. This might be due to the high pressure and shear rate inside the shunts, the thrombi tend to be more arterial in character (Chi et al. 1999).

References and Further Reading

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Chi L, Rebello S and Lucchesi BR (1999) In vivo models of thrombosis. In: U'Prichard ACG and Gallagher KP (eds) Anti-thrombotics. Springer, Berlin/Heidelberg, pp 101–127

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Yokoyama T, Kelly AB, Marzec UM, Hanson SR, Kunitada S, Harker LA (1995) Antithrombotic effects of orally active synthetic antagonist of activated factor X in nonhuman primates. Circulation 92:485–491

11.4.4.6 Thread-Induced Venous Thrombosis

PURPOSE AND RATIONALE

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducing cotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it growing to a maximum mass after 2–3 h. The prolonged nonocclusive character of thrombogenesis in this model focuses on progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

PROCEDURE

Rabbits weighing between 2.5 and 3.5 kg are anesthetized with isofluorane inhalation anesthesia, and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240, inner diameter 1.67 mm) of 14-cm length is filled with isotonic saline, and a copper wire with five fixed cotton threads (length 6 cm) is inserted into the tube (after determination of the net weight of the cotton threads). A laparotomy is performed and the V. cava and V. iliaca are dissected free from surrounded tissue. The test agent is administered by a rabbit intragastric tube 60 min (depending on the ex vivo study) prior to initiation of thrombus formation. Blood samples will be measured at 60, 90, 120, 150, and 210 min after oral administration of the test compound.

Thrombus formation is induced by the inserting the thrombosis catheter into the caval vein via the V. iliaca (7 cm). Then, the copper wire is pushed forward 3 cm to liberate the cotton threads into the vessel lumen. One hundred fifty minutes after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed, longitudinally opened, and the content blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

EVALUATION

- Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread
- Mean arterial blood pressure (MAP)
- APTT, HepTest, antiFIIa and antiFXa activities

CRITICAL ASSESSMENT OF THE METHOD

The composition of the cotton threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes thus being in accordance with human deep vein thrombosis structure. Nonocclusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors, and thrombin inhibitors (Hollenbach et al. 1994, 1995).

MODIFICATIONS OF THE METHOD

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

References and Further Reading

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11.4.4.7 Thrombus Formation on Superfused Tendon

PURPOSE AND RATIONALE

In all models that include vessel wall damage, blood gets in contact with adhesive proteins of the subendothelial matrix, i.e., von Willebrand factor, collagens, fibronectin, laminin, and others. Gryglewski et al. (1978) described an in vivo method where blood of an unanesthetized animal is in contact ex vivo with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs based on the formation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

PROCEDURE

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37°C), blood was separated into two streams, each flowing at a speed of 3 ml/min superfusing in parallel two twin strips of the central part of longitudinally cut rabbit Achilles tendon (30×3 mm). The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air, and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

EVALUATION

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of antiaggregatory activity.

References and Further Reading

Gryglewski RJ, Korbut R, Ocetkiewicz A, Stachura J (1978) In vivo method for quantitation of anti-platelet potency of drugs. Naunyn-Schmiedeberg's Arch Pharmacol 302:25–30

11.4.5 Stasis-Induced Thrombosis (Wessler Model)

PURPOSE AND RATIONALE

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952; 1953, 1955a, 1957; Wessler et al. 1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances which can

be used as procoagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

PROCEDURE

Anesthetized rabbits are fixed in supine position on a temperature-controlled (37°C) heating table. Following cannulation of both carotid arteries (the left in cranial direction) and the right V. femoral, segments of 2-cm length of the two external jugular veins are exposed and isolated between two loose sutures. 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 s followed by injection of 0.5 ml physiological saline within 15 s. Forty-five seconds later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 s before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

EVALUATION

The size of the clots is assessed using a score system: (0, blood only; 1, very small clot piece(s), filling out at most 1/4 of the vessel; 2, larger clot piece(s), filling out at most 1/2 of the vessel; 3, very large clot(s), filling out at most 3/4 of the vessel; 4, one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum–maximum). Thrombus weight is given as mean±SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric *U*-test of Mann and Whitney (thrombus score) or Student's *t*-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p<0.05$.

CRITICAL ASSESSMENT OF THE METHOD

Breddin (1989) described the Wessler model because of its static character as the retransformation of an in vitro experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation in vivo. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

MODIFICATIONS OF THE METHOD

There are a number of different procoagulant agents that had been used to induce thrombosis in this model, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates, and factor X_a. (Aronson and Thomas 1985; Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem, some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or postthrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal antihuman PAI-1 antibody, and Biemond et al. (1996) compared the effect of thrombin and factor X_a inhibitors with a low-molecular-weight heparin.

11.4.5.1 Venous Reperfusion Model

New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue, and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ^{131}I-radiolabeled fibrinogen (final radioactivity,

approximately 25 mCi/ml). One hundred fifty microliters of this blood is then aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol $CaCl_2$, and 200 µl of the clotting blood is immediately injected into the isolated segment. Thirty minutes after clot injection, the vessel clamps are removed and blood flow is restored. ^{125}I-radiolabeled fibrinogen (approximately 5 µCi) is injected through the cannula in the carotid artery (in case of the fibrinolysis studies, immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ^{131}I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ^{125}I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed as variance analysis and the Newman-Keuls test. Statistical significance is expressed at the level of $p<0.05$.

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11.4.6 Disseminated Intravascular Coagulation (DIC) Model

PURPOSE AND RATIONALE

Another model that is also used widely in rats and mice is a model of systemic thrombosis or disseminated intravascular coagulation (DIC) that is induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (Herbert et al. 1996; Yamazaki et al. 1994; Sato et al. 1998). After systemic administration of the thrombogenic stimulus, this model can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is the thrombus mass, but when stasis is not used, the readouts are fibrin degradation products, fibrinogen, platelet count, PT, and APTT, among others. As shown by the many and varied parameters, when used without stenosis, the postexperimental analysis can be time-consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations such as the ability to withdraw multiple blood samples over the course of the experiment and the difference in activity of at least some FXa inhibitors in human compared to rat plasma in vitro require that compounds be characterized further in more advanced in vivo models of thrombosis.

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11.4.7 Microvascular Thrombosis in Trauma Models

PURPOSE AND RATIONALE

Successful replantation of amputated extremities is dependent in large degree on maintaining the microcirculation. A number of models have been developed in which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (Fu et al. 1997; Korompilias et al. 1997; Stockmans et al. 1997). In the model of Stockmans (1997), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1,500 g/mm^2, is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for a period of 30 s as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over a period of 35 m by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias (1997) and Fu (1997) examine the formation of arterial thrombosis in rats and rabbits, respectively. In these models, either the rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points postanastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

11.4.8 Cardiopulmonary Bypass Models

PURPOSE AND RATIONALE

Cardiopulmonary bypass (CPB) models have been described in baboons (Van Wyk et al. 1998), swine (Dewanjee et al. 1996), and dogs (Henny et al. 1985). In each model, the variables that can affect the hemostatic system such as anesthesia, shear stresses caused by the CPB pumps and the exposure of plasma components and blood cells to foreign surfaces (catheters, oxygenators, etc.) are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet function are altered. The effectiveness of direct thrombin inhibitors (Van Wyk, et al. 1998), LMWHs (Murray 1985), and heparinoids (Henny et al. 1985) has been compared to standard heparin. Endpoints have included the measurement of plasmatic anticoagulant levels, the histological determination of microthrombi deposition in various organs, the formation of blood clots in the components of the extracorporeal circuit, and the deposition of radiolabeled platelets in various organs and on the components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatibility of components used to maintain extracorporeal circulation. For detailed protocols and evaluations, see Callas et al. (1995), Carrie et al. (1994), Fu et al. (1997), Korompilias et al. (1997), Meuleman et al. (1991), Millet et al. (1994), Stockmans et al. (1997), Vlasuk et al. (1991), Walenga et al. (1987), and Wessler et al. (1959).

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11.4.9 Extracorporeal Thrombosis Models

PURPOSE AND RATIONALE

These models employ passing blood over a section of damaged vessel (or other selected substrates) and recording the thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (Badimon and Badimon 1989). This model is interesting because the results can be directly compared to the in vivo deep arterial injury model (Wysokinski et al. 1996) results and to results from a similar extracorporeal model used in humans (Dangas et al. 1998; Ørvim et al. 1995). Dangas et al. (1998) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet glycoprotein IIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvim et al. (1995) also used this model in humans to evaluate the antithrombotic efficacy of rTAP, but instead of evaluating the compound after administration of rTAP to the patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue-factor-coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant. Although this model does not completely represent pathological intravascular thrombus formation, the use of this "human model" of thrombosis may be very useful in developing new drugs because it directly evaluates the ex vivo antithrombotic effect of a drug in flowing human blood.

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11.4.10 Experimental Thrombocytopenia or Leukocytopenia

PURPOSE AND RATIONALE

Intravenous administration of collagen, arachidonic acid, ADP, PAF (platelet-activating factor), or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravessel aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leukocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leukocytopenia as a consequence of in vivo platelet or leukocyte stimulation.

PROCEDURE

11.4.10.1 Materials and Solutions

Substances used to induce thrombocytopenia/leukocytopenia (intravenous administration)	
In rabbits: Arachidonic acid (Sigma)	1 mg/kg
Collagen (Hormonchemie)	30 µg/ml
In mice: Collagen + adrenaline (Hormonchemie)	90 µg/kg +20 µg/kg
In hamsters: Collagen +adrenaline	50 µg/kg +10 µg/kg
In guinea pigs: PAF (Paf-acether, Bachem)	0.03–0.04 µg/kg
Thrombin (Hoffman-LaRoche)	60 U/kg
Anesthetics: Pentobarbital sodium (i.p.)	30 mg/kg
Xylazine (i.m.)	8 mg/kg
Urethane (i.p.)	1.5 g/kg
Platelet analyzer: Sysmex microcellcounter F-800	

Male guinea pigs (Pirbright White) weighing 300–600 g, male NMRI mice (25–36 g), or Chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated, and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun® (i.m.) and placed on an electrically warmed table at 37°C. The carotid artery is cannulated for blood withdrawal, and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen +adrenaline (injection of the mixture of both within 10 s) or PAF or thrombin. In mice, collagen+adrenaline are injected into a tail vein.

Approximately 50–100 µl blood is collected into potassium-EDTA-coated tubes at times −1, 1, and 2 min (guinea pigs and mice) or 5, 10, and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 h after withdrawal in 10 µl samples of whole blood using a microcell counter suitable for blood of various animal species.

EVALUATION

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100%.

Percent inhibition of thrombocytopenia (or leukocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The method of collagen+epinephrine–induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis/thrombosis. A recent example is the Gas6 −/− mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G protein G(z) (Yang et al. 2000b). The advantage of the method for this purpose is the simple experimental procedure and the small volume of blood necessary. In general, application of the method in small animals (mice,

hamsters) needs only small amounts of drug substance. The model is a useful first step of in vivo antithrombotic efficacy of antiplatelet drugs.

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11.4.11 Collagenase-Induced Thrombocytopenia

Purpose and Rationale

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before.

PROCEDURE

11.4.11.1 Materials and Solutions

Anesthetic: pentobarbital sodium (i.p.)	60 mg/kg
Heparin (Liquemin®) (i.v.)	500 U/kg
To induce thrombocytopenia (intravenous administration): Collagenase (E.C. 3.4.24.3), (Boehringer, Mannheim)	10 mg/ml/kg
Platelet analyzer:Sysmex microcellcounter F-800	

Male Sprague–Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal, and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin, and 20 min later, approximately 100 µl blood is collected (initial value). Ten minutes later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20, and 30 min following the injection of collagenase, samples of approximately 100 µl blood is collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10 µl samples of whole blood within 1 h after blood withdrawal using a microcell counter. See Völkl and Dierichs (1986) for details.

EVALUATION

1. The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set 100%.
2. Percent inhibition of thrombocytopenia is calculated in treated relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

References and Further Reading

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11.4.12 Reversible Intravital Aggregation of Platelets

PURPOSE AND RATIONALE

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation in vivo. ADP, (adenosine diphosphate), PAF (platelet activating factor), arachidonic acid, thrombin, and collagen are known to induce platelet aggregation. In the following procedure, labeled platelets are

continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation promoting agents produces an increase in counts in A and a fall in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The in vivo method can be used to evaluate platelet antiaggregatory properties of test compounds.

PROCEDURE

11.4.12.1 Preparation of Labeled Platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at 240 g for 10 min, the platelet-rich plasma (PRP) is transferred into a tube and suspended in calcium-free Tyrode solution containing 250 ng/ml PGE_1. The suspension is centrifuged at 640 g for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium-free Tyrode solution containing 250 ng/ml PGE_1. 51Cr is added to 1 ml of the platelet suspension. Following a 20-min incubation period at 37°C, the suspension is again centrifuged at 640 g for 10 min. The supernatant is removed and the labeled platelets are finally resuspended in 1 ml calcium-free Tyrode solution containing 250 ng/ml PGE_1.

11.4.12.2 In Vivo Experiment

Male Sprague–Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anesthetized with pentobarbital sodium (30 mg/kg i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labeled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One hour after administration of labeled platelets (when counts in A and B have stabilized), the aggregation promoting agent (ADP, PAF, arachidonic acid, thrombin, or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 h after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin) or 1 h (collagen) after compound administration, another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

Standard compound

- PGI_2 (prostacyclin)

EVALUATION

The microcomputer continuously reveals information about aggregation and desegregation of labeled platelets.

The following parameters are recorded:

- A = counts over thorax
- B = counts over abdomen
- Difference: $A-B$
- Ratio: A/B.

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's *t*-test.

MODIFICATION OF THE METHOD

Oyekan and Botting (1986) described a method for monitoring platelet aggregation in vivo in rats using platelets labeled with indium^{3+} oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected ^{111}indium-labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

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11.4.13 Microfluidic Devices for Studies of Shear-Dependent Platelet Adhesion

Combination of in vivo models and ex vivo flow chambers allow for manipulation of shear stresses (1–3), controlled addition of platelet stimuli (4, 5),

and evaluation of various pharmacological agents (6). Flow chambers for studying platelet rolling, adhesion, and aggregation over immobilized ligands have been critical in understanding shear-dependent receptor-ligand dynamics (7–9). However, most parallel-plate and annular flow chambers require milliliters of fluid to perform experiments over a relevant time scale. This volume of blood is easily obtained from human subjects but requires pooling or dilution of murine whole blood owing to the relatively low total blood volume of a single animal (~1 ml) (10, 11). The cost of pooling is difficult when using genetically modified mice to obtain data for a single experimental condition in a parallel-plate flow chamber. Thus, current flow chamber designs are poorly suited for research with murine blood. Studies of dynamic platelet adhesion assays in microfluidic devices reduce the blood volume requirements to less than 0.1 ml per assay, making the assays compatible with samples of whole blood obtained from a single mouse. Certain devices have an array of eight flow chambers with shear stress varying by a factor of about two between adjacent chambers, covering a 100-fold range from low venous to arterial. Other devices allow for simultaneous high-resolution fluorescence imaging of dynamic adhesion of platelets from two different blood samples. Adhesion of platelets in the devices to EC monolayer or common ECM substrate coatings can be carried out. These microfluidic systems, with the requirement of small blood volume, allow for investigating blood from wild-type mice and from different genetically modified mouse strains under different shear, which might also allow for adhesion tests in clinical settings with blood from neonates and children.

11.4.13.1 Experimental Protocol

Platelets were visualized by adding 2 μM mepacrine to whole blood to label dense granules where at this concentration no effect on either platelet or leukocyte functions (12, 13). Test compounds are added 15–30 min prior to onset of blood flow through the device. To coat the glass substrata of the microfluidic devices with physiological matrices, the microchannels were filled with 10–20 μg/ml fibrinogen, 100–300 μg/ml acid-soluble type I collagen, or 3–10 μg/ml vWF and incubated for 1 h at room temperature. Subsequently, the devices are rinsed with 20 mM Hepes, pH 7.4 and then blocked with 1–3% serum albumin for 30 min. The flow through the device can be stopped by clamping the Tygon tubing. Use 100–200 μl of human or mouse blood into a 1-cc plastic syringe through a Tygon tubing line, which is then connected to the device inlet.

11.4.13.2 Image Acquisition and Analysis

The microfluidic device is mounted on a mechanical stage of inverted fluorescence microscope. Fluorescence microscopy with light source and a GFP filter set (Ex470/Em525) and the images of the platelets are acquired. The stage could be programmed to move in periodic scanning loops between test chambers 1–8, with eight stops to take a fluorescence image of each chamber.

The microfluidic device could also be used to study platelet-leukocyte or other heterotypic cell interactions.

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11.5 Bleeding Models

11.5.1 Subaqueous Tail Bleeding Time in Rodents

PURPOSE AND RATIONALE

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

PROCEDURE

Anesthetized rats are fixed in supine position on a temperature-controlled (37°C) heating table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37°C).

EVALUATION

The time until bleeding stops is determined within a maximum observation time of 600 s.

CRITICAL ASSESSMENT OF THE METHOD

There are numerous variables that can influence rodents' bleeding time measurements as discussed by Dejana et al. (1979): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, and procedure of injury (Simplate method, transsection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay conditions (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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11.5.2 Arterial Bleeding Time in Mesentery

PURPOSE AND RATIONALE

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated that inhibit thrombus formation thus prolonging arterial bleeding time. The test is used to detect agents that interfere with primary hemostasis in small arteries.

PROCEDURE

Male Sprague–Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37°C.

The abdomen is opened by a midline incision, and the mesentery is lifted to display the mesenteric

arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode solution maintained at 37°C. Bleeding times are determined with small mesenteric arteries (125–250 μm external diameter) at the junction of mesentery with intestines. Adipose tissue surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16×5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

EVALUATION

1. Mean values of bleeding times [s] are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.
2. The significance of the results is assessed with the unpaired Student's *t*-test.
3. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.

For further details on methods and evaluations of various mechanisms or agents, see the following: Butler et al. (1982), Dejana et al. (1979), and Zawilska et al. (1982).

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11.5.3 Template Bleeding Time Method

PURPOSE AND RATIONALE

The template bleeding time method is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the development of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method that enables the Simplate technique as a method to analyze the bleeding time in the oral mucosa of dogs.

PROCEDURE

The dog is positioned in sternal or lateral recumbency. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buckle mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co., Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 s. The endpoint for each bleeding is determined when the filter paper no longer develops a red crescent.

EVALUATION

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between 2 and 4 min.

CRITICAL ASSESSMENT OF THE METHOD

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989; Klement et al. 1998). Brassard and Meyers (1991) describe the buckle mucosa bleeding time as a test that is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979; Lind 1991).

MODIFICATIONS OF THE METHOD

The Simplate device can also be used to perform incisions at the shaved inner ear of rabbits taking care to avoid major vessels. The normal range of bleeding

time in anesthetized rabbits is approximately 100 s (77 ± 4 s, $n = 20$) in our own laboratory.

Klement et al. (1998) described another ear bleeding model in anesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37°C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade avoiding major vessels, and the ear was immediately reimmersed in saline. At different times thereafter (5–30 min), aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine-type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between 2 and 8 min.

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11.6 Genetic Models of Hemostasis and Thrombosis

PURPOSE AND RATIONALE

Recent advances in genetic molecular biology have provided tools allowing scientists to design genetically altered animals that are deficient in certain proteins involved in thrombosis and hemostasis (so-called knockouts, or "nulls") (Carmeliet and Collen 1999; Pearson and Ginsburg 1999). These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examining the phenotype (e.g., spontaneous bleeding, platelet defect, prolonged bleeding after surgical incision, etc.) of a specific knockout strain, scientists can identify the role of the knocked out protein. Then, if the phenotype is favorable (e.g., not lethal), pharmacological agents can be designed to mimic the knockout. More recently, novel gene medicine approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process. The majority of these gene knockouts result in mice that develop normally, are born in the expected Mendelian ratios, and are viable as defined by the ability to survive to adulthood. Although seemingly normal, these knockout mice display alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, vWF, and β_3 integrin (Bi et al. 1996; Denis et al. 1998; Hodivala-Dilke et al. 1999; Wang et al. 1997) results in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knockout models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann thrombasthenia, respectively). In addition, deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally but are unable to survive the perinatal period due to severe hemorrhage, in most cases due to the trauma of birth.

Genetic knockouts have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of the $ß_3$ integrin (Hodivala-Dilke et al. 1999) or of $G_{\propto q}$ (Offermanns et al. 1997) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein-coding region in the $ß_3$ integrin carboxy tail, $ß_3$-DiY, at sites that are thought to be phosphorylated upon platelet activation also results in unstable platelet aggregation (Law et al. 1999). Deletion of various receptors

such as thromboxane A_2, P-selectin, P2Y1, and PAR-3 demonstrates diminished responses to some agonists while other platelet responses are intact (Thomas et al. 1998; Subramaniam et al. 1996; Leon et al. 1999; Kahn et al. 1998). Deletion of PAR-3, another thrombin receptor in mice, has little effect on hemostasis. This indicated the presence of yet another thrombin receptor in platelets and led to the identification of PAR-4 (Kahn et al. 1998).

Given that knockouts of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (Bugge et al. 1995; Ploplis et al. 1995), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and combined t-PA/u-PA knockout (Carmeliet et al. 1994) result in mice that demonstrate impaired fibrinolysis, susceptibility for thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice may provide a good model of myocardial infarction and heart failure caused by thrombosis (Christie et al. 1999). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, demonstrate no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (Carmeliet et al. 1993), suggesting that inhibition of PAI-1 might be a promising approach for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several of these genes are important in embryonic development. For example, deletion of tissue factor (Bugge et al. 1996a; Toomey et al. 1996; Carmeliet et al. 1996), tissue factor pathway inhibitor (Huang et al. 1997), or thrombomodulin (Healy et al. 1995) results in an embryonic lethal phenotype. These and other (Connolly et al. 1996; Cui et al. 1996) hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. Results of these studies have provided rationale and impetus for attacking certain targets pharmacologically. These types of models have also provided excellent model systems for studying novel treatments for human diseases. For example, these models provided exceptional systems for studying gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the FIX gene and its promoter, results in mice that mimic the human phenotype of hemophilia B (Lin et al. 1997). When these mice are treated with adenoviral-mediated transfer of human FIX, the bleeding diathesis is fully corrected (Kung et al. 1998). Similarly, selectively bred dogs that have a characteristic point mutation in the sequence encoding the catalytic domain of FIX also have a severe hemophilia B that is phenotypically similar to the human disease (Evans et al. 1989). When adeno-associated virus-mediated canine FIX gene was administered to these dogs intramuscularly, therapeutic levels of FIX were measured for up to 17 months (Herzog et al. 1999). Clinically relevant partial recovery of whole blood clotting time and APTT was also observed over this prolonged period. These data provided support for initiating the first study of adeno-associated virus-mediated FIX gene transfer in humans (Kay et al. 2000). Preliminary results from this clinical study provided evidence for expression of FIX in the three hemophilia patients studied and also provided favorable safety data to substantiate studying this therapy at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these experiments have provided pharmacological, pharmacokinetic, and safety data that were extremely useful in developing this approach and designing safe clinical trials.

Gene therapy approaches to rescuing patients with bleeding diatheses are further advanced than gene therapy for thrombotic indications. However, promising preclinical data indicate that local overexpression of thrombomodulin (Waugh et al. 1999a) or tissue plasminogen activator (Waugh et al. 1999b) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of tissue factor pathway inhibitor prevented thrombus formation in balloon-injured porcine carotid arteries (Zoldhelyi et al. 2000). These and other studies (Vassalli and Dichek 1997) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully

optimized for pharmacokinetics, safety, and efficacy in laboratory animal studies prior to administration to humans.

11.6.1 Genetically Modified Animals

Development and application of animal models of thrombosis has played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. The development and application of small and large animal models of thrombosis to the discovery and development of novel antithrombotic agents is described in this review. The methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/hemostasis is presented using gene therapy for hemophilia as an example of how animal models have aided in the development of therapies that are presently being evaluated clinically.

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11.6.2 Knockout Mice

PURPOSE AND RATIONALE

Genetically modified animals, in particular knockout mice, help to understand the role of various factors in blood clotting, thrombolysis, and platelet function. They are useful to verify the mode of action of new drugs.

11.6.2.1 Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ~10% die shortly after birth and another 40% around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples fail to clot, or support platelet aggregation in vitro (Suh et al. 1995).

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11.6.2.2 Factor II (Prothrombin)

Phenotype

Partial embryonic lethality: 50% between embryonic day (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage few days after birth; factor II is important in maintaining vascular integrity during development as well as postnatal life (Sun et al. 1998; Xu et al. 1998).

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11.6.3 Factor V

Phenotype

Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature; the remaining 50% progress normally to term but die from massive hemorrhage within 2 h of birth, more severe in mouse than in human (Cui et al. 1996; Yang et al. 2000a).

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11.6.3.1 Factor VII

Phenotype

Develop normally but suffer fatal perinatal bleeding (Rosen et al. 1997).

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11.6.3.2 Factor VIII

Phenotype

Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness, or reduced activity during the first year of life; have residual clotting activity (APTT) as shown by Bi et al. (1995).

References and Further Reading

Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazaazian HH (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10:119–121

11.6.3.3 Factor IX

Phenotype

Factor IX coagulant activities (APTT): +/+ 92%, +/− 53%, −/− <5%; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized (Kundu et al. 1998; Wang et al. 1997).

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11.6.3.4 Factor X

Phenotype

Partial embryonic lethality (1/3 died on E11.5–12.5); fatal neonatal bleeding between postnatal day (P) 5–20 as shown by Dewerchin et al. (2000).

References and Further Reading

Dewerchin M, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

11.6.3.5 Factor XI

Phenotype

APTT prolonged in −/− (158–200 s) compared with +/+ (25–34 s) and +/− (40–61 s); no factor XI activity and antigen did not result in intrauterine death, −/− similar bleeding as +/+ with a tendency to prolongation (Gailani et al. 1997).

References and Further Reading

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

11.6.3.6 TF (Tissue Factor)

Phenotype

Abnormal circulation from yolk sac to embryo∼E8.5 leading to embryo wasting and death; TF has a role in blood vessel development (Bugge et al. 1996a; Carmeliet et al. 1996; Toomey et al. 1996, 1997).

References and Further Reading

Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci U S A 93:6258–6263

Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlanderen I, Demunck H, Kasper M, Breier G, Evrard P,

Müller M, Risau W, Edgington T and Collen D (1996) Role of tissue factor in embryonic blood vessel development. Nature 383:73–75
Toomey JR, Kratzer KE, Lasky NM, Stanton JL, Broze GJ (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587
Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 94:6922–6926

11.6.3.7 TFPI (Tissue Factor Pathway Inhibitor)

Phenotype

None survive the neonatal period; 60% die between E9.5 and 11.5 with signs of yolk sac hemorrhage (Huang et al. 1997).

References and Further Reading

Huang ZF, Higuchi D, Lasky N, Broze GJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

11.6.3.8 Thrombin Receptor

Phenotype

Fifty percent die at E9–10; 50% survive and become grossly normal adult mice with no bleeding diathesis; −/− platelets strongly respond to thrombin; −/− fibroblast lose their ability to respond to thrombin→second TR must exist as shown by Connolly et al. (1996) and by Darrow et al. (1996).

References and Further Reading

Connolly AJ, Ishihara H, Kahn ML, Farese RV Jr., Coughlin SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519
Darrow AL, Fung-Leung WP, YE RD, Santulli RJ, Cheung WM, Derian CK, Burns CL, Damiano BP, Zhou L, Keenan CM, Peterson PA, Andrade-Gordon P (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76:860–866

11.6.3.9 Thrombomodulin

Phenotype

Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/− mice develop normal without thrombotic complications (Christie et al. 1999; Healy et al. 1995, 1998; Weiler-Guettler et al. 1998).

References and Further Reading

Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539
Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854
Healy AM, Hancock WW, Christie PD, Rayburn HB, Rosenberg RD (1998) Intravascular coagulation activation in a murine model of thrombomodulin deficiency: effects of lesion size, age, and hypoxia on fibrin deposition. Blood 92:4188–4197
Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW (1998) A targeted point mutation in thrombomodulin generates viable mice with a prothrombotic state. J Clin Invest 101:1983–1991

11.6.3.10 Protein C

Phenotype

KO mice appeared to develop normally macroscopically but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 h after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable suggesting fibrinogen depletion and secondary consumptive coagulopathy (Jalbert et al. 1998).

References and Further Reading

Jalbert LR, Rosen ED, Moons L, Chan JCY, Carmeliet P (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

11.6.3.11 Plasminogen

Phenotype

Severe spontaneous thrombosis; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; impaired skin healing; reduced macrophage and keratinocyte migration (Bugge et al. 1995; Ploplis et al. 1995).

References and Further Reading

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807
Ploplis VA, Carmeliet P, Vazirzadeh S, Van Vlaenderen I, Moons L (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

11.6.3.12 Alpha$_2$-Antiplasmin

Phenotype

Normal fertility, viability, and development; no bleeding disorder; spontaneous lysis of injected clots→enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS (Lijnen et al. 1999).

References and Further Reading

Lijnen HR, Okada K, Matsuo O Collen D, Dewerchin M (1999) alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

11.6.3.13 T-PA (Tissue-Type Plasminogen Activator)

Phenotype

Extensive spontaneous fibrin deposition; severe spontaneous thrombosis; impaired neointima formation; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; abnormal tissue remodelling (Carmeliet et al. 1998; Christie et al. 1999).

References and Further Reading

Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

11.6.3.14 PAI-1 (Plasminogen Activator Inhibitor 1)

Phenotype

Reduced thrombotic incidence; no bleeding; accelerated neointima formation; reduced lung inflammation; reduced atherosclerosis. Detailed studies on PAI-1 are reported by Carmeliet et al. (1993), Eitzman et al. (1996), Erickson et al. (1990), Kawasaki et al. (2000), and Pinsky et al. (1998).

References and Further Reading

Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, de Mol M, Mulligan RC, Collen D (1993) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. J Clin Invest 92:2756–2760

Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or over-express the murine plasminogen activator inhibitor-1 gene. J Clin Invest 97:232–237

Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR (1990) Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 346:74–76

Kawasaki T, Dewerchin M, Lijnen HR, Vermylen J, Hoylaerts MF (2000) Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. Blood 96:153–60

Pinsky DJ, Liao H, Lawson CA, Yan SF, Chen J (1998) Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J Clin Invest 102:919–928

11.6.3.15 Vitronectin

Phenotype

Normal development, fertility, and survival; serum is completely deficient in "serum spreading factor" and plasminogen activator inhibitor 1 binding activities; delayed arterial and venous thrombus formation (Eitzman et al. 2000; Zheng et al. 1995).

References and Further Reading

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogenactivator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Sunders TL, Camper SA, Samuelson LC, Ginsburg D (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci USA 92:12426–12430

11.6.3.16 Urokinase, U-PA (Urinary-Type Plasminogen Activator)

Phenotype

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, shortened life span; spontaneous fibrin deposits more extensively and in more organs (Carmeliet et al. 1998; Heckel et al. 1990).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intra-abdominal bleeding directly related to transgene expression in the liver and elevated

plasma u-PA levels. Fifty percent die between 3 and 84 h after birth; severe hypofibrinogenemia, loss of clotting function.

References and Further Reading

Carmeliet P, Schoonjans L, Kieckens L, Ream B; Degen J (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 62:447–456

11.6.3.17 UPAR (Urinary-Type Plasminogen Activator Receptor)

Phenotype

Phenotype normal; attenuated thrombocytopenia and mortality associated with severe malaria (Bugge et al. 1995, 1996b; Dewerchin et al. 1996; Piguet et al. 2000).

References and Further Reading

Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, Degen JL (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Biol Chem 270:16886–16894

Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL (1996) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904

Dewerchin M, van Nuffelen A, Wallays G, Bouché, Moons L, Carmeliet P, Mulligan RCM, Collen D (1996) Generation and characterization of urokinase receptor deficient mice. J Clin Invest 97:870–878

Piguet PF, Da-Laperrousaz C, Vesin C, Tacchini-Cottier F, Senaldi G, Grau GE (2000) Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. Infect Immun 68:3822–3829

11.6.3.18 Gas 6 (Growth Arrest–Specific Gene 6 Product)

Phenotype

Mice are viable, fertile, appear normal; do not suffer spontaneous bleeding or thrombosis; and have normal tail bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (Angelillo-Scherrer et al. 2001).

References and Further Reading

Angelillo-Scherrer A, DeFrutos PG, Aparicio C, Melis E, Savi P, Lupu F, Dewerchin M, Hoylaerts MF, Herbert J-M, Collen D, Dahlbaeck B, Carmeliet P (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

11.6.3.19 GPIbalpha (Glycoprotein Ib Alpha, Part of the GP Ib-V–IX Complex)

Phenotype

Bleeding, thrombocytopenia, and giant platelets (similar to human Bernard Soulier syndrome). See Ware et al. (2000) for details.

References and Further Reading

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

11.6.3.20 GPV (Glycoprotein V, Part of the GP Ib-V–IX Complex)

Phenotype

Increased thrombin responsiveness, GpV−/− platelets are normal in size, normal amounts in GpIb-IX, functional in vWF binding; platelets are hyperresponsive to thrombin→increased aggregation response; shorter bleeding time; →GpV = negative modulator of platelet function (Ramakrishnan et al. 1999).

References and Further Reading

Ramakrishnan V, Reeves PS, DeGuzman F, Deshpande U, Ministri-Madrid K (1999) Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

11.6.3.21 Gpiib (Integrin Alpha IIb, Glycoprotein IIb, Part of the GP IIB–IIIa Complex)

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in man; platelets failed to bind fibrinogen, to aggregate, and to retract a fibrinogen clot; α-granules do not contain fibrinogen (Tronik-Le Roux et al. 2000).

References and Further Reading

Tronik-Le Roux D, Roullot V, Poujol C, Kortulewski T, Nurden P, Marguerie G (2000) Thrombasthenic mice generated by replacement of the integrin alpha$_{IIb}$ gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

11.6.3.22 GP IIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GP IIb–IIIa Complex)

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in man, e.g., defective platelet aggregation, clot retraction; spontaneous bleeding, prolonged bleeding times; dysfunctional osteoclasts, development of osteosclerosis with age (Hodivala-Dilke et al. 1999; McHugh et al. 2000).

References and Further Reading

Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

McHugh KP, Hodivala-Dilke K, Zheng M-H, Namba N, Lam J (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

11.6.3.23 GP IIa (Glycoprotein IIa, Integrin Beta 1, Part of the GP Ia–IIa Complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally; platelet count is normal. Collagen-induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major in vivo defects (Nieswandt et al. 2001).

References and Further Reading

Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JWM, Zirngibl H, Faessler R (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

11.6.3.24 VWF (Von Willebrand Factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF; highly prolonged bleeding time, hemorrhage, spontaneous bleeding; mice useful for investigating the role of vWF; delayed platelet adhesion in ferric-chloride-induced arteriolar injury (Denis et al. 1998; Ni et al. 2000).

References and Further Reading

Denis C, Methia N, Frenette PS, Rayburn M, Ullman-Cullere M (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:952–959

Ni H, Denis CV, Subbarao S, Degen JL, Sato TN, Hynes RO, Wagner DD (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

11.6.3.25 Thromboxane A2 Receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (Thomas et al. 1998).

References and Further Reading

Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001

11.6.3.26 Prostacyclin Receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses (Murata et al. 1997).

11.6.3.27 References and Further Reading

Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

11.6.3.28 PECAM (Platelet Endothelial Cell Adhesion Molecule)

Phenotype

Normal platelet aggregation; prolonged bleeding time as described by Duncan et al. (1999) and by Mahooti et al. (2000).

References and Further Reading

Duncan GS, Andrew DP, Takimoto H, Kaufman SA, Yoshida H (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162: 3022–3030

Mahooti S, Graesser D, Patil S, Newman P, Duncan G, Mak T, Madri JA (2000) PECAM-1 (CD 31) expression modulates bleeding tome in vivo. Am J Pathol 157:75–81

11.6.3.29 Pallid (Pa)

Phenotype

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky-Pudlak syndrome (the beige mouse is a model of the Chediak-Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution, kidney lysosomal enzyme elevation serum alpha 1 antitrypsin deficiency, and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino-acid protein pallidin that interacts with syntaxin 13, a protein-mediating vesicle docking and fusion (Huang et al. 1999).

References and Further Reading

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 23:329–332

11.6.3.30 G Alpha(q) (Guanyl Nucleotide Binding Protein G Alpha Q)

Phenotype

Defective aggregation in response ADP, TXA2, thrombin, collagen; shape change normal (Offermans et al. 1997; Ohlmann et al. 2000).

References and Further Reading

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186

Ohlmann P, Eckly A, Freund M, Cazenave JP, Offermanns S, Gachet C (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. Blood 96:2134–2139

11.6.3.31 G Z (Member of the Gi Family of G Proteins)

Phenotype

Impaired platelet aggregation to epinephrine; resistance to fatal thromboembolism; exaggerated response to cocaine, reduced effect of morphine and antidepressant drugs (Yang et al. 2000b).

References and Further Reading

Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA, Brass LF (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

11.6.3.32 Phospholipase C Gamma

Phenotype

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fcgamma receptor signaling, therefore, loss of collagen-induced platelet aggregation (Wang et al. 2000).

References and Further Reading

Wang D, Feng J, Wen R, Marine JC, Sangster MY, Parganas E, Hoffmeyer A, Jackson CW, Cleveland JL, Murray PJ, Ihle JN (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

11.6.3.33 CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)

Phenotype

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature in vivo. Platelets fail to aggregate to standard agonists in vitro associated with purinergic P2Y1 receptor desensitization; fibrin deposition at multiple organ sites (Enjyoji et al. 1999).

References and Further Reading

Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5:1010–1017

11.6.3.34 Protein Kinase, cGMP-Dependent, Type 1

Phenotype

Viable, fertile; unresponsive to cGMP and NO; defective VASP-phosphorylation; increased adhesion and aggregation of platelets in vivo in ischemic/reperfused mesenteric microcirculation; no compensation by cAMP kinase system (Massberg et al. 1999).

References and Further Reading

Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Faessler R, Ruth P, Krombach F, Hofmann F (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. J Exp Med 189:1255–1264

11.6.3.35 Vasodilator-Stimulated Phosphoprotein (VASP)

Phenotype

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen/thrombin activation, impaired cyclic nucleotide–mediated inhibition of platelet activation (Aszodi et al. 1999; Hauser et al. 1999).

References and Further Reading

Aszodi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KF, Kehrel B, Offermanns S, Faessler R (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMVBO J 18:37–48

Hauser W, Knobeloch KP, Eigenthaler M, Gambaryan S, Krenn V (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125

11.6.3.36 Arachidonate 12-Lipoxygenase (P-12LO)

Phenotype

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in ex vivo assays and increased mortality in an ADP-induced mouse model of thromboembolism (Chen et al. 1994; Johnson et al. 1998).

References and Further Reading

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95: 3100–3105

11.6.3.37 Arachidonate 5-Lipoxygenase (P-5LO)

Phenotype

Develop normally and are healthy. No difference in their reaction to endotoxin shock; however resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced (Chen et al. 1994; Johnson et al. 1998).

References and Further Reading

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

11.6.3.38 Thrombopoietin

Phenotype

TPO −/− and c-mpl −/−: both exhibit a 90% reduction in megakaryocyte and platelet levels; but even with these small platelet levels, the mice do not have excessive bleeding; all platelets which are present are morphologically normal+functionally; in vivo TPO is required for control of megakaryocyte and platelet number but not for their maturation (Lawler et al. 1998).

References and Further Reading

Lawler J, Sunday M, Thibert V, Duquette M, George EL (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

11.6.3.39 Thrombospondin-1

Phenotype

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-1 is involved in normal lung homeostasis (Lawler et al. 1998).

References and Further Reading

Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, Hynes RO (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Mouse knockout models of virtually all of the known hemostatic factors have been reported, as shown in the table below.

Genetic models of thrombosis and hemostasis

Knockout coagulation	Viable	Embryonic development/ survival	References
Protein C	No	Normal perinatal death	Jalbert et al. (1998)
Fibrinogen	Yes	Normal perinatal death	Suh et al. (1995)
Fibrinogen-QAGVD	Yes	Normal	Suh et al. (1995)
fV	No	Partial embryonic loss Perinatal death	Cui et al. (1996)
FVII	Yes	Normal perinatal death	Rosen et al. (1997)
fVIII	Yes	Normal	Bi et al. (1996)
fIX	Yes	Normal	Wang et al. (1997)
fXI	Yes	Normal	Gailani et al. (1997)
Tissue factor	No	Lethal	Toomey et al. (1996) Bugge et al. (1996a)
TFPI	No	Lethal	Huang et al. (1997)
vWF	Yes	Normal	Denis et al. (1998)
Prothrombin	No	Partial embryonic loss perinatal death	Xue et al. (1998) Sun et al. (1998)
Fibrinolytic			
u-Pa & t-PA	Yes	Normal growth retardation	Carmeliet et al. (1994)
uPAR	Yes	Normal	Dewerchin et al. (2000) Bugge et al. (1995)
Plasminogen	Yes	Normal growth retardation	Bugge et al. (1995) Ploplis et al. (1995)
PA-I	Yes	Normal	Carmeliet et al. (1993)
Thrombomodulin	No	Lethal	Healy et al. (1995)
Platelet			
β_3	Yes	Normal partial embryonic loss	Hodivala-Dilke et al. (1999)
β_3-DiYF	Yes	Normal	Law et al. (1999)
P-Selectin	Yes	Normal	Subramaniam et al. (1996)
PAR-1	Yes	Normal	Connolly et al. (1996)
PAR-3	Yes	Normal	Kahn et al. (1998)
$G_{\alpha q}$	Yes	Normal perinatal death	Offermans et al. (1997)
TXA_2 receptor	Yes	Normal	Thomas et al. (1998)
P2Y1	Yes	Normal	Leon et al. (1999)

References and Further Reading

Bi L, Sarkar R, Naas T, Lawler AM, Pain J, Shumaker SL, Bedian V (1996). Further characterization of Factor VIII-deficient mice created by gene targeting: RNA and protein studies. Blood 88:3446–3450

Bugge TH, Suh TT, Flick MJ, Daugherty CC, Romer J, Solberg H, Ellis V, Dano K, Degen JL (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Bio Chem 270: 16886–16894

Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmback K, Danton MJS, Colbert MC, Witte DP, Fujikawa K, Davie EW, Degen JL (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

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Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC, Collen D (1993) Plasminogen activator inhibitor-1 gene-deficient mice. J Clin Invest 92:2756–2760

Connolly AJ, Ishihara H, Kahn ML, Farese RV Jr, Coughlin SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D (1996) Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68

Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, Wagner DD (1998) A mouse model of severe von

Willebrand disease: Defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529
Dewerchin M, Liang Z, Moons L, Carmeliet P, Castellino FJ, Collen D, Rosen ED (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190
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11.7 Critical Issues in Experimental Models

11.7.1 The Use of Positive Control

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, low-molecular-weight heparins, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel antithrombotic agents should certainly be demanded to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose–response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as APTT, PT, template bleeding time, or other more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (1996b), who compared the antithrombotic efficacy of argatroban and dalteparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early in vivo evaluation of compounds that demonstrate acceptable in vitro potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it

will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which polyantithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

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11.7.2 Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well established in terms of efficacy, the preclinical tests for evaluating safety, i.e., bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic or thrombolytic therapy stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhages are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (Stieg and Kase 1998; Sloan and Gore 1992). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays varies from compound-to-compound so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (Bernardi et al. 1993; Bick 1995; Rodgers and Levin 1990). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg i.v. bolus plus 0.2 mg/kg/h (Antman et al. 1996; GUSTO Investigators 1996). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/h), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with Xa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provides valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

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11.7.3 Selection of Models Based on Species-Dependent Pharmacology/Physiology

As alluded to earlier, species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (Cox et al. 1992) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (Bostwick et al. 1996; Cook et al. 1993; Panzer-Knodle et al. 1993). This discovery leads to the widespread use of larger animals (particularly in dogs, whose platelet response to GPIIb/IIIa antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course, the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (1996) administered a GPIIb/IIIa antagonist orally and intravenously to rats and then mixed the platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs. The mixture was then evaluated in an agonist-induced platelet aggregation assay, and the resulting inhibition of canine platelet aggregation (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065 is much more potent against human FXa (Ki = 78 nM) than against rabbit (Ki = 102 nM) and rat (Ki = 1980 nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, PTx2, was 0.52 μM) and in squirrel monkey plasma (PTx2 = 0.46μM) but was much less potent in rabbit, dog, and rat plasma (PTx2 = 1.5, 6.5, and 22.2 μM, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (Tidwell et al. 1980; Nutt et al. 1991; Taniuchi et al. 1998). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by in vitro coagulation or platelet aggregation tests prior to evaluation in animal models.

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11.7.4 Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans (Kararli 1995). Due to resource issues (mainly compound availability) and animal care and use considerations, small rodents, such as rats, are usually considered for primary in vivo evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or nonhuman primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of FXa inhibitor, YM-60828, have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4, 33, 7, and 20% in rats, guinea pigs, Beagle dogs, and squirrel monkeys, respectively. Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (Sanderson et al. 1998) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

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11.7.5 Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. The influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in the table below, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g., toxicity, narrow therapeutic window, or undesirable pharmacokinetics or pharmacodynamics) that are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. For detailed applications, see the following references: Bugge et al. (1995), Bugge et al. (1996a), Carmeliet et al. (1993), Carmeliet et al. (1994), Carmeliet et al., (1996), Christie et al. (1999), Connolly et al. (1996), Cui et al., (1996), Evans et al. (1989), Healy et al. (1995),

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Animal models of thrombosis and their clinical correlates

Compound	Preclinical animal model	Preclinical results	Reference	Clinical indication	Clinical result	Reference
Recombinant tissue plasminogen activator (activase)	Rabbit pulmonary artery thrombosis	Lysis of preformed pulmonary thrombus	Matsuo et al. (1981)	Acute myocardial infarction—thrombolysis	Improved recanalization	Collen et al. (1984)
Abciximab (ReoPro)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Coller et al. (1986)	High-risk coronary angioplasty	Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization	EPIC Investigators (1994)
Tirofiban (aggrestat)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Lynch et al. (1995)	Unstable angina	Reduction in death, myocardial infarction, refractory ischemia	Prism Investigators (1998)
Eptifibitide (Integrilin)	TPA-induced coronary thrombolysis	Significant improvement in lysis of occlusive thrombus	Nicolini et al. (1994)	Acute myocardial infarction—thrombolysis with tPA	Improvement in incidence and speed of reperfusion	Ohman et al. (1997)
Enoxaparin (Lovenox)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Leadley et al. (1998)	Unstable angina	Significant decrease in death, myocardial infarction, and need for revascularization at 30 days	Cohen et al. (1998)
Hirudin (Refludan)	Rabbit jugular vein thrombus growth	Inhibition of thrombus growth compared to standard heparin	Agnelli et al. (1990)	Deep vein thrombosis after total hip replacement	Significantly decreased rate of DVT	Eriksson et al. (1997)
Argatroban	Canine coronary artery electrolytic injury (TPA-induced thrombolysis)	Accelerated reperfusion and prevented reocclusion	Fitzgerald et al. (1989)	Unstable angina	No episodes of MI during drug infusion	Gold et al. (1993)

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Ocular Safety Tests

12

Beat P. Mertz

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12.1 General Considerations

Ocular drugs, cosmetics, or all chemicals which can come in contact with the eye carry the potential of harming various ocular structures. Thus, their possible toxic effects on ocular structures must be evaluated in tests before use in humans.

At present, it has been suggested that this be done in three steps. First, a thorough review of the literature of similar compounds should be conducted to obtain theoretical knowledge about ocular toxicity. Second, tests of this substance are performed in various in vitro procedures to obtain possible serious toxic effects, which might stop further testing. Third, if no serious toxic effects have been observed, tests are conducted in the eyes of one animal followed by subsequent tests in more animals. Fourth, the substance of an ocular drug will then be subjected to clinical trials. An important feature of these preclinical tests is that they correctly predict or are in agreement with findings in humans. A recent study reviewed the principles and the available evidence that support the design and conduct of preclinical studies in a way that permits effective and safe first-dose studies of potential new medicines in humans (Greaves 2004). Another study concluded that there is indeed a relatively good agreement between certain preclinical and clinical observations (Dart 2003).

A comprehensive review of various in vitro and in vivo procedures was published by Wilhelmus in 2001.

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12.2 Dendritic Cell Culture

PURPOSE AND RATIONALE

This test uses an in vitro human dendritic cell culture to obtain information of the potential for various chemicals to induce allergic contact dermatitis. This test is used as an alternative to the local lymph node assay (LLNA) to minimize or replace the use of live animal testing for predicting skin sensitization (Kimber et al. 2002, see below). The test allows for evaluation of skin sensitization by examining the presence of cell surface markers on peripheral blood mononuclear cell (PBMC)-derived dendritic cells (DC) that are known to be involved in the development of allergic contact dermatitis.

PROCEDURE

The method contains three stages including first establishing a cell line, followed by test chemical exposure, and finally evaluating for expression of cell surface markers. To establish a cell line, human leukocyte preparations are attained from a plasma distributor. The leukocyte preparations, as described by Ryan et al. (2004), are diluted with an equal part of complete medium (RPMI 1640 containing 1 × L-glutamine 1 × penicillin-streptomycin-neomycin antibiotic mixture), 30 μ2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum. The diluted preparation is layered onto a Ficoll-Paque gradient to attain the PBMC. According to protocol, the PBMC concentration is adjusted with complete medium, and a proportion of the cell suspension is plated in T75 flasks and incubated for 2 h at 37°C/CO_2. Nonadherent cells are removed and discarded. A mixture containing 10 mL complete medium with 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) is added to the adherent cells remaining in the flask. The flasks are incubated for 48 h at 37 °C/CO_2. Following incubation, the cells are collected, centrifuged, and resuspended in fresh complete medium containing GM-CSF and IL-4 for another incubation for 72 h. Residual T cells and B cells are removed from the cultures on day 5 by two passages over CD2 (pan T cell) and CD19 (pan B cell) immunomagnetic beads. The T- and B-cell-depleted DC are resuspended in fresh cytokine containing complete medium, replated, and incubated for 48 h.

The prepared cell line is collected on the seventh day, washed, resuspended in complete medium containing GM-CSF and IL-4 and 2 mL, and plated in the wells of a six-well culture plate. One milliliter of test chemical also prepared in complete medium with GM-CSF and IL-4 is added to each well, and the plate is incubated for 24 h. Viability of the DC after test chemical exposure is then assessed by propidium iodide dye exclusion using a Coulter Epics XL flow cytometer (Ryan et al. 2004).

EVALUATION

Measurement of cell surface marker expression by flow cytometry after exposure to chemical allergens has been previously established by Ryan et al. (2004). Expression is assessed by flow cytometry using saturating concentrations of fluorochrome-conjugated monoclonal antibodies such as CD86-fluorescein isothiocyanate (FITC), CD83-FITC, CD40-phycoerythrin (PE), CD54-PE, CD80-FITC, and CD1a-FITC. The measurement of the flow cytometer represents the fluorescence intensities of the surface markers. Single parameter histograms are produced and analyzed for changes in mean fluorescence intensity (MFI) of allergen-treated DC as a percentage of the MFI of an untreated control DC (Ryan et al. 2004).

CRITICAL ASSESSMENT OF THE METHOD

Cytotoxicity may decrease cell surface expression and transcription of markers. Thus, if concentration of test chemical induces low cell viability, then measurement of cell surface expression and transcription is limited. In addition, donor variability may introduce differences in fluorochrome-conjugated monoclonal antibody expression (Aiba et al. 1997).

PBMC-derived DC serve as a surrogate marker for Langerhans cells (LC), and the chemical allergen-induced changes in cell surface markers of DC produce a similar pattern to those that occur in LC which are the antigen-presenting cell in the skin that plays a key role in the development of allergic contact dermatitis (Ryan et al. 2005).

MODIFICATION OF THE METHOD

The fluorochrome-conjugated monoclonal antibodies used may be varied. Investigation on one, a combination, or all may be performed. In addition, RNA isolation to confirm the quality of RNA and GeneChip microarray for transcript profiling may be performed after flow cytometry evaluation.

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12.3 Murine Local Lymph Node Assay

PURPOSE AND RATIONALE

This test using animals seems to be the most frequently used predictive test for the identification of skin-sensitizing chemicals or for chemicals prone to cause allergic contact dermatitis. While it is most frequently used for general dermatological investigations, results can be applied to the eyelids as well (Kimber and Basketter 1992; Kimber et al. 1994).

PROCEDURE

Mice (often female mice of the CBA/CA or CBA/J strains) are exposed on the dorsum of both ears with 25 μL of two to five concentrations of the test chemical or to the same volume of the vehicle. Treatment is performed daily for 3 consecutive days. Five days following the start of the test, all mice are injected via the tail vein with 250 μL of phosphate buffered saline, which contains 20 μc Ci of tritiated methyl thymidine. Five hours later, the animals are sacrificed. The draining auricular lymph nodes are excised and pooled. A cell suspension is prepared by gentle disaggregation through a 200-mesh stainless steel gauze. The cells are washed twice with phosphate buffered saline and precipitated with 5% trichloroacetic acid at 4°C. After 12 h, the pellets are resuspended in 1 mL of trichloroacetic acid, and this volume is transferred to scintillation vials. Incorporation of tritiated methyl thymidine is determined.

EVALUATION

Activity is measured according to the increase in lymph node proliferation measured in methyl thymidine incorporation. Chemicals, which cause a threefold or greater response than the vehicle, are considered to have skin-sensitizing potential.

CRITICAL ASSESSMENT OF THE METHOD

Allergens can cause vastly different responses in individuals or the allergic response is very individualized. Nevertheless, this method will identify the majority of skin-sensitizing chemicals and has shown good agreement with human patch tests (Schneider and Akkan 2004).

MODIFICATION OF THE METHOD

The test is performed by using single lymph nodes from individual animals, which allows statistical evaluation of the results (Kimber et al. 1995).

A modification of the procedure uses 125-iododeoxy-uridine but seems to give the same results (Kimber et al. 1995).

A nonradioactive assay has been developed based on the release of IL-2 from lymph nodes of chemically exposed lymph nodes (Hariya et al. 1999). However, this assay is only valid for strong but not weak sensitizing chemicals.

A more recently developed nonradioactive test uses the incorporation of 5-bromo-2'-deoxyuridine as a measure of cell proliferation (Takeyoshi et al. 2004).

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12.4 Isolated Chicken Eye

In 2008, the National Institute of Environmental Health Sciences (NIEHS) in the USA, and in 2009, WHO, both accepted and adopted recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for two methods that can reduce live animal use for ocular safety testing.

The two alternative test methods, the isolated chicken eye (ICE) assay and the bovine corneal opacity and permeability (BCOP) assay, do not involve the use of live animals. These are the first scientifically valid alternative methods to gain US regulatory acceptance for ocular safety testing.

However, due to space limitations, please refer to OECD guideline 438, dated September 7, 2009, for more detailed methodology and evaluation criteria. This section can only summarize purpose and procedure for the reader to consider when confronted with an anticipated ocular corrosive and severe irritant. The other alternative, BCOP, is briefly presented as an option.

PURPOSE AND RATIONALE

The isolated chicken eye (ICE) test method is an in vitro test method that can be used to classify substances as ocular corrosives and severe irritants (EU 2001; UN 2003; US EPA 1996). For the purpose of this description, severe irritants are defined as those that induce ocular lesions that persist in the rabbit for at least 21 days after administration. While it is not considered valid as a complete replacement for the in vivo rabbit eye test such as the Draize test, the ICE is recommended for use as part of a tiered testing strategy for regulatory classification and labeling within a specific applicability domain (ESAC 2007; ICCVAM 2007). If a positive response is obtained using either of the two newly approved alternative methods, the product can be labeled as causing irreversible or severe eye damage (EC 2006) and no live animal testing will be required. If the response is negative, the product is then tested in an animal to confirm that it does not cause severe or irreversible damage, as outlined in OECD Test Guideline 405 (OECD 2002).

The procedures used evaluate the potential ocular corrosivity or severe irritancy of a test substance as measured by its ability to induce toxicity in an enucleated chicken eye. Toxic effects to the cornea are measured by (1) a qualitative assessment of opacity, (2) a qualitative assessment of damage to epithelium based on application of fluorescein to the eye (fluorescein retention), (3) a quantitative measurement of increased thickness (swelling), and (4) a qualitative evaluation of macroscopic morphological damage to the surface. The corneal opacity, swelling, and damage assessments following exposure to a test substance are assessed individually and then combined to derive an eye irritancy classification.

PROCEDURE

Treated corneas are evaluated pretreatment and starting at 30, 75, 120, 180, and 240 min (±5 min) after the posttreatment rinse. The endpoints evaluated are corneal opacity, swelling, fluorescein retention, and morphological effects (e.g., pitting or loosening of the epithelium). All of the endpoints, with the exception of fluorescein retention (which is determined only at pretreatment and 30 min after test substance exposure) are determined at each of the above time points. Photographs are advisable to document corneal opacity, fluorescein retention, morphological effects, and, if conducted, histopathology.

After the final examination at 4 h, users are encouraged to preserve eyes in an appropriate fixative (e.g., neutral buffered formalin) for possible histopathological examination. Corneal swelling is determined from corneal thickness measurements made with an optical pachymeter on a slit lamp microscope. It is expressed as a percentage and is calculated from corneal thickness measurements according to the following formula:

$$\left(\frac{\textit{corneal thickness at time } t - \textit{corneal thickness at time} = 0}{\textit{corneal thickness at time} = 0}\right) \times 100$$

Table 12.1 Corneal opacity scores

Score	Observation
0	No opacity
0.5	Very faint opacity
1	Scattered or diffuse areas; details of the iris are clearly visible
2	Easily discernible translucent area; details of the iris are slightly obscured
3	Severe corneal opacity; no specific details of the iris are visible; size of the pupil is barely discernible
4	Complete corneal opacity; iris invisible

Table 12.2 Fluorescein retention scores

Score	Observation
0	No fluorescein retention
0.5	Very minor single cell staining
1	Single cell staining scattered throughout the treated area of the cornea
2	Focal or confluent dense single cell staining
3	Confluent large areas of the cornea retaining fluorescein

The mean percentage of corneal swelling for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal swelling, as observed at any time point, an overall category score is then given for each test substance. Corneal opacity is calculated by using the area of the cornea that is most densely opacified for scoring. The mean corneal opacity value for all test eyes is calculated for all observation time points. Based on the highest mean score for corneal opacity, as observed at any time point, an overall category score is then given for each test substance (Table 12.1).

The mean fluorescein retention value for all test eyes is calculated for the 30-min observation time point only, which is used for the overall category score given for each test substance (Table 12.2).

Morphological effects include "pitting" of corneal epithelial cells, "loosening" of epithelium, "roughening" of the corneal surface, and "sticking" of the test substance to the cornea. These findings can vary in severity and may occur simultaneously. The classification of these findings is subjective according to the interpretation of the investigator.

EVALUATION

The ICE test method is an organotypic model that provides short-term maintenance of the chicken eye in vitro. In this test method, damage by the test substance is assessed by determination of corneal swelling, opacity, and fluorescein retention. While the latter two parameters involve a qualitative assessment, analysis of corneal swelling provides for a quantitative assessment. Each measurement is either converted into a quantitative score used to calculate an overall irritation index or assigned a qualitative categorization that is used to assign an in vitro ocular corrosivity and severe irritancy classification. Either of these outcomes can then be used to predict the in vivo ocular corrosivity and severe irritation potential of a test substance.

The overall in vitro irritancy classification for a test substance is assessed by reading the irritancy classification that corresponds to the combination of categories obtained for corneal swelling, corneal opacity, and fluorescein retention and applying the overall in vitro irritancy classification scheme. The ICE test method has an overall accuracy of 83–87%, a false positive rate of 6–8%, and a false negative rate of 41–50% for the identification of ocular corrosives and severe irritants, when compared to in vivo rabbit eye test method data.

CRITICAL ASSESSMENT OF THE METHOD

A limitation of the test method is that, although it takes into account some of the ocular effects evaluated in the rabbit ocular irritancy test method and to some degree their severity, it does not consider conjunctival and iridal injuries. Also, although the reversibility of corneal lesions cannot be evaluated per se in the ICE test method, it has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury can be used to distinguish between irreversible and reversible effects (Maurer et al. 2002).

Finally, the ICE test method does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure. Based on the purpose of this assay (i.e., to identify ocular corrosives/severe irritants only), false negative rates are not critical since such substances would be subsequently tested in rabbits or with other adequately validated in vitro tests, depending on regulatory requirements, using a sequential testing strategy in a weight-of-evidence approach. However, investigators could consider using this test method for testing all types of substances (including formulations), whereby a positive result could be accepted as indicative of an ocular corrosive or severe irritant response.

MODIFICATION OF THE METHOD

Even if an ocular corrosive or severe irritant classification is not obtained for a test substance, ICE data can be useful in conjunction with test data from the in vivo rabbit eye test or from an adequately validated in vitro test to further evaluate the usefulness and limitations of the ICE test method for identifying nonsevere irritants and nonirritants.

Furthermore, the aforementioned bovine corneal opacity and permeability (BCOP) assay is an approved in vitro test method that can be used under the same circumstances and same specific limitations to classify substances as ocular corrosives and severe irritants, in a similar but somewhat less reliable manner (cf. OCDE guideline 437 dated September 7, 2009). Toxic effects to the cornea are measured by (1) decreased light transmission (opacity) and (2) increased passage of sodium fluorescein dye (permeability). The opacity and permeability assessments of the cornea following exposure to a test substance are combined to derive an in vitro irritancy score (IVIS), which is used to classify the irritancy level of the test substance.

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12.5 The Draize Test

PURPOSE AND RATIONALE

This test uses the eyes of a live animal, preferentially rabbits, to obtain information of the possible ocular toxicity of various chemicals. This test is most closely related to the human situation where these chemicals will later be used as ocular drugs or cosmetics or where the human eye might be exposed accidentally to these substances.

This is a classical test developed about 60 years ago (Draize et al. 1944). For further description of methods see Chaps. 8 "Peripheral Nervous System" and 15 "Skin Pharmacology".

PROCEDURE

For acute toxicity studies, 0.1 mL of a solution or ointment of the test substance is instilled into the conjunctival sacs of three to six rabbits. Readings are then taken 1, 24, and 48 h after instillation and are evaluated. If residual injuries still exist, another reading is performed after 96 h.

EVALUATION

The evaluation occurs according to a slight variation of the scoring system developed by Friedenwald et al. (1944) and presented in abbreviated form in Table 12.3.

CRITICAL ASSESSMENT OF THE METHOD

The eyes of the rabbit differ in certain aspects from the eyes of humans. They are more sensitive, have a lower tear production and blink frequency, and posses a nictitating membrane. Nevertheless, the Draize test predicts human ocular toxicity correctly in 85% cases but overestimates in 10% and underestimates in 5% (Gad and Chengelis 1991). In addition, ethical concerns have been raised in the use of animals, and benefit vs. risk of these tests for the protection of the human eye must be carefully evaluated.

MODIFICATION OF THE METHOD

Although not mentioned directly for the eye in the original paper, modifications suggested for skin testing are also applicable to the eye. These include varying the dose, using multiple applications, and washing the eyes after an application to study reversibility of observed toxicity (Draize et al. 1944).

Table 12.3 The total score is the sum of all the individual scores

Part	Tissue score
Cornea	(A) Opacities 1 or 2
	(B) Area of cornea involved 3 or 4
	Score equals A × B × 5; maximum = 80
Iris	(A) Folds, swelling, injections 1
	No reaction to light, hemorrhage 2
	Score A × 5; maximum = 10
Conjunctivae	(A) Redness, beefy red 1, 2, or 3
	(B) Chemosis, swelling, lid closing 1, 2, 3, or 4
	(C) Discharge, amounts 1, 2, or 3
	Score (A + B + C) × 2; maximum = 20

Instead of applying the test substance topically, the substance can be injected into the eye and they can then be observed for pathological changes (Veckeneer et al. 2001).

This test has been used since then many times, each with slight variations, for instance, exposure of only one eye to the test substance and no exposure of the other eye or exposure of the other eye to the vehicle. To minimize discomfort to the animal, a local anesthetic is sometimes instilled before use of the test substance. The qualitative scoring system has been extended using also measures of eye blinks or wipes. Examinations can also include magnifying glasses, slit lamp examination, fluorescein staining, and photodocumentation. After observed toxicity, animals are sacrificed; the eyes are removed and subjected to microscopic and histological examinations.

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Side Effects of Central Analgesic Drugs

13

Lars Arendt-Nielsen

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13.1 General Considerations

Morphine, as the prototype of a central analgesic drug, has characteristic adverse effects that are relevant to the treatment of patients. Important among these effects are the following:

- Respiratory depression
- Decrease of body temperature
- Tolerance
- Physical dependence
- Abuse liability

Since the ratio between therapeutic effect and adverse effects varies among opioids, great effort has been made to synthesize compounds with a better ratio of antinociceptive activity versus adverse side effects. This effort to discover better, safer compounds necessitated the development of laboratory methods that are sensitive to and, therefore, could be used to quantify the adverse effects of opioids. Moreover, the discovery of several distinct types of receptors that can interact with opioids or with endogenous peptides (e.g., μ, κ, and δ receptors) allows a more selective classification of agonists and antagonists.

13.2 Test for Respiratory Depression

PURPOSE AND RATIONALE

Respiratory depression is one of the most prominent adverse effects of μ opioid agonists (e.g., morphine). The frequency of breathing and the inspiratory volume can be affected differently by drugs and have to be measured.

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PROCEDURE

Male or female rabbits with a body weight between 2.5 and 3 kg are placed in restraining cages. A mask is placed over the nose of the animals which is connected with a valve opening on exhaling and closing on inhaling. The frequency of breathing is recorded from the opening and closing of the valve. The inspiratory volume is measured with a gasometer. Intravenous injection of morphine in doses between 1 and 10 mg/kg results in a dose-dependent decrease in respiratory frequency and respiratory volume. The doses are increased logarithmically after the effect of the preceding dose has subsided.

EVALUATION

Three animals are used for the test compound and the standard. Dose–response curves of the effect on respiratory frequency and volume are compared. While μ opioid agonists decrease respiratory function, κ opioid agonists either increase or have no effect on respiratory function. The magnitude of respiratory depression produced by μ opioid agonists is related to their efficacy at opioid receptors with low-efficacy agonists such as nalbuphine having much less effect on respiration as compared to morphine.

MODIFICATIONS OF THE METHOD

Nelson and Elliott (1967) compared the effects of morphine, morphinone, and thebaine on respiration and oxygen consumption in rats, and Murphy et al. (1995) provided a method for distinguishing central and peripheral mechanisms of respiratory depression in rats.

Ling et al. (1983, 1985) measured blood gas values (pO_2, pCO_2, pH) in unrestrained rats via an arterial cannula. pO_2 and pH decreased, whereas pCO_2 increased after morphine.

Studies in rhesus monkeys have compared the respiratory depressant effects of opioids to their effects on other behavior (Howell et al. 1988; Butelman et al. 1993; Gerak et al. 1994).

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13.3 Decrease of Body Temperature

Opioids can have profound effects on body temperature; however, depending on the particular conditions under which the compound is evaluated, either increases or decreases in body temperature can be observed. Route of administration, dose, age of the subject, as well as ambient temperature contribute to the type of response opioids exert on body temperature. Thus, in rats, a single i.p. injection of morphine can produce hypo- or hyperthermia; restraint exaggerates the former response and attenuates the latter. Since this adverse side effect of opioid drugs is shared by several other compounds with no central antinociceptive activity, the test cannot be regarded as specific.

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13.4 Methods for the Study of Tolerance

PURPOSE AND RATIONALE

Repeated tolerance treatment with opioid agonists can decrease sensitivity to drug effects, thereby limiting the effectiveness of analgesics and requiring an increase in dose for recovery of antinociceptive effects. The radiant heat or the hot plate method for testing antinociceptive activity in mice is adapted to measure drug-induced changes in the sensitivity to a noxious stimulus.

PROCEDURE

Male mice (10–12 per condition) with an initial weight of 18–20 g are used. They are placed in restraining cages. A noxious stimulus is produced by an intense light beam directed to the proximal part of the tail. The subject can respond to this stimulus by flicking its tail. The reaction time, the interval between stimulus onset and response, is automatically measured. A maximum time of exposure to the stimulus (e.g., 12 s cutoff time) prevents tissue damage. Prior to drug administration, two control measures of reaction time are obtained for each animal. After administration of the drug, the test is repeated 15, 30, and 60 min after subcutaneous injection or 30, 60, and 120 min after oral administration. In this way, time of peak activity can be determined. Mice showing a reaction time of the average control value plus two times the standard deviation in the control experiment are regarded as positive. Complete dose–response curves are determined and ED_{50} values are calculated. Subsequently, the animals are treated for 5 days once everyday with a dose which is four times higher than the ED_{50} in the first experiment. On the following day, dose–response curves are determined using at least three doses. The ED_{50} is calculated again.

EVALUATION

Reduced effectiveness of a fixed dose and/or the need for larger doses to obtain a constant response indicates the development of tolerance. ED_{50} values obtained before and after repeated daily treatment are compared to assess the magnitude of tolerance.

CRITICAL ASSESSMENT OF THE METHOD

Tolerance is observed not only with opioid agonists but also with many other drugs including barbiturates, benzodiazepines, and ethanol. The measurement of antinociception after single and repeated administration, therefore, has to be regarded as a primary test. Moreover, a decrease in the potency of a drug after daily drug treatment, while providing evidence for tolerance, does not give insight to the mechanism by which tolerance has developed (i.e., pharmacodynamic or pharmacokinetic). Demonstration that the antinociceptive effects of a new drug do not decrease after repeated daily treatment with high doses indicates that it is not necessary to escalate dose in order to maintain effectiveness and represents the first step for establishing the absence of tolerance liability.

MODIFICATIONS OF THE METHOD

Other authors (e.g., Glassman 1971) injected the dose which induced a full antinociceptive effect in mice twice daily for a period of 21 days and evaluated the stepwise decay of effectiveness. After 21 days, the effect of 10 mg/kg morphine or 30 mg/kg meperidine i.p. decreased to approximately 50% of the value of the first day.

Langerman et al. (1995) evaluated the acute tolerance to continuous morphine infusion up to 8 h in the rat with various doses using the hot plate and the tail flick assay. Tolerance was observed with the hot plate assay, but not with the tail flick assay, suggesting tolerance development at a supraspinal site.

Smith et al. (2003) used twice-daily injections of morphine and implantation of morphine-containing pellets to study mechanisms of opioid tolerance in mice.

Riba et al. (2002) showed that the role of δ opioid receptors in modifying the antinociceptive effects of μ opioid agonists changes during morphine tolerance.

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13.5 Tests for Physical Dependence

PURPOSE AND RATIONALE

Withdrawal and physical dependence phenomena, either after abrupt cessation of chronic treatment or after administration of a pharmacologic antagonist (e.g., naltrexone), can be observed in a variety of nonhuman species. Importantly, the withdrawal that emerges in nonhumans topographically resembles important features of withdrawal in humans. On this basis, tests for drug dependence and withdrawal have been developed for monkeys (Seevers 1963; Seevers and Deneau 1963; Aceto 1990; Woods et al. 1993), dogs (Martin et al. 1974, 1976), rats (Buckett 1964; Cowan et al. 1988), and mice (Way et al. 1969; VonVoigtlander and Lewis 1983). Two general approaches are used to evaluate physical dependence potential: primary physical dependence and single-dose substitution. In the former, the test substance is administered repeatedly over days, and the assessment of dependence (i.e., withdrawal) occurs either after discontinuation of drug treatment or by administration of a pharmacologic antagonist. Precipitated withdrawal studies are warranted only when the mechanism or site of action of the test substance is known and when an appropriate pharmacologic antagonist is available. In a single-dose substitution study, a reference substance (e.g., morphine) is administered repeatedly over days; after discontinuation of treatment with the reference substance, and when reliable withdrawal signs have emerged, the test substance is assessed for its ability to attenuate withdrawal signs. In this type of study, the test substance can be administered just once to assess its acute withdrawal-reversing effects or can be administered repeated over days (i.e., replace the reference substance) and subsequently discontinued followed by assessment of withdrawal signs.

A well-established in vitro procedure has also been used to test for opioid dependence (i.e., antagonist-precipitated withdrawal) in opioid-treated guinea pig ileum (Rodríguez et al. 1978; Collier et al. 1979; Cruz et al. 1991).

13.5.1 Opioid Withdrawal Responses in the Guinea Pig Ileum Made Dependent In Vitro

A 40-cm long segment of the small intestine of male guinea pigs weighing 600–900 g is removed and placed in a low-magnesium Krebs solution. The terminal section of the guinea pig ileum is used after discarding the portion of 10 cm closest to the ileocecal junction. The ileum is cut in eight 3-cm long segments. The intestinal content is gently removed with the aid of a glass rod. To produce opioid dependence, segments are incubated in 500 mL Erlenmeyer flasks containing 480 nM morphine in 250 mL Krebs solution saturated with a 95% O_2/5% CO_2 gas mixture at a temperature ranging between 4°C and 6°C for 1–48 h. One hour before completion of the incubation time, the segments are removed, placed in glass chambers with 50 mL Krebs solution bubbled with 95% O_2/5% CO_2 gas mixture at 36°C, and mounted on a vertical Nichrome electrode with one edge fixed to the chamber plug and the opposite fixed to an isometrical force transducer (Grass FT 03) connected to a polygraph for recording the contractile activity of the longitudinal muscle. The ilea are set up with an initial tension of 1 g and left for a period of 30 min for stabilization. Thereafter, all segments are electrically stimulated with supramaximal rectangular pulses (10–40 V) of 0.5-ms duration at a frequency of 0.1 H.

Five min before naloxone administration, the electrical stimulation is suspended. The response to naloxone is recorded by administration of up to 100 nM. The response to the antagonist is recorded for 20 min, and thereafter, the electrical stimulation is reinitiated and maintained for 10 min.

Thirty-five minutes after naloxone administration, various doses of nicotine are administered to provide a positive control. For comparisons, a concentration–response curve for nicotine (1, 3.1, 5.6, 10, 31, and 56 μM) is obtained in untreated ilea.

Moreover, the concentration–response curve for nicotine is obtained in ilea that are (1) exposed to 10 nM naloxone for 20 min, (2) exposed to 480 nM of morphine for 1 h, or (3) pretreated for 10 min with 3 or 10 nM of naloxone and exposed to 480 nM of morphine for 1 h. The response to nicotine is attenuated after pretreatment with morphine, and this attenuation is dose-dependently antagonized by naloxone.

There is a correlation between the response to supramaximal electrical stimulation and the withdrawal response (contraction) precipitated with 100 nM naloxone as well as a correlation between withdrawal and nicotine response after long-term exposure (12–48 h) with 480 nM morphine.

13.5.2 Test for Physical Dependence in Rats

Male albino rats receive either morphine or saline i.p. twice daily. The starting dose of morphine is 20 mg/kg, followed by 40 mg/kg increments daily until by day 11 the level is 420 mg/kg. Maintenance at 400 mg/kg is continued through day 20. The test compound is similarly administered to groups of 10 rats each. The daily increments have to be adjusted to a maximum level that is not lethal for the duration of the experiment.

Primary physical dependence capacity is measured on days 11 and 17 when all animals receive an injection of 10 mg/kg of naltrexone or naloxone i.p. in the morning. Signs of withdrawal are recorded during a 30–60-min period. Rats are scored for the presence or absence of withdrawal signs (e.g., diarrhea, wet-dog-type shaking) using standardized scoring.

A single-dose substitution study substitutes either a single dose or multiple doses (from day 20 through day 23) of the test compound in morphine-dependent rats; scoring for suppression of withdrawal occurs on days 20–23 and after discontinuation of the test substance.

13.5.3 Test for Physical Dependence in Monkeys

Groups of three to four rhesus monkeys (3–6 kg body weight) receive morphine four times daily (s.c. or i.m.) beginning with a dose of 1.0 mg/kg. Progressively, the unit dose is increased to a final dose of 3.2 mg/kg/6 h. The test substance is similarly administered to groups of three to four monkeys. For the test compound, the daily increments in drug administration are adjusted to a maximally tolerated (nontoxic) dose and frequency of injection. Both groups of monkeys are then maintained at their appropriate dose levels for a minimum of 112 days. On days 35, 60, and 91, 1 mg/kg of naltrexone or naloxone is administered (s.c. or i.m.) in the morning. On days 50 and 112, all doses are omitted for 24 h. Signs of withdrawal are recorded during a 30–60-min period using standardized scoring (e.g., Katz 1986; Brandt and France 1998).

CRITICAL ASSESSMENT OF THE METHOD

The emergence of withdrawal signs after discontinuation of drug treatment is dependent of the duration of action of the treatment compound. Thus, after discontinuation of morphine treatment, withdrawal reliably emerges within 24 h. For drugs with an unusually long duration of action (e.g., buprenorphine), observations for withdrawal signs need to occur over longer periods of time (e.g., several days). Opioid antagonists will precipitate withdrawal in animals treated with opioid agonists. If a test substance has actions at nonopioid receptors, a negative result with naltrexone or naloxone in a precipitated withdrawal study will not provide useful information regarding dependence potential. Thus, both precipitated withdrawal and abstinence-induced withdrawal need to be studied for test compounds.

Rhesus monkeys have been used extensively for assessing physical dependence potential of opioid agonists. An excellent correlation between humans and rhesus monkeys has been shown regarding the physical dependence liability of opioids, although there are some compounds for which the relative potency between humans and monkeys is not what is predicted from other data. Nonhuman species can also be used to assess physical dependence potential of other classes of drugs, including sedative/hypnotics. Physical dependence potential alone cannot be assumed to predict abuse liability because some drugs that are not abused (e.g., κ opioids) can produce marked physical dependence (Gmerek et al. 1987).

MODIFICATIONS OF THE METHOD

Mouse jumping as a simple screening method to estimate the physical dependence capacity of opioid

agonists has been recommended by Saelens et al. (1971). Mice receive seven i.p. injections over 2 days. The test compound is given at doses increasing in multiples of two until a maximally tolerated dose is reached. Two hours after the last injection, the animals receive an i.p. injection of 100 mg/kg naloxone and are placed individually into glass cylinders. The number of jumps is recorded during 10 min.

Kest et al. (2002) compared naloxone-precipitated withdrawal jumping in several strains of mice after acute or multiple injections of morphine or after chronic infusion of morphine with osmotic minipump.

Yoshimura et al. (1993) studied the physical dependence on morphine induced in dogs via the use of mini-osmotic pumps. Naloxone-precipitated withdrawal signs were recorded such as hyperactivity, biting, digging, tremors, nausea, hyperthermia, and increased wakefulness and by EEG activation in the amygdala and hippocampus, followed by a dissociation of the EEG in the cortex (fast wave) from that in the limbic (slow wave) system, increased heart rate, and raised blood pressure. Withdrawal signs were more severe in animals with mini-osmotic pumps than in those receiving the same dose by syringe injections.

Pierce and Raper (1995) studied the effects of laboratory handling procedures on naloxone-precipitated withdrawal behavior in morphine-dependent rats, and Gellert and Holtzman (1978) used access to drug in drinking solutions to study morphine dependence and withdrawal in rats.

Pierce et al. (1996) used slow-release emulsion formulations of methadone to induce dependence in rats. Withdrawal was induced following i.p. challenge with either naloxone or saline, and dependence was assessed in terms of the presence or absence of characteristic withdrawal signs.

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13.6 Tests for Abuse Liability

13.6.1 General Considerations

Drug abuse liability often occurs in the absence of physical dependence. Various terms have been used to describe abuse-related phenomena that are not specifically linked to physical dependence (Deneau 1964), e.g., psychological dependence, and laboratory procedures have been developed that are predictive of abuse-related effects in humans. For example, based on the early observations of Olds (Olds et al. 1956; Olds 1979) on intracranial self-stimulation, procedures have been developed for studying drug-induced changes in brain-stimulation reward (Kornetsky and Bain 1990). Moreover, self-administration procedures are used widely to study the reinforcing effects of a variety of drugs (Deneau et al. 1969; Hoffmeister 1979; Littmann et al. 1979; Woolverton and Schuster 1983; Bozarth 1987; Meisch and Carroll 1987; Weeks and Collins 1987; Yokel 1987; Woolverton and Nader 1990), and, more recently, conditioned place preference has been used in studies on abuse liability. Furthermore, drug discrimination procedures can be used to complement other assays of abuse liability; discrimination procedures have the advantage of having a high degree of pharmacologic selectivity (Holtzman 1983; Brady et al. 1987; Colpaert 1987; Overton 1987; Hoffmeister 1988; Holtzman 1990). A general discussion of abuse liability assessment, as it relates opioid analgesics as well as nonanalgesic drugs, appears elsewhere in this volume (Porsolt et al. 2002).

13.6.2 Drug Discrimination Studies

PURPOSE AND RATIONALE

Many drug discrimination laboratories have used simple two-choice discrimination methods to investigate the mechanism or site of action of new compounds by examining those compounds in animals trained to discriminate a reference substance and known drug of abuse (Shannon and Holtzman 1976; Holtzman 1983; Brady et al. 1987; Shannon and Holtzman 1986; Colpaert 1987; Overton 1987; Hoffmeister 1988; Carboni et al. 1989; Holtzman 1990).

PROCEDURE

Rats are trained to press one of two choice levers to avoid or to escape electric foot shock which is delivered intermittently beginning 5 s after the start of the trial. The occurrence of a trial is signaled by the illumination of a light in the operant chamber. A third (observing) lever is mounted in the wall of the chamber opposite the two choice levers and must be pressed before the choice response is made. This contingency prevents the rat from persevering on a single response lever; thus, the choice response in each trial is relatively independent of the consequences of choice responses in the preceding trials of the session. The rats are tested in 20-trial sessions. Animals are trained to discriminate a prototype of the drug of interest. Morphine and fentanyl have served well as training drugs for exploring the discriminative effects of classical μ opioid drugs. Training often occurs more rapidly when the dose of the training drug is the largest dose that does not disrupt behavior. For discrimination training, the animal is placed in the operant chamber and trained to perform the required response, initially under a schedule of continuous reinforcement where a single response on either lever postpones or terminates shock. As performance increases, the response requirement is increased progressively across days (e.g., to a maximum of 10 [fixed ratio 10]) and discrimination training commences, whereby responding on one lever postpones or terminates shock. In two-choice procedures, the left and the right choice lever are designated for drug and vehicle, respectively, for half of the animals in a group; the lever designation is

reversed for the other half of the animals. Acquisition of the discrimination is a function of the drug, training dose, and the number of training sessions. Training continues until the subject reaches predetermined performance criteria, which typically could be as follows: at least 80% of the total session responses on the injection (drug or vehicle) appropriate lever and less than one fixed-ratio value (e.g., 10) of responses on the injection-inappropriate lever prior to delivery of the first reinforcer (e.g., shock postponement or termination) for 6 consecutive training sessions. A morphine discrimination can be established in rats, according to these criteria, in 6–12 weeks. Once stable discrimination performance has been achieved, tests of generalization to novel drugs can be interposed among the training sessions. During test sessions, the reinforcer is available after completion of the response requirement on either lever. Complete dose–response curves for the training drug (e.g., morphine) and the test drug are obtained. In cases where the test drug does not produce responding on the training-drug-appropriate lever, the test drug should be evaluated up to doses that decrease rates of lever pressing or until other behavioral effects are observed, in order to insure that the compound is evaluated up to behaviorally active doses.

EVALUATION

Results of the stimulus-generalization test usually are evaluated with the quantitative or graded method, whereby the amount of responding on the training-drug-associated lever is expressed as a percentage of the total number of responses during a test (i.e., responding on the drug-appropriate lever plus responding on the vehicle-appropriate lever). This percentage is than compared with the percentage of drug-appropriate responses normally engendered by the training drug (reference standard). The discriminative stimulus effects of the test drug substitute for those of the training drug if the maximal percentages of drug-appropriate responding are not significantly different from each other. When stimulus control of behavior transfers from one drug to another, it can be inferred that the test drug produced discriminative effects that are similar to those of the training drug. An advantage of this procedure is that it is pharmacologically very selective and that the discriminative stimulus effects of drugs are related to and predictive of subjective effects in humans.

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures display a high degree of pharmacologic selectivity. While test drugs that resemble the training drug result in dose-dependent drug-appropriate lever selection, drugs that are pharmacologically dissimilar to the training drug typically cause responding on the choice lever that is appropriate for the drug vehicle, up to behaviorally active doses. The pharmacologic selectivity of these procedures permits differentiation not only among compounds acting on different receptors or neurochemical systems (e.g., dopamine receptors vs opioid receptors) but also among compounds acting on different subtypes of receptors within the same receptor class (e.g., μ vs κ opioid receptors). Because of the importance of pharmacokinetic factors to the overall abuse liability of drugs, and because drug discrimination procedures are relatively insensitive to pharmacokinetic factors, as compared to self-administration procedures, positive results from a drug discrimination study are not in themselves sufficient to predict abuse liability. Along with other measures of drug action, results of drug discrimination studies are used to predict the likelihood of new compounds having abuse liability. Typically, self-administration data are used along with drug discrimination data, since these two assays are sensitive to different, though related aspects of drug activity.

MODIFICATIONS OF THE METHOD

Drug discrimination studies are performed in a variety of species including squirrel monkeys, rhesus monkeys, pigeons, gerbils, and mice (Hein et al. 1981; Herling and Woods 1981; Bertalmio et al. 1982; Dykstra et al. 1987, 1988; France and Woods 1993; France et al. 1994, 1995; Jarbe and Swedberg 1998; Shelton et al. 2004; Stolerman et al. 2004). Operant responding can be maintained with different reinforcers, including food, liquids, and aversive stimuli (e.g., electric shock). The pharmacologic selectivity of drug discrimination procedures is particularly evident with opioid agonists that bind selectively to different receptor subtypes. For example, monkeys trained to discriminate injections of the μ agonists codeine, etorphine, or alfentanil generalize to other μ agonists and not to nonopioid drugs, to opioid antagonists, or to opioid agonists that produce their behavioral effects through other (non-μ) opioid receptors. Conversely, monkeys trained to discriminate a κ agonist such as

ethylketocyclazocine or U-50,488 generalize to κ opioid agonists and not to μ opioid agonists, opioid antagonists, or nonopioid drugs (Woods et al. 1993; France et al. 1994).

Meert et al. (1989) used drug discrimination studies to characterize risperidone as an antagonist of LSD.

Meert and Janssen (1989) and Meert et al. (1990) showed differences between ritanserin and chlordiazepoxide in drug discrimination procedures.

Δ^9-Tetrahydrocannabinol discrimination in rats has been proposed as model for cannabis intoxication in humans (Balster and Prescott 1990).

An attempt was made to measure opiate abstinence responses in the guinea pig ileum made dependent in vitro (Cruz et al. 1991).

The drug discrimination method has also been applied to study anxiolytic drugs using pentylenetetrazol at subconvulsive doses (Sherman and Lal 1979, 1980; Sherman et al. 1979; Lal and Sherman 1980).

The conditioned taste aversion procedure has been described as a more rapid alternative to two-lever operant procedures in drug discrimination research (Garcia et al. 1955; van Heest et al. 1992).

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13.6.3 Conditioned Place Preference Paradigm

PURPOSE AND RATIONALE

The conditioned place preference paradigm has been used widely to examine behavioral actions that are thought to be related to positive reinforcing effects, as measured by other procedures, such as self-administration (van der Kooy 1987; Hoffman 1989; Tzschenke 1998; Self and Stein 1992). Particular environmental stimuli are paired with the presence or absence of a presumed reinforcer (e.g., drug or

food), and later, in the absence of that reinforcer, animals are tested for their preference for either environment.

PROCEDURE

To induce place preference with food, a food-restricted animal is exposed to an experimental chamber that consists of two compartments (which differ in floor texture and wall color) and that are separated by a removable barrier. In some iterations of this procedure, the two compartments are joined by a small tunnel or a third (neutral) compartment. On alternate days, the animal is confined to one or the other compartment, with food available in only one of the compartments. Thus, food is selectively paired with one of the distinctive environments. After several (e.g., 4 in each compartment for a total of 8) conditioning sessions, the animal is placed in the same chamber without the barrier in place (for procedures that use a third [neutral] compartment, the animal is placed in that compartment and otherwise in the middle of the chamber). In the absence of the reinforcer (e.g., food), animals demonstrate a relative increase in the amount of time spent in the environment that was paired with food as compared to the compartment that was not paired with food. Place conditioning with drugs is conceptually similar and involves the differential pairing of drug effect with one compartment and the absence of drug effect (vehicle) with the other. Drugs can be administered by various different routes (Amalric et al. 1987; Bals-Kubik et al. 1988, 1990; Iwamoto 1988; Shippenberg and Herz 1987), and usually animals are placed in the chamber immediately after drug administration for a 40-min conditioning session.

Male Sprague-Dawley rats weighing 250–300 g are used for these studies. When drugs are to be administered intracerebroventricularly, rats are anesthetized with 60 mg/kg i.p. sodium hexobarbital, and 23-gauge guide cannulae aimed at the lateral ventricle ($AP = -0.9$ mm, L = +1.5 mm, $DV = 3.5$ mm) (Paxinos and Watson 1982) are stereotaxically implanted; conditioning commences 1 week later.

The apparatus consists of 30 × 60 × 30 cm Plexiglas boxes with a clear Plexiglas front. For conditioning sessions, each box is divided into two equal-sized compartments by means of a sliding wall. One compartment is white with a textured floor, the other black with a smooth floor. For testing, the central wall is raised 12 cm above the floor to allow passage from one compartment to the other.

Conditioning sessions are conducted once a day for 8 days and consist of administering drug or its vehicle on alternate days. The rats are immediately confined to one compartment of the box following drug injection and to the other compartment following vehicle injection. All conditioning sessions last 40 min. Test sessions are carried out 1 day after the last training session and in the absence of drug. The rats are placed in a neutral position (either in the center or in the neutral compartment) of the test box and allowed free access to both sides of the box for 15 min. A video camera with integrated stopwatch is used for data recording. Alternatively, photocells mounted along the sides of each compartment can be used to electronically monitor the location of the subject in the apparatus. The time spent in each compartment is assessed by visual analysis of the recorded videotape or by data collected through photocell beam breaks.

For intracerebroventricular injections, a 30-gauge injection needle is attached to a microsyringe via polyethylene tubing. The drug solutions are administered over a 60-s period, and the injection needles are left in place for an additional 30 s to ensure complete delivery of the solution. For antagonism tests, groups of rats receive an intracerebroventricularly injection of the antagonist (naltrexone or naloxone) or vehicle 10 min before the microinjection of the conditioning drug. At the end of the experiments, the rats are anesthetized and sacrificed by decapitation. The brains are removed and sectioned in a cryostat to verify the location of the cannulae. Alternatively, antagonists can be administered systemically.

EVALUATION

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place and are expressed as means ± SE. In cases where animals show a bias toward one compartment prior to conditioning, drug conditioning can be established with the nonpreferred compartment, thereby increasing the confidence that preference for that compartment is specifically related to drug administration. Dose–response curves are analyzed with a one-way ANOVA. The Wilcoxon test, in which the time spent in the drug associated place is compared to that in the vehicle-paired place, is used to determine whether individual doses produce significant conditioning. A one-way ANOVA followed by the Student Newman–Keuls test is used to

determine the statistical significance of effects of the antagonist pretreatment.

Unlike drug discrimination, conditioned place preference is not pharmacologically selective insofar as drugs from many different classes (e.g., opioids, ethanol, stimulants) generate positive results. Generally, there is a strong positive correlation between drugs that can be used to establish conditioned place preference and those that are positive reinforcers by other measures (e.g., i.v. self-administration); however, one of the most effective reinforcers in self-administration studies, that is also widely abused by humans, does not unanimously generate strong conditioned place preference in nonhumans – cocaine. Thus, results from conditioned place preference studies should be used in concert with results from other measures of reinforcing effects (e.g., self-administration). For the purpose of opioids, in general, μ agonists are effective for establishing place preference, whereas κ agonists are not. In fact, μ antagonists or κ agonists can generate place aversion (e.g., Sante et al. 2000).

MODIFICATIONS OF THE METHOD

In order to distinguish place preference and place aversion, place-conditioning behavior can be expressed by a difference in preference pre- and postconditioning, where post- and prevalues are the difference in time spent in the preferred and the nonpreferred sides in the postconditioning and preconditioning tests, respectively. Positive values indicate preference and negative values aversion (Kitaichi et al. 1996). For nonbiased procedures, where animals do not show an inherent preference for either compartment, results are presented simply as a difference score (i.e., time spent in the drug-paired compartment minus time spent in the vehicle-paired compartment).

In addition to place preference, others (Mucha and Herz 1985; Broadbent et al. 2002) used taste preference conditioning.

Cunningham (Bormann and Cunningham 1998; Gabriel et al. 2004) used the same chamber for training and testing with the exception that floor texture varied according to treatment condition. Thus, drug and vehicle were paired with different floor textures, and during test sessions, the time spent on each section of a floor comprising the two different textures (half of the floor with each) was used as an index of preference or aversion. This procedure has the advantage that the size of the test chamber is not different from the size of the training chamber.

Perks and Clifton (1997) used sucrose solution to generate a place preference which was subsequently devalued using a LiCl taste aversion procedure.

Brockwell et al. (1996) described a computerized system for the simultaneous monitoring of place conditioning and locomotor activity in rats consisting of four independent conditioning boxes, each equipped with six pairs of photosensors connected to an experiment controller, an electronic board containing a microprocessor and a programmable timer for storing both instructions and data.

Steinpreis et al. (1996) investigated place preference in Sprague-Dawley rats treated with graded i.p. doses of methadone. Place preference for methadone peaked at 4 mg/kg, and aversion was produced at 10 mg/kg.

Using the conditioned place preference paradigm, Mamoon et al. (1995) assessed the rewarding properties of butorphanol in comparison to morphine after unilateral microinjections into the ventral tegmental area of male Lewis rats.

Gaiardi et al. (1997) assessed rewarding and aversive effects of buprenorphine by place preference and taste aversion conditioning. After subcutaneous doses of 0.025, 0.050, and 0.100 mg/kg, buprenorphine caused a significant increase in the amount of time spent on the drug-paired compartment, but no significant decrease of saccharin consumption. Rewarding and aversive effects did not occur within a similar dose range.

Contarino et al. (1997) found no tolerance to the rewarding effects of morphine.

Tsuji et al. (1996) studied the effect of microinjections of GABA agonists and antagonists into the ventral tegmental area of Sprague-Dawley rats on morphine-induced place preference.

Sufka (1994) recommended the conditioned place preference paradigm as a novel approach for assessing effects of opioids in chronic pain induced in rats by unilateral injections of Freund's adjuvant into the hind paw.

Conditioned place avoidance was found after naloxone which was attenuated by clonidine (Kosten 1994).

In addition to morphine and other μ opioid agonists, other drugs with known or putative abuse liability

were tested in the place-conditioning paradigm, e.g., cocaine (Lepore et al. 1995; Suzuki and Misawa 1995; Calcagnetti et al. 1996; Martin-Iverson and Reimer 1996; Martin-Iverson et al. 1997), caffeine (Brockwell et al. 1991; Brockwell and Beninger 1996), cannabinoids (Lepore et al. 1995; Sañudo-Peña et al. 1997), LSD (Parker 1996), methamphetamine (Suzuki and Misawa 1995), amphetamine (Hoffman and Donovan 1995; Turenne et al. 1996), methylphenidate (Gatley et al. 1996), fenfluramine (Davies and Parker 1993), 7-OH-DPAT (Khroyan et al. 1995; Chaperon and Thiébot 1996), gamma-hydroxybutyric acid (Martellotta et al. 1997), propofol (Pain et al. 1997), and NMDA receptor antagonists (Steinpreis et al. 1995; Papp et al. 1996).

Furthermore, 5-HT_3 receptor antagonists (Acquas et al. 1990), 5-HT_3 receptor agonists (Higgins et al. 1993), dopamine release inhibitors (Schechter and Meehan 1994), D1 receptor antagonists (Acquas and Di Chiara 1994), D3 receptor–preferring agonists (Khroyan et al. 1997), and antiemetic agents (Frisch et al. 1995) were studied in the place-conditioning paradigm.

Suzuki et al. (1991, 1993) and Del Poso et al. (1996) studied opioid-induced place preference and Bechtholt et al. (2004) studied the effects of handling on conditioned place aversion and conditioned place preference by ethanol in mice.

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Endocrine Pharmacology

14

Jürgen Sandow

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14.1 General Considerations

14.1.1 Introduction

Studies on endocrine safety pharmacology are part of the "supplemental safety pharmacology studies" described in the ICH guideline S7A. These supplemental studies are performed in order to identify the risk of adverse effects on organ system functions which are not addressed by the general methods of the core battery (ICH 2001; Valentin et al. 2005; Porsolt 2006). Supplemental studies are often required as a consequence of endocrine-related findings in repeated-dose toxicity studies. Such findings may be organ weight changes, histopathology, abnormal findings of biochemistry, and changes in biomarkers which were monitored due to the established pharmacological profile. Frequently, there are unexpected findings related to the gonadal system (testis and ovaries in adult rats), the adrenocortical system (stress-related findings, adrenal hyperplasia, increasing blood pressure), the thyroid gland (thyroid hormone abnormalities, histopathology), the endocrine or exocrine pancreas (impaired glucose control, pancreatitis), changes of electrolytes and calcium-regulating hormones (e.g., calcitonin), and biomarkers of bone turnover. Changes may be related to hormone-producing organs and to hormone-responsive tissues. Such changes may appear at the time of performing regulatory toxicology studies or may be anticipated from conspicuous findings of receptor profiling and early toxicology.

Interpretation of the findings is a difficult matter, the key issue being risk assessment and relevance of predictions for therapeutic problems. The relevance for initiating human phase I trials needs to be assessed as well as possible implications for phase II studies (Baldrick 2008; Bass et al. 2009). The specific pharmacology of the test substance is often studied with particular reference to the proposed therapeutic indication(s). When this does not include an endocrine disorder or therapeutic condition, potential problems need to be anticipated from structural indications, results of early profiling, and repeated-dose toxicology studies. Frequently, decisions on extended endocrine safety pharmacology are taken retrospectively due to unexpected findings. In case of compounds with established endocrine activity, there is often a reliable prediction that hormone-responsive organ systems may be affected. This will inform about the inclusion of targeted investigation by repeated-dose studies, preferentially coordinated with the regulatory toxicology studies (Hayes 2001). Endocrine activities may be anticipated from in vitro findings of the early evaluation in a general pharmacology screen (Spielmann 2005; Suter 2006). The pharmacology profiling of new drugs is nowadays predominantly performed by in vitro testing (Faller et al. 2006). The application of receptor screening may be quite an effective procedure for prediction of hormonal activity; however, the final assessment can only be done by animal studies due to the complexities of signal transduction and interaction of hormonal systems (cross talk). It will be necessary to consider the level of exposure (MacDonald et al. 1994; Heinrich-Hirsch et al. 2001; Singh 2006), to establish a relation of the effects observed to the predicted therapeutic concentrations. The established primary interaction of hormones and substances with hormonal activity with an ever increasing set of membrane receptors and nuclear receptors, as well as binding proteins and enzymes, can be approached by a number of high-throughput procedures and also by specific in vitro testing (Suter 2006; Sanderson 2006). This evidence will be available at that time of designing the final strategy for toxicological evaluation of a drug candidate, including endocrine safety pharmacology. For all activities predicted from the in vitro testing, some evidence for hormone-related effects should be available from the early animal studies. At this stage, a dialogue is needed for the toxicologists and the pharmacologists. Animal studies in rodents are by preference with repeated-dose administration for several days, to allow for early feedback effects and adjustment of receptors. Identification of consistent biological response very much facilitates the safety pharmacology strategy.

Endocrine safety pharmacology studies are performed for compounds designed to enter clinical pharmacology phase I (Valentin et al. 2005, 2009). They are needed to detect undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above (ICH-guideline S7A, 2001). The main objective is to decide whether the test compound can be administered to humans, which effects need to be monitored in clinical pharmacology and which clinical investigations (including monitoring of biomarkers) are

required for a risk evaluation (Williams et al. 2006; Frank and Hargreaves 2003). In endocrine pharmacology, the design of specific studies will be based on the individual properties and intended clinical uses of the test substance. When planning a toxicology program, safety pharmacology endpoints (hormone determinations, biochemical parameters, biomarkers, dynamic function tests) can be incorporated in repeated-dose toxicology, in relation to toxicokinetics and whenever possible in a similar manner as known from clinical studies. Where this is not feasible, endpoints related to suspected hormone activity should be evaluated in satellite safety pharmacology and in mechanistic toxicology studies (Harvey et al. 1999a; Bass et al. 2009).

Endocrine safety pharmacology studies investigate the pharmacodynamic effects of a substance on physiological functions in relation to exposure in the biologically effective ("therapeutic") range and above (ICH S7A). They are usually initiated (a) to evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies and (b) to investigate the mechanism of the adverse pharmacodynamic effects observed and/or suspected. Evidence from preclinical pharmacology needs to be available to estimate biologically effective dosage (physiological range). The toxicology concept to establish the NOEL level (no observable effect) is helpful for environmental agents and chemicals, whereas for the safety pharmacology, an effective dose range is mandatory. The approach to predictive findings in toxicology with relevance for clinical pharmacology is essentially based on studies in animals (VanderGreef and McBurney 2005; Collis 2006) and includes an innovative search for mechanisms of toxicity, directly related to the effects found in regulatory toxicology studies (Liebler and Guengerich 2005; Ettlin et al. 2010a, b). The endocrine profile is increasingly important for peptides and proteins produced by biotechnology (Cavagnaro 2002), a development which was predicted at an early time by Zbinden (1990, 1991) and requires targeted animal studies.

Endocrine toxicology is extensively reviewed and discussed in many textbooks (Witorsch 1995; Harvey et al. 1999a; Thomas and Thomas 1999; Eldridge and Stevens 2010); much of the evidence is derived from studies in occupational toxicology and on chemicals with endocrine-disrupting activities (Ashby 2000; Riecke and Stahlmann 2000). There has been remarkable progress on the development of tumor models with intent to early prediction of safety issues (Arp 1999; Jacobson-Kram et al. 2004; Andersen and Krewski 2009), as well as in vitro testing targeted to prediction of toxicology (Dykens and Will 2007; Walmsley and Billinton 2011).

In this chapter, pharmacological methods are described which can be applied to the characterization of candidate compounds, their effects on endocrine functions (hormone secretion and synthesis), and their effect on target organ functions. Most frequently, these effects are directed to the reproductive system, thyroid gland, adrenal glands, pituitary hormones and gastrointestinal hormones, and hormonal factors which regulate growth and metabolism. There is a close relation to pharmacological methods designed for carbohydrate and lipid metabolism, insulin resistance, atherosclerosis, and obesity (Vogel 2008a, b, c).

Due to the complexity of endocrine investigations and the time and expense of classical assays for hormone effects, animal bioassays are no longer recommended for a standard protocol. Evidence for endocrine risks is usually obtained from in vitro studies on hormone receptors and enzymes, which should lead to the inclusion of specific study parameters in the regulatory toxicology studies. In some instances, a targeted investigation is performed for the release or inhibition of secretion of specific hormones. This may be done by single-dose tests measuring the hormone response and by acute changes in the serum concentration or associated tissue contents of hormones. Some of the classical bioassays, described in detail by Vogel (2008c), can be used for the safety pharmacological characterization. In the present section, reference is made to the selection of procedures and assays, which may be required to meet the ICH guideline S7A (2001). These studies are appropriate in the context of regulatory toxicology studies when added to the standard protocol and when monitored in satellite studies on the interference with hormone systems and hormone-responsive target organs (Harvey et al. 1999a).

14.1.2 Regulatory Toxicology Studies

The unexpected evidence in the early toxicology studies is often provided by organ weight changes (frequently testes or ovaries, thyroid gland, or adrenal glands), by significant changes in clinical chemistry

(e.g., of enzymes or of electrolytes), and very frequently by histological observations which need an explanation and interpretation. In regulatory studies, the animals should not be overburdened with supplementary examinations. Satellite groups treated with similar dosage are needed frequently to obtain serum and tissue samples and to be used for ex vivo studies. Satellite groups will also provide the necessary specimens for specific investigation, for example, of immunohistochemistry and molecular biology. With paired organs (testes, ovaries, adrenals), access to sufficient samples should represent no problem, for example, anterior pituitaries in rats may be bisected by a median sagittal cut to obtain tissue for hormone analysis, in addition to standard histology.

Some changes in hormone contents may already be detected in regulatory toxicology studies by inclusion of additional analytical parameters in the study protocol. Such hormone determinations are mostly related to the anterior pituitary, thyroid gland, adrenals, and reproductive system and less frequently to changes in growth hormone and prolactin secretion, to the assessment of endocrine pancreas function (antidiabetic agents), or to biomarkers of bone turnover (e.g., bisphosphonates, calciferols). Interference with glucose regulation may require that frequent samples are taken, animals compensate for hyperglycemia, and show no related symptoms unless dynamic function tests are applied (OGT). Major changes in electrolyte balance are readily detected with interim collection of urine specimens under controlled conditions (metabolism cages, see Sect.14.4.2); they may be found in serum samples acquired at autopsy, with adequate sampling and immediate processing. Organ weight changes and findings in histopathology may require a follow-up endocrine analysis in a mechanistic study using, for example, immunostaining and markers of cell proliferation (for instance staining for Ki 67, a nuclear protein that is associated with cellular proliferation). Frequent findings in animal studies which may not necessarily be related to similar effects in humans are thyroid enlargement, changes in ovarian or testis morphology (for instance Leydig cell hyperplasia), adrenal hyperplasia, pheochromocytoma in rats, or pancreatic tumors (e.g., beta cell hyperplasia). There is an extensive literature on the relevance of findings in toxicology studies, which may not be necessarily related to similar observations in humans and may require interpretation of their relevance for phase I studies (Thomas 1996; Capen 1998; Neubert et al. 1999; Thomas and Thomas 1999; Harvey et al. 1999a; Eldridge and Stevens 2010).

The observations are mostly from 14 to 28-day studies, less frequently from 90-day or 6-month treatments. Estrogenic effects are indicated by uterine weight, androgenic effects by epididymal weight, or more frequently by histology of the testis (Sellers et al. 2007). In satellite studies, it is useful to obtain and store a sufficient blood sample volume from treated animals and controls to be able to search for distinct hormonal changes, retrospectively, in explanation of unexpected findings. This is recommended for long-term studies where blood sampling can be performed at some time during the study without interfering with the final condition of animals at autopsy. It is advisable to perform an evaluation similar to the procedure outlined in Vogel (2008c) (Endocrine Profiling). In view of the progress in molecular biology, it may also be advisable to acquire sample suitable for, for example, genomic analysis.

14.1.3 Mechanistic Studies

A clearly defined set of questions arises from drug candidates which belong to a class that has already shown some endocrine effect or is suspected of having endocrine-mediated effects (Harvey et al. 1999b). No general recommendation can be given for the situation when specific endocrine investigations need to be performed in separate non-GLP studies. Such studies are summarized as mechanistic studies.

A detailed ex vivo biochemical analysis of drug-induced changes requires hormone determinations in brain, hypothalamic and pituitary tissue, peripheral target organs, and measurement of circulating plasma concentrations of hormones and hormone-dependent substances. Many of the analytical hormone assays are now available from commercial suppliers, mostly with adequate validation of their performance. There is a problem of species specificity which needs to be considered and confirmed before applying methods which may be suitable for clinical use (human hormones) but not for the rat or other animal species. In each case, it is advisable to validate applicability in a small study before embarking on large and expensive toxicology studies. Hypothalamic hormones in tissue samples are identified by direct measurement (for

instance, TRH, GnRH, somatostatin, GHRH, CRF). There are strict conditions for sample acquisition at autopsy: tissue samples need to be frozen immediately; enzyme inhibitors need to be added during processing in most instances. Interpretation of results of hypothalamic hormone content is always required with due consideration of related changes in serum concentration of pituitary hormones, pituitary hormone contents, organ weight changes and histological findings.

The suprahypothalamic neurotransmitter level can be assessed by a determination of catecholamines in circumscribed brain areas; the technique requires preparation of frozen tissue and isolation of specific nuclei by the micropunch technique. The catecholamines and indolamines can be measured by radioenzymatic methods and by a high-pressure liquid chromatography (HPLC) with electrochemical detection. These mechanistic investigations are difficult and time consuming; they are mostly initiated due to questions arising from the receptor interaction profile of the drug candidate. Such studies may be required to prove that receptor interactions of hypothalamic hormones at the neurotransmitter level are related to the functional state of endocrine organ functions (functional expression, e.g., for changes in prolactin secretion). Mostly, however, the peripheral effects of such neurotransmitter (for instance the effect on prolactin secretion) are sufficiently distinct and may be considered as biomarkers.

14.1.3.1 Mechanistic Studies (Single Dose)

Such studies refer in general to endocrine experiments directed at measuring the time action profile of specific hormones (Sandow 1979) in the context of establishing the dose range for investigations (physiological and supraphysiological concentrations). Reference values should be available at least for rats and dogs; dynamic function tests using an established stimulation or inhibition test are recommended. For such acute experiments, standard operating procedures are established based on the secretory kinetics of the hormones to be measured (Heinrich-Hirsch et al. 2001). When studies are performed in anesthetized animals, the anesthetic has a marked effect on hormone secretion. In some cases, animals prepared with indwelling cannulas will be required for meaningful assessment. The problem of episodic and pulsatile hormone secretion may be avoided in anesthetized animals; selection of a suitable time of the day with reference to chronobiology is recommended, and this may be different for specific hormones. Retroorbital punction in unanesthetized rats was found to be a suitable procedure for the gonadotropins and the thyroid hormones. The procedure is not suitable for growth hormone and prolactin secretion studies. The rat has limitations of sampling (retroorbital or tail vein). Dogs provide better experimental conditions for frequent sampling; they need to be trained for some time before experimentation to reduce stress-related effects. The investigator will need to know about the physiology of hormone secretion in the species to be examined; consultation with an endocrine pharmacologist is helpful, and reference may also be made to similar procedures which are established for the human (website www.endotext.org). As an example, measuring the serum concentrations of corticotropin (ACTH) is extremely difficult in dogs because they react strongly to the stress of the sampling procedure and to any changes in the laboratory environment during the experiment.

For the pituitary hormones, pancreatic hormones, and biomarkers for bone changes (including calcitropic hormones), analytical methods are in general available with species-specific reagents (mouse, rat, dog, monkey) and to a limited extent for rabbits and other species. It is recommended to perform pilot experiments for validation of commercial hormone assays. Steroid hormones can be measured readily by an array of analytical methods. Application of gene arrays and studies on gene expression are recommended for repeated-dose exposure when a steady state with meaningful changes is read (3–7 days duration).

14.1.3.2 Mechanistic Studies (Repeated Dose)

These studies are designed to investigate the context of changes, adapting experimental conditions to the questions raised by the regulatory studies (Harvey et al. 1999a). The dose range should include the anticipated therapeutic range (physiological range) and does not need to include very high doses. The purpose is to compare baseline conditions with changes observed by stimulation or inhibition of target hormone secretion after 7–28 days of treatment. Several time points need to be included, and pilot experiments are recommended unless the laboratory has some experience with the particular hormone determination. At the

end of a repeated-dose study of some duration, an important decision for the last treatment is at which time to sacrifice the animals in relation to final treatment injection. This item is important both to measure serum concentrations and to detect valid changes in the tissue content of hormones at autopsy. Changes in pituitary hormone concentration or that of other target organs are valuable indicators of the extent of the endocrine effect. Changes in tissue content do occur within hours after treatment; they may not be detected 24 h after the last treatment. This is the usual interval for autopsy in regulatory studies; the interval may not be suitable for mechanistic studies.

The biochemical parameters for evaluation of changes in hypothalamic-pituitary-target organ function are derived from hormone concentrations in tissue and should be related to those found in serum or plasma. Tissue and plasma concentration are influenced by time of the day (circadian rhythm of the hormone to be measured, chronobiology), the stress of handling, and the technique of sacrificing the animals. Plasma levels are more susceptible to modification by stress than tissue concentrations. During prolonged periods of sampling, tissue content may also change due to stress preceding autopsy. Decapitation is recommended as the established method of sacrificing animals with a minimum of endocrine interference. This refers to decapitation in a quiet operating room separate from the animal facility, preferably in small groups of animals transferred to that room immediately before the procedure. Studies on organ tissues ex vivo may be considered, for instance incubation of the testis or ovary with hormones for stimulation (functional assays).

In regulatory studies and in mechanistic studies, one may prefer to assess changes in tissue content for hormones like corticotropin (ACTH), prolactin, and growth hormone (GH), rather than serum concentrations. The tissue content of these hormones changes slowly and in a characteristic direction, whereas their plasma levels may fluctuate widely both with handling stress and due to circadian rhythm. This also indicates that for mechanistic studies, it is important to select time points of investigation for which comparative laboratory data are available and reliable publications with evidence that circadian rhythm does not interfere during the selected time period. In general, practical lab work will determine the timing, and the light schedule in the animal facility will serve as a synchronizer for adrenocortical steroids.

For the more stable secretion of thyroid-stimulating hormone (TSH, thyrotropin) and the gonadotropin (LH and FSH), plasma determinations are adequate provided that autopsy is completed within a short time and the time of autopsy is selected based on data which minimize artifacts due to circadian hormonal changes. In our laboratory, rats are sacrificed by decapitation (one experimental group at a time), and autopsies are performed during the morning period of 08:00–10:00. This is different from mechanistic studies, where it may be necessary to measure changes in hormone secretion spaced at increasing intervals after treatment or to obtain sequential tissue samples at the related time points. These recommendations are to some extent valid for basal tissue concentrations of gonadal steroids and adrenal steroids, which show considerable circadian variability.

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14.2 Endocrine Profile

This is a repeated-dose test which is applied for the detection of biological effects by organ weight changes, changes in specific hormones and biomarkers, and other parameters to be added when considered helpful. The final protocol of this test is designed at the time of starting repeated-dose toxicology studies. The available information is related to in vitro pharmacology (in particular receptor screening and enzyme tests) and to single-dose or multiple-dose animal studies performed with the test substance.

The endocrine profile of a drug candidate is determined in a repeated-dose study of at least 7 days duration (Sandow 1979; Vogel 2008b). The study plan is similar to a toxicology study of 14–28 days duration in two animal species, that is, the rat and dog. The test is not suitable for mice. The S7A guidelines state "effects of the test compound on organ systems not investigated elsewhere should be assessed when there is a reason for concern." Reasons for concern are frequently related to the reproductive system (male and female gonadal function), the adrenal system (adrenocortical function), the thyroid gland, the endocrine and exocrine pancreas, and the biomarkers of bone turnover.

Concern may be caused by previous toxicology studies with findings of histopathology, clinical chemistry, or organ weight changes. For the toxicology studies, it is helpful to determine weights of all endocrine organs (Sellers et al. 2007) including the pituitary gland.

The purpose of the endocrine profile is to inform on the design of repeated-dose toxicology studies. When a distinct endocrine profile of the drug candidate is present before starting the toxicology studies, a targeted investigation can be added to the standard toxicology protocol, satellite groups may be added, and specific mechanistic studies may be initiated in the context of the toxicology program.

In the endocrine profile, the repeated-dose administration with a design and dose selection similar to toxicology studies is preferred. Studies of up to 4 weeks' duration need to include analytical determination of serum/plasma concentrations of hormones, basal concentrations at several time points during the study, and dynamic function tests for the response of hormone secretion (e.g., LHRH, TRH, CRF/CHRH, monoiodotyrosine MIT for prolactin secretion). At autopsy, tissue samples are stored for hormone contents of endocrine organs and hormone-dependent tissues (e.g., hypothalamus, pituitary gland, reproductive organs, pancreas). In specific instances, tissue may be process by molecular biology methods. This is more in the domain of mechanistic studies when mechanisms of toxic effects observed need to be identified or excluded. In the endocrine profile, at least 7 days of treatment are mandatory to allow for adaptation and response of hormone-dependent organs to the treatment. Repeated daily dosing (once or twice per day) ensures that a steady state is reached. Selection of at least two doses is recommended for a sufficient effect size to be achieved and to detect biologically relevant/statistically significant differences between controls and treatment groups. A dose range similar to the anticipated regulatory toxicology studies is recommended.

The inclusion of untreated controls or a vehicle control group is mandatory. The additional inclusion of comparator groups (drug of known endocrine activity) may be considered. In the majority of cases, this may not be necessary, because there is an extensive database in the literature about the effect size of established drugs that affect the endocrine systems. This agrees with the guideline statement "in well-characterised in-vivo test systems, positive controls may not be necessary."

14.2.1 Endocrine System Evaluation in Rats (Endocrine Profile)

PURPOSE AND RATIONALE

This study is designed for specific endocrine evaluation, a test substance which has shown some effects on endocrine systems during the preceding toxicology evaluation (28 days treatment in rats, or in dogs), with no distinct profile related to a specific endocrine system.

This study is generally performed in rats, less frequently in dogs. In rats, extensive reference data are available for hormones and substances acting on hormone systems, including serum concentrations and tissue contents. There is considerable technical difficulty to adapt this method to mice. In the endocrine profile, several endocrine systems are examined simultaneously, preferably in the rat strain that is also used in the regulatory toxicology studies. Parameters to be investigated (see Table 14.1) are organ weight changes, histology or histopathology of hormone-producing organs and hormone-responsive tissues, specific clinical chemistry (biomarkers), and hormone concentrations. During the course of the study and (to be considered) 24 h before autopsy, hormone concentrations in blood and tissue are determined (related to function of the reproductive system, thyroid gland, adrenals, endocrine and exocrine pancreas, etc.). A particular design of this study may include specific effects on the endocrine pancreas (Vogel 2008a), for example, insulin receptor signaling, IGF-I receptor signaling, and immunohistochemistry of the pancreas. This design is applied for antidiabetic agents.

PROCEDURE

Adult male and female rats, body weight preferably 200 g, are randomly assigned to control and treatment groups, at least six animals per group and at least two doses. The dose range for treatment is selected based on pharmacodynamic information (dose-response curves from single-dose studies, in vitro findings using receptor affinity and receptor signaling, biomarkers, or enzymes). The low dose should elicit the primary pharmacodynamic effect in the test species (preferably related to the proposed therapeutic effect in humans, on target); the high dose should elicit the adverse effect(s) anticipated based on previous study results in vitro and in vivo (supraphysiological dose

Table 14.1 Endocrine profile, study parameters, and hormone systems to be investigated during repeated-dose treatment and at autopsy. Optional biomarkers to be included in the investigation during the study and at autopsy

Endocrine system	Optional biomarker
Pituitary-adrenal axis	
Pituitary weight	Corticotropin-releasing hormone in hypothalamus
Adrenal weight	Pituitary content of adrenocorticotropin and vasopressin
Thymus weight	Adrenal content of corticosterone and aldosterone
	Corticosterone and aldosterone blood level
	Electrolyte concentrations in serum (sodium, potassium chloride)
	Urinary excretion of corticosterone and aldosterone
Pituitary-gonadal axis	*Male reproduction*
Pituitary weight	LH-RH in hypothalamus
Weight of testes	Pituitary content of FSH, LH, and prolactin
Weight of seminal vesicles	Blood levels of FSH, LH, and prolactin
Weight of ventral prostate	Testosterone content in testes and serum
Weight of levator ani muscle	Growth hormone in pituitary and serum
	Female reproduction
Pituitary weight	LH-RH in hypothalamus
Weight of ovaries	Pituitary content of FSH, LH, and prolactin
Weight of uterus	Blood levels of FSH, LH, and prolactin
	Content of estradiol and progesterone in ovaries and serum
Pituitary-thyroid axis	
Pituitary weight	TRH in hypothalamus
Thyroid weight	Pituitary content of thyrotropin
	Serum content of thyrotropin, T3, and T4

range, not maximum tolerated dose). In case of two test doses, the dose increment should be at least fivefold. In accordance with guideline ICH S7A, the high test dose should produce moderate adverse effects in this study or in other studies of similar route and duration, and the adverse effects can include dose-limiting pharmacodynamic effects or other toxicity.

The duration of the treatment period should be at least 7 days, to allow for a steady state to be established. It may be extended to 28 days, based on findings of a preceding toxicology study with observations of concern, which did not include satellite groups for specific investigation of hormone-related mechanisms.

At suitable time points during the treatment period (e.g., once per week), the hormone concentrations in controls and treatment groups may be determined at baseline (always at the same time of the day, to avoid confounding effects of diurnal hormone fluctuations). Blood sampling should be done sparingly (once per week, e.g., dynamic function tests) and discontinued during the last week of the study period. A final sample 24 h before autopsy may be considered. Dynamic function tests (stimulation or inhibition) are performed at 7 days intervals, by using established diagnostic reagents that act on hormone secretion (e.g., LHRH, TRH, CRF/ACTH, somatostatin). In a similar manner as for human diagnostic procedures, reagents may be combined when their hormonal effects do not interfere (e.g., combined TRH/LHRH test). It is recommended to perform preliminary investigations to become familiar with the test and validate the analytical method selected.

Measurement of the effects by a noninvasive procedure is often possible. A preferred procedure is to use metabolism pages for collection of urine samples during the night period (activity period of rats) and urinary excretion data, when possible with correction for creatinine excretion. This approach is useful in pharmacokinetics (Sandow et al. 1990) as well as pharmacology. The noninvasive procedure is particularly helpful in the assessment of adrenocortical function in rats (Hilfenhaus 1977; Hilfenhaus and Herting 1980; Sarrieau and Mormède 1998; Eriksson et al. 2004). During the treatment period, collection of urine samples may be performed in metabolism cages during the night period, in rats which have been adapted to this procedure, at least 3 days before the first diagnostic sampling. Sampling in metabolism cages may also be included in regulatory toxicology studies. Biomarkers in urine samples are analyzed, for example, for endogenous compounds (noninvasive diagnostic procedure), electrolytes, and for the test substance excreted in the urine (pharmacokinetics). An alternative noninvasive method for the assessment of adrenal steroid excretion is collection of feces and analysis of steroid metabolites. This approach has been applied to an astounding number of animal species in veterinary medicine and zoology (Ganswindt et al. 2003); it is however time consuming and unusual.

At the end of the treatment period, groups of animals are killed under minimum stress conditions, preferably by rapid decapitation. The time point "24 hours after last dosing" is not always suitable. When changes in pituitary hormone contents need to be assessed, the time point "2 hours after last dosing" is preferable. For ex vivo studies (in vitro test on organs or cell preparations from organs) suitable interval after last treatment need to be selected. Satellite groups may be included to determine the exact time course of changes after dosing.

Endocrine organs are dissected out, and their weight is recorded. In the case of paired organs, one organ is assigned to histology (with suitable fixation), and the other organ is immediately frozen for further hormone analysis, receptor determination, or molecular biology (gene expression profiling). Organs of interest are the adrenal glands, thyroid gland, male reproductive organs (testes, seminal vesicles, ventral prostate, levator ani muscle), and female reproductive organs (uterus, ovaries).

Specific dissection is performed on the brain and pituitary gland. Hypothalamic fragments may be dissected and shock frozen immediately in liquid nitrogen, for later extraction and determination of hypothalamic peptides, and cerebral cortex fragments are analyzed for control. The pituitary gland is dissected out, and the posterior pituitary is stored separately. The anterior pituitary is halved by a median sagittal cut to obtain paired tissue samples for histology (fixation) and for subsequent analysis of hormone contents (stored frozen at −20°C until hormone assay).

At the time of autopsy, other tissues of interest may be obtained (e.g., pancreas, liver, gastrointestinal tract) and processed either for histology or biochemical analysis. The decision on the supplementary selections depends on preexisting information about possible endocrine-mediated adverse effects or toxic pharmacodynamic effects known for other members of the group of compounds investigated.

The selection of the blood sampling procedure depends on the hormone to be measured. Most hormones can be reliably measured, for example, during brief carbon monoxide anesthesia or preferably during brief ether anesthesia by retroorbital venipuncture. Repeated blood sampling is not advisable in this method. Time-action profiles may be established in satellite groups with indwelling catheters, to be sampled for several hours during anesthesia with pentobarbital or ketamine. Experience with the effects of the anesthetic on the hormone to be analyzed is required.

EVALUATION

For all quantitative parameters such as organ weights, group means are calculated, and the significance of differences is assessed by the appropriate statistical methods.

Histology may be reported in descriptive terms, evaluated by semiquantitative methods or by computer-assisted histomorphometric methods (to be used for quantitative statistical analysis). For advanced methods of in situ hybridization and gene expression, a description of the observed changes is generally appropriate together with quantitative data generated by computer-assisted analysis of gene expression profiles.

MODIFICATIONS OF THE METHOD

In each case, this method needs to be adapted by establishing the dose response for new test substance (dose level and duration of action), summarizing the biological/biochemical information which gave "reasons for concern" in the planning of the study. It is helpful to have early pharmacokinetic data for assessment of exposure (MacDonald et al. 1994). The age of the test animals (or initial body weight) may be selected according to the objective of investigation. Adult animals (160–200 g initial body weight) are recommended. However, the method is also applicable to studies on pubertal development (30–40 g initial body weight). For compounds which affect, for example, body composition (antiobesic drugs) or bone density (e.g., bisphosphonates), the selection of rats 300–400 g body weight is preferable, and markedly longer treatment periods may be required (mechanistic studies beyond the endocrine profile design).

CRITICAL ASSESSMENT OF THE METHOD

The endocrine profile is a flexible approach to the investigation of several hormonal systems in one study. It is closely related to the design of subchronic toxicology studies; the specific focus on endocrine systems is very helpful in the interpretation of histological findings and organ weight changes indicative of toxicology. Toxicologist and pharmacologist should convene for the design of such studies because there are characteristic limitations of regulatory toxicology, which require satellite studies of closely related

design. Interpretation of a perceived risk for the human is primarily directed at the result of hormone assays and biomarkers, which might be included in the phase I and II clinical trials for targeted risk assessment (translational design). The spectrum of toxic changes in hormone-dependent organ systems has been reviewed and is available, with reference to the pituitary gland (Tucker 1999), thyroid and parathyroid (Capen 1999), male reproductive system (Sikka 1999), adrenocortical function (Hinson and Raven 1999), and functions of the endocrine and exocrine pancreas (Wilson and Longnecker 1999). The long-term effects of comparative endocrine carcinogenesis have been reviewed for specific instances (Gopinath 1999). These effects however remain to be addressed when results from 6- to 12-month toxicology studies become available. Their interpretation is a considerable challenge because many tumors found in rodents are not relevant for the clinical use of the same compounds, when long-term clinical observations are compared with the initial findings of concern.

14.2.1.1 New Evidence from Environmental Toxicology

A very extensive body of knowledge has been accumulated by toxicologists in programs supported by the OECD and the Environmental Protection Agency (EPA). Many of the classical endocrine bioassays have been reevaluated for the assessment of chemicals with endocrine disruptor characteristics, resulting from large-scale production of chemical products initially found to be toxic for the ecosystem, as well as for reproductive function in humans. Industrial toxicology has provided the initiative for studying many of the well-known steroid compounds as well as thyroid hormones for their effects on mammalian systems. The section on chronic treatment of rats is of particular interest for assessment of endocrine safety pharmacology, both with reference for the analytical parameters and biomarkers included and in particular for the long-term treatment effects. The characteristic difference in relation to endocrine safety pharmacology is the determination of no-observable-effect levels (NOEL) and the use of maximum tolerated doses (O'Connor et al. 2000, 2002a; Kang et al. 2004). Classical bioassays studied extensively with a number of modifications are the Hershberger assay in castrated male rats (Freyberger et al. 2007; Shin et al. 2007; Yamasaki et al. 2004) and the uterotrophic assay in immature rats (Yamasaki et al. 2003). An assay in intact male rats for antiandrogenic activity has been evaluated (O'Connor et al. 2002b; Freyberger and Ahr 2004).

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14.2.2 Determination of Hypothalamic Hormones

PURPOSE AND RATIONALE

Many tests substances change the secretion of hypothalamic hormones, either by direct action or more generally by their feedback effects at the hypothalamic level. Under these conditions, the tissue concentration in hypothalamic specimens of treated rats is of interest, especially because several hypothalamic peptide assays are available and can be measured in the same specimen. The circulating concentrations for hypothalamic hormones (peptides) are difficult to measure, due to analytical problems of low concentration, rapid inactivation by enzymes, and pulsatile fluctuation. Circulating concentrations would also change immediately due to the interference of anesthesia and of the autopsy procedure. Tissue concentrations are readily measured in specimens taken rapidly and stored at frozen until assay, preferably adding enzyme inhibitors. It is therefore recommended to take samples of hypothalamic tissue and control samples of cerebral cortical tissue when the effect of a drug candidate on hypothalamic function is to be investigated.

PROCEDURE

Specific dissection is performed on the brain (cerebral cortex, the reference tissue), the hypothalamus (of rats), and pituitary gland. Hypothalamic fragments are dissected out and shock frozen immediately in liquid nitrogen, for later extraction and determination of hypothalamic peptides. The procedure is suitable for the rat and for the rabbit. Immediately after sacrifice, the skull is opened, the brain is removed, and a piece of cerebral cortex and the hypothalamic fragment at the base of the brain is clipped with scissors and stored on dry ice or in liquid nitrogen. Specimens may be shock frozen and homogenized immediately in an ice-cold buffer containing peptidase inhibitors (e.g., bacitracin or aprotinin). Specimens of cerebral cortical tissue are analyzed as controls. The pituitary gland is then dissected out and stored separately, after removing the posterior pituitary (neurohypophyseal hormones).

There are numerous publications which refer to the measurement of luteinizing hormone-releasing hormone (LHRH; Adams and Spies 1981a, b; Adams et al. 1981; Aubert et al. 1980; Berault et al. 1983; Catt et al. 1980; Chan et al. 1981; Clayton 1982a, b; Clayton and Catt 1979; Clayton et al. 1979a, b, 1980; Conn and Hazum 1981; Conn et al. 1981; Dalkin et al. 1981; Dufau et al. 1979; Hazum 1981; Heber and Odell 1978; Kuhl et al. 1980; Loughlin et al. 1981; Loumaye and Catt 1982; Naor et al. 1981; Perrin et al. 1980; Sandow 1982; Sandow et al. 1979, 1981), corticotropin-releasing hormone (CRH; Aguilera et al. 1986; Brown et al. 1988; De Souza and Battaglia 1988; De Souza 1987; Flores et al. 1990; Grino et al. 1986; Hauger et al. 1987, 1988; Millan et al. 1986; Perrin et al. 1986), and growth hormone-releasing hormone (GHRH; Abribat et al. 1990; Audhya et al. 1985; Bohlen et al. 1984; Gelato et al. 1985).

EVALUATION

The hypothalamic peptide concentration in the specimens is determined by radioimmunoassay, using a synthetic reference peptide for the standard curve. Several commercial assays are available for hypothalamic peptides (e.g., http://www.abnova.com). An alternative method is the determination of peptide content in tissue extracts by an HPLC method or by mass spectrometry.

Group means are calculated, and appropriate tests for significance are applied.

MODIFICATIONS OF THE METHOD

In the context of safety pharmacology, hypothalamic hormone concentrations are measured after repeated-dose treatment, to assess due to direct action or feedback effects elicited by the drug candidate. These measurements can be included in the conventional subacute toxicology protocols, after some practice of hypothalamic dissection.

CRITICAL ASSESSMENT OF THE METHOD

The main difficulty is experienced with rapid processing of the tissue samples and selection of an enzyme inhibitor to prevent spontaneous degradation by tissue peptidases (Elkabes et al. 1981; Griffiths and Kelly 1979; Hazum et al. 1981; Horsthemke and Bauer 1981, 1982; Horsthemke et al. 1981; McDermott et al. 1982; Posner et al. 1982). The result needs to be assessed in context with changes in pituitary hormone contents; frequently, the adaptation to treatment at the pituitary level is more informative than the level of the hypothalamic tissue contents.

In many research publications, receptors for hypothalamic peptides have been described at the endocrine target organ level, for example, LHRH receptor in the gonads (Harwood et al. 1980a, b; Pieper et al. 1980; Sharpe 1982; Sharpe and Fraser 1980) and in nonendocrine tissues, for example, the gastrointestinal tract (LHRH and GHRH receptors, Bruhn et al. 1985). These studies, however, have identified no major regulatory mechanisms which could be activated by test compounds to be developed for phase I clinical pharmacology. Application of hypothalamic hormone measurement in safety pharmacology should therefore be restricted to the level of information required for understanding interaction with the secretion of pituitary hormones.

The determination of neurotransmitter concentrations at the suprahypothalamic level is much too complex for an inclusion in safety pharmacology studies and remains an area for research.

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CRH – Corticotropin Releasing Hormone

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GH-RH – Growth Hormone Releasing Hormone

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14.2.3 Determination of Pituitary Hormone Contents

- Gonadotropins (LH and FSH)
- Thyrotropin (TSH)
- Growth hormone (GH, somatotropin) and insulin-like growth factor (IGF-1)
- Prolactin (PRL)
- Corticotropin (ACTH) and melanotropin (MSH)

PURPOSE AND RATIONALE

This section describes the measurement of pituitary hormones in the context of repeated-dose studies of 1–4 weeks' duration (endocrine profile or regulatory toxicology). In endocrine safety pharmacology studies in rats, it is advisable to determine pituitary hormone concentrations at autopsy (24 h after last treatment injection). Specific rat pituitary hormone assays are commercially available (e.g., Amersham hormone assays at http://www.gelifesciences.com) from the National Hormone and Peptide Program (http://www.humc.edu/hormones/material.html). The time point 2 h after last dosing is in general suitable, when important and characteristic changes in pituitary hormone contents need to be assessed. This will however require

the inclusion of satellite groups in regulatory toxicology studies or specific mechanistic studies.

For toxicology studies in dogs, preparation of the pituitary glands at autopsy may be performed, but this is time consuming and less advisable because of the small numbers of animals in regulatory toxicology studies. In general, homologous assays are recommended. For the rat luteinizing hormone (Daane and Parlow 1971; Niswender et al. 1968; Seki et al. 1971), rat follicle-stimulating hormone (Beastall et al. 1987; Midgley 1967) and rat thyroid-stimulating hormone (Kieffer et al. 1975; Garcia et al. 1976, 1977; DeVito et al. 1989) are glycoprotein hormones exhibiting species specificity. For pharmacological experiments in rats, the homologous assays are preferred. Reagents and advice on procedures were provided by the National Pituitary Agency, Bethesda, Md. and adapted from the standard operating procedure in safety pharmacology. For rat growth hormone (Schalch and Reichlin 1966) and rat prolactin (Niswender et al. 1969; McNeilly and Friesen 1978), specific RIA and ELISA assays are commercially available (e.g., http://www.genwaybio.com). Determination of corticotropin (ACTH) is less frequently required; commercial ELISA assays are available (e.g., http://www.calbiotech.com). For specific research investigation, there are several other methods available, such as assays for melanotropins and other specific pituitary peptides. In each case, it is recommended to perform a preliminary validation of the assay procedure and reagents to make sure that rat hormones are adequately recognized. Sample acquisition and processing may have special requirements which need to be practiced.

PROCEDURE

The skull is opened by scissors, the pituitary gland is dissected out, the posterior pituitary is stored separately (assays for vasopressin and oxytocin are rarely required), and the anterior pituitary is halved by a median sagittal cut to obtain separate tissue samples for histology (fixation) and for subsequent analysis of hormone contents (stored frozen at $-20°C$ until hormone assay). Pituitary glands are homogenized in phosphate-buffered saline pH 7.4 and diluted to the concentration required for the radioimmunoassay. The initial homogenate may be stored deep-frozen for sequential assay of several hormones.

The recommendation for investigation of the paired pituitary halves may be particularly helpful for specific investigation of immunocytochemistry and molecular biology methods.

EVALUATION

Hormone concentrations in dilutions of pituitary homogenates are calculated from standard curves using the appropriate reference standard for rat pituitary hormones, and sample data are calculated as hormone per pituitary gland or per pituitary weight (mg). Group means are calculated, and the significance of differences is assessed by the appropriate statistical methods.

MODIFICATIONS OF THE METHOD

This approach provides a fast and flexible assessment of several pituitary hormones, and results may be compared with morphometric evaluation and immunohistochemistry for several hormones of interest.

CRITICAL ASSESSMENT OF THE METHOD

Changes in pituitary hormone contents are frequently characteristic and may be followed up by measurement of hormone secretion profiles if required. Analytical methods for the rat are often also applicable to mice. For toxicology studies in dogs, preparation of the pituitary glands at autopsy may be performed, but is time consuming and less advisable because of the small numbers of animals in such studies.

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14.2.4 Dynamic Function Tests

The changes in baseline hormone secretion are useful for interpretation of drug effects; it is however often advisable to include dynamic function tests based on stimulation of pituitary hormone secretion or of gonadal and adrenal hormone secretion. Examples for pituitary testing and preclinical procedures to be applied have been mentioned above (Melmed and Kleinberg 2008). Typical pituitary function tests are the TRH test for TSH secretion, the LHRH test for secretion of FSH and LH, and the monoiodotyrosine (MIT) test for prolactin secretion. Synthetic CRF may be injected to stimulate secretion of corticotropin (ACTH); more frequently, the injection of corticotropin is used to stimulate the adrenal directly. At the target organ level, similar function tests can be performed, for example, using thyrotropin (TSH) to stimulate thyroid hormone secretion (T4 and T3), human chorionic gonadotropin (hCG) to stimulate testosterone or ovarian steroid secretion, and corticotropin (ACTH) to stimulate adrenal steroids secretion. All reagents for these tests are readily available for tests in rats and dogs; the tests can be built into a standard toxicology protocol, several days before the end of the study. Conversely, the inhibition of enhanced hormone may be tested in treated animals at the end of the study, for example, with inhibitors of prolactin secretion (bromocriptine); this is usually reserved for mechanistic studies. The application of such dynamic function tests in the endocrine diagnostics of pituitary adenoma and responsiveness to therapeutic peptides has been extensively reviewed (Arvat et al. 2001; Babic et al. 2011; Chanson and Salenave 2004; Emeric-Sauval 1986; Ho et al. 1985; Vasquez and Greenblatt 1985).

CRITICAL ASSESSMENT OF THE METHOD

There is one advantage in applying dynamic function tests; they can be included in the safety pharmacology study after 2 or 3 weeks of treatment and may then indicate dose-related changes in pituitary responsiveness caused by the drug candidate.

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14.2.5 Gonadotropin Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

Anterior pituitaries may be incubated directly, kept in culture, or used for cell lines (Mittler and Meites 1964, 1966; Mittler et al. 1970; Sandow et al. 1972) in order to study the synthesis and release of gonadotropins in response to LH-RH.

PROCEDURE

Female Sprague-Dawley rats weighing 100–150 g are used as donors. Each anterior pituitary is removed and cut into four to six pieces of approximately equal size. The cultures are performed in 3.5–1-cm sterile disposable plastic Petri dishes each containing 3 ml medium consisting of nine parts DIFCO medium 199 and one part of newborn calf serum. Twenty-five U/ml penicillin and 25 μg/ml streptomycin are added. In each dish, the explants are supported at the gas interface. An atmosphere of 95% oxygen and 5% carbon dioxide and a temperature of 36°C are maintained. Opposite sides of the same pituitaries provide matched control and experimental preparations. The pituitaries are incubated for a total time of 5 days. After the first 2 days, the medium is removed and discarded. Fresh medium is then added with the LH-RH solutions. Approximately 12 h after the first change of medium and addition of LH-RH, media are removed and frozen. Fresh medium with LH-RH is again added; this procedure is repeated until six samples of medium representing the last 3 days of culture are obtained. Media are assayed for LH content by radioimmunoassay according to Niswender et al. (1968) and for FSH content according to Parlow (1964).

For preparation of pituitary cells, there are several modifications which have been successfully applied, enrichment of specific cell types has been reported, and cell lines in culture are more stable and practical than the freshly dispersed pituitary tissue.

EVALUATION

Several increasing concentrations of test preparation and LH-RH standard preparation are tested, and dose-response curves are obtained allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Pituitary halves or quarters were initially used for the assay of LH-RH as well as for the assay of TRH; cultures of enzymatically dispersed anterior pituitary cells from rats were also used (Vale et al. 1972; Martin and Sattler 1979). Loughlin et al. (1981) used perifused pituitary cultures as model for LH-RH regulation of LH secretion. O'Conner and Lapp (1984) studied the effect of pulse frequency and duration of luteinizing hormone-releasing hormone in anterior pituitary cells attached to Cytodex I beads. Functional integrity of anterior pituitary cells separated by a density gradient has been studied (Scheikl-Lenz et al. 1985). These studies have however shown that the time course of the pituitary response in vitro is quite different from the response observed in experimental animals. Receptor-binding ability to rat pituitary and human breast cancer membranes of different agonists and antagonists of luteinizing hormone-releasing hormone was studied by Fekete et al. (1989). Vigh and Schally (1984) and Czernus and Schally (1991) described in detail a cell superfusion system consisting of a Sephadex column with dispersed pituitary cells. LH response of anterior pituitary cells to 3-min exposure to various concentrations of LHRH at 30-min intervals as well as growth hormone response to human growth hormone-releasing hormone (GHRH) resulted in excellent dose-response curves. The effect of growth hormone-releasing hormone was inhibited by somatostatin. Likewise, the effect of LHRH was inhibited by pretreatment with LHRH antagonists.

The method is suitable for studies of interaction with synthetic steroid hormones and endogenous compounds which are modulating the response to releasing hormones (pituitary feedback studies).

CRITICAL ASSESSMENT OF THE METHOD

This stimulation of LH release and FSH release from pituitary cells in vitro does not reflect the time course of the pituitary hormone response found in vivo. The pituitary incubation methods are however useful early safety evaluation of compounds that have shown endocrine activity in the initial toxicology evaluation in rats, and the incubation methods are also useful for comparison of compounds and potency estimates.

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14.2.6 TSH Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

The in vitro bioassay method of Saffran and Schally (1955a, b), developed for detecting CRF activity in hypothalamic extracts, was modified to measure TRH activity in vitro (Guillemin et al. 1963; Bowers et al. 1965; Schally and Redding 1967). The assay is also applicable to other hypothalamic hormones, to their analogues, and to the testing of compounds which modulate the pituitary response to hypothalamic hormones, for example, gonadal steroids and adrenal steroids as well as synthetic compounds with endocrine-modulating activity.

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 g serve as donors. After removal, each pituitary is cut in half, transferred to a 15-ml beaker containing 1.5-ml Krebs-Ringer bicarbonate medium with 200 mg% glucose, and incubated for three 60-min periods. The media used in the first 2 incubations are discarded. At the beginning of the third incubation period, various amounts of test preparation or TRH standard are added to individual beakers. At the end of the third incubation period, the media from both control and experimental beakers are carefully aspirated. The remaining pituitary tissue may be stored frozen for later determination of hormone contents. The incubation media are assayed by RIA for content of TSH and other pituitary hormones of interest.

EVALUATION

Dose-response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

For the assay of TRH analogues, cultures of enzymatically dispersed anterior pituitary cells from rats can be used instead of pituitary halves (Vale et al. 1972).

Barros et al. (1986) studied the effect of TRH on cultured GH3 rat anterior pituitary cells using the whole-cell voltage clamp technique.

CRITICAL ASSESSMENT OF THE METHOD

The method of testing in vitro on dispersed pituitary cells is essentially as static method for the comparison of hormones and hormone analogues. It has also been applied to the testing and pituitary tissue ex vivo, from animals that have undergone a period of treatment with compounds that are known to modify the pituitary response, for example, synthetic corticoids.

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14.2.7 GH Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

For testing of pituitary cell stimulation, isolated pituitary glands or cell cultures may be used. In the investigation of growth hormone regulation, antagonism of GH release may be tested by adding somatostatin analogues at increasing concentrations in the presence of a standard concentration of GHRH. Human GHRH and the related peptides with growth hormone-releasing activity may be tested. The effect of GHRH analogues can be tested avoiding the interference of counterregulatory somatostatin secretion which limits the duration of GH release in vivo.

PROCEDURE

The pituitaries of male Sprague-Dawley rats weighing about 100 g are quickly removed after decapitation. The posterior lobe is discarded, and the anterior lobe is divided into two halves by a midsagittal cut. Five bisected hemipituitaries are incubated in plastic vials containing 4 ml TCM 199 with 0.1% BSA, 15 μg/ml penicillin, and 25 μg/ml streptomycin. The vials are gassed with 95% O_2 and 5% CO_2. After 30 min of control incubation, the medium is changed, and various doses of standard and test substances are added for an incubation of 90 min. GH content in the medium and in the pituitary tissue after incubation is determined by a specific radioimmunoassay (Schalch and Reichlin 1966). Other of hormones may be tested in the same procedure, for example, prolactin.

EVALUATION

Dose-response curves are established for standard and test compounds measuring GH release into the medium and GH depletion from the pituitaries. Potency ratios with confidence limits may be calculated from dose-concentration curves.

MODIFICATIONS OF THE METHOD

Superfused pituitary cells may be used in measuring activity and duration of effect, as well as interaction of stimulatory and inhibitory factors. Growth hormone-releasing factor from tumors in human pancreas and from rat hypothalami as well as analogues of growth hormone-releasing hormone were evaluated in a superfused pituitary cell system (Vigh and Schally 1984; Czernus and Schally 1991; Halmos et al. 1993). Anterior pituitaries of two young adult male Sprague-Dawley rats were digested with 0.5% collagenase CLS2 (Worthington) for 50 min. After incubation, the fragments were digested into cell clusters (5–40 cells) by mechanical dispersion, transferred onto two columns, and allowed to sediment simultaneously with 0.8-ml Sephadex G-10. The dead volume of the system was set to 1 ml. Medium 199 containing BSA (2.5 g/l), $NaHCO_3$ (2.2 g/l), and gentamicin sulfate (85 μg/ml) was equilibrated with a mixture of 95% air and 5% CO_2 and used as the culture medium. The medium was pumped at a flow rate of 0.33 ml/min. During an overnight recovery period, the baseline stabilized and the cells regained their full responsiveness. The samples were then infused through a four-way valve at $5 \times 10 - 10$ M concentration for 3 min (one fraction) at 45-min intervals. Rat GH was determined by

radioimmunoassay. The same system was used by Rekasi and Schally (1993) and Kovács et al. (1996a) to evaluate the activity of growth hormone-releasing hormone antagonists. For determination of the antagonistic activity, the cells were exposed to GHRH antagonist simultaneously with GHRH or to GHRH antagonist combined with 100-mM KCl (controls for potassium stimulated GH secretion) for 3 min. After 30 min, the duration of the inhibitory effect of GHRH antagonist was also tested by repeated 3-min infusions of 10^{-9} M GHRH. Using this system with pituitaries of transgenic mice overexpressing the human GHRH gene, Kovács et al. (1997) evaluated the effects of growth hormone-releasing hormone antagonists. Horváth et al. (1995) also determined cAMP release from superfused rat pituitary cells stimulated by growth hormone-releasing hormone.

GH release was determined using cultured rat pituitary cells (Brazeau et al. 1982; Perkins et al. 1983; Scheikl-Lenz et al. 1985). Pituitary cells were prepared by enzyme dispersion with collagenase, DNAase and pancreatin. The cells were cultured for 3 days in microbiological Petri dishes in Dulbecco's modified essential medium with 20-mM HEPES, 15% fetal calf serum, 100 mU/ml penicillin-G, and 100 µg/ml streptomycin at 37°C and 10% CO_2.

Cheng et al. (1993) tested time- and dose-dependent growth hormone release by a non-peptidyl growth hormone secretagogue in rat pituitary cells. Sanchez-Hormigo et al. (1998) tested growth hormone-releasing hexapeptide on growth hormone secretion from cultured porcine somatotropes.

As a follow-up from the in vitro pituitary cells studies, Kovács et al. (1996b) measured the effects of chronic administration of a growth hormone-releasing hormone agonist on body weight, tibia length, and tail length in growth hormone-deficient (monosodium glutamate-lesioned) rats. Jacks et al. (1996) evaluated an orally active growth hormone secretagogue in dogs. Serum growth hormone levels were dose-dependently increased after oral and intravenous administration. Moreover, an increase of insulin-like growth factor and serum cortisol was found. Clearly, the pituitary incubation method can only identify compounds which are suitable for further in vivo characterization, because the responses found in test animals at the target organ level are vastly different from those found at the pituitary cell level.

CRITICAL ASSESSMENT OF THE METHOD

The advantage of pituitary cell incubation with test compounds of suspected endocrine activity is that useful initial information can be provided, especially for compounds that show neuroendocrine modulating activity, for example, neuroleptics and dopamine receptor agonists. The response at a clearly defined cellular level needs then to be compared with the response in intact animals, where multiple feedback loops are operating and may markedly change the response profile when the test result is compared in vitro and in vivo. The possibility of using pituitary cells ex vivo from treated animals should always be kept in mind.

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14.2.8 Determination of Gonadal and Adrenal Steroid Hormones

PURPOSE AND RATIONALE

The gonadal steroid hormones are not species specific and can be readily determined by analytical method in mice, rats, rabbits, dogs, and other animal species used in safety evaluation. There have been many developments in technology of steroid hormone assays, in part to improve clinical diagnostics and to a large extent for research applications (Bock 2000; Henley et al. 2005; Wu 2006). Many standard assay procedures are available from commercial suppliers ("Internet Resources"). For investigation of receptor concentrations in tissue samples (Lieberman 2001) and for circulating concentrations, many methods are available (Wheeler and Morley Hutchinson 2005). In the endocrine profile protocol in rats, assays for testosterone, estradiol, and progesterone are usually required for the interpretation of effects found in the reproductive system (by organ weight changes or by histology). Reproductive organ weights (testes, seminal vesicles, uterus, and ovaries) are recorded at the end of the study. In general, effects of the test compound will be related to changes in steroid biosynthesis. In rat and dog repeated-dose studies, it is advisable to correlate the changes in organ weights with circulating concentrations of target organ hormones. In male rats, serum testosterone is determined at time of autopsy, 2 or 24 h after last treatment. In case of relevant changes, serum LH may also be determined. In female rats, serum estradiol and progesterone are determined; there is however the problem of the ovarian hormone concentrations changing with the estrous cycle. Their determination in a general procedure is relevant when major changes are found in weight of the uterus and the ovaries. Determination effects on testosterone have been helpful in toxicology studies, whereas estradiol determination in female dogs was not useful.

PROCEDURE

Blood samples are taken during the study or at autopsy. Gonadal steroid hormones are determined by specific immunoassays requiring very small sample volumes; extraction procedures are no longer needed. Steroid receptors can be determined in tissue sample storage at autopsy, for later selection of necessary investigations.

EVALUATION

Hormone concentrations are calculated from standard curves using the appropriate steroids as reference standards. Sample data are calculated as hormone concentrations in serum or per unit tissue weight (mg), when gonadal tissue is extracted. Group means are calculated, and the significance of differences is assessed by the appropriate statistical methods.

MODIFICATIONS OF THE METHOD

For the steroid hormone assays, there are many suppliers nowadays, and an Internet search for suitable reagents and methods is indicated. Due to frequent changes in suppliers, it is recommended to search for a suitable steroid assay method at the time of planning and endocrine safety pharmacology study.

CRITICAL ASSESSMENT OF THE METHOD

In the general procedure in female rats, determination of serum estradiol is useful when changes in the reproductive system are detected; concentrations may be evaluated in relation to the rat estrous cycle. In adult male rats, changes in reproductive organ weights are distinctive and generally do not require an additional measurement of testosterone for confirmation. For repeated-dose studies in dogs, testosterone measurement was found useful, whereas measurement of estradiol and progesterone concentrations did not contribute, due to the specific problems of the dog estrous cycle and due to low circulating estradiol concentrations. An alternative approach is the processing of gonadal tissue by ex vivo procedures or in vitro methods (Powlin et al. 1998), extensively applied for the characterization of endocrine-disrupting chemicals.

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Internet Resources

http://themedicalbiochemistrypage.org/steroid-hormones.php

http://www.rapidtest.com/index.php?product=Steroid-Assays&cat=106

http://www.diagnosticsproductguide.com/bguide/User/Company/1957/Diagnostic-Systems-Laboratories-Inc

14.3 Hypothalamic-Pituitary-Gonadal Function

The investigations addressed here are directed at changes in gonadal function, which are either predicted or anticipated from the specific pharmacology studies with the test compound (see reviews by DeKretser (1993), Findlay (1994)). Early indications may come from receptor screening data (Sedlak et al. 2011; Sonneveld et al. 2005; Sonneveld et al. 2006; Wilkinson et al. 2008), from in vitro assays (Lamb and Chapin 1993; Scrimshaw and Lester 2004) and targeted cell-based assays (pubmed/PubChem Bioassay). There is now a wide array of sophisticated and complex research methods, including studies on mechanisms in transgenic animals (Walters et al. 2010; Zhou 2010). This includes the application of nuclear receptor technology (Chen 2008) in the search for and profiling of new chemical entities (NCEs). Application of these methods is in the research domain, whereas the safety pharmacology needs to address questions which arise from the proposed clinical indication, directed at the response of the gonadal system preferably in intact animals (mechanistic studies), with a view to understanding of hormone-dependent regulations initiated or modified by the tests compound. In particular, this includes evaluation of feedback effects which cannot be investigated by in vitro systems (Collis 2006) and findings from early exploratory toxicology studies (Kramer et al. 2007; Bass et al. 2009).

There are many methods in early toxicology of lead compounds and xenobiotics which rely on application of nuclear receptor technology (Lu et al. 2006; Houck and Kavlock 2008; Lazar 2011) and address effects on gonadal steroid biosynthesis (Sanderson 2006). There is also an extensive knowledge comprising methods for and results of assessment of endocrine-disrupting xenobiotics (Steinberger and Klinefelter 1993; Zenick et al. 1994; Hess 1998; Moffit et al. 2007; Wong and Cheng 2011; Shanle and Xu 2011). Mechanisms of male reproductive toxicology identified by classical toxicology have been reviewed extensively (Mattison 1983; Working 1989; Chapin and Williams 1989). One interesting example is the effect of ketoconazole on the male reproductive system due to inhibition of steroid biosynthesis (Sikka et al. 1985; Bhasin et al. 1986). The advancement in

technology for multiple steroid determination from mammalian blood samples (namely, experimental animals) is very impressive and for specific questions may become of practical relevance in toxicology research (Koren et al. 2012), both for gonadal function and for evaluation of adrenal steroids. For safety pharmacology, it is preferable to rely on conventional methods for gonadal steroids.

14.3.1 Repeated Dose Study in Male and Female Rat

PURPOSE AND RATIONALE

These investigations directed at gonadal function are repeated-dose studies of similar design as the regulatory toxicology studies in rats (see Sect. 14.2). The purpose is to detect changes in the secretion of gonadotropins, gonadal steroids, and associated organ weight changes. The findings need to be interpreted in the context of reproductive toxicology, which is addressed by the regulatory studies (Neubert et al. 1999; Dorato and Buckley 2006). The duration of treatment can vary from 7 to 28 days, depending on the study objective. Issues of safety pharmacology to be addressed are in the following:

Group 1: direct effects on female and male reproductive tract, due to gonadal steroid activity (direct hormone action, by interaction with nuclear hormone receptors)

Group 2: indirect effects caused by the secretion of gonadotropins and their effect on female and male reproductive tract (by interaction with gonadotropin receptors), including feedback activation or inhibition at the level of the pituitary gland

Group 3: hypothalamic or suprahypothalamic effects caused by increased secretion and synthesis of hypothalamic peptides, which stimulate secretion of gonadotropins. The hypothalamic effects may be mediated, for example, by feedback of gonadal steroids

In group 3, the gonadotropin release will induce the secretion of gonadal steroids. This is the most relevant level accessible to investigation in vitro, ex vivo, and by repeated-dose animal study. When complex hormone interactions are present, for instance, by induced changes in the secretion and synthesis of prolactin, detailed investigation of ovarian function and steroid-induced effects on the mammary gland will be required, by histology and by proliferation markers. There is considerable overlap with the findings of reproduction toxicology, and the investigation of pituitary-gonadal function should always be in the context of ovarian morphology/histology, vaginal cytology, and uterine weight/histology. Treatment periods of 4 weeks are preferred for these investigations. In male rats, weight changes of the testis and epididymis and associated histological findings need to be addressed. The related in vitro methods are directed at the Sertoli cell (FSH mediated) and Leydig cell (LH mediated, DeKretser 1993).

PROCEDURE

Male and female rats of suitable age are assigned to groups of six to eight animals, including control groups. The selection of age group depends on study objectives, generally adult animals; however, the method is also applicable to the assessment of the effect on pubertal development. For safety pharmacology, initial body weight 100–200 g is preferred, and duration of treatment should be 28 days (in some cases, a 7-day treatment period for exploratory determination of gonadotropins and gonadal steroids is acceptable, when clear indications about the anticipated effects are available from previous in vitro pharmacology investigation and from structure activity considerations). The strain of rats needs to be the same as used in the regulatory toxicology of the same institution, to avoid problems caused by strain-specific reactions, as found with, for example, Sprague-Dawley rats versus Fischer rats (Weisenberg et al. 1987; Sandusky et al. 1988; Chandra et al. 1993; Fort et al. 1995).

For specific questions, selection of rats of 30-g initial body weight or 300-g initial body weight may be advisable (concerning pubertal development or effects on the reproductive system of animals having reached full maturation, weight-plateaued female animals, and ovariectomized adult females for bone-related investigation).

The dose range is selected based on the biological dose which is known to induce an effect related to the proposed therapeutic indication (low dose or "therapeutic dose"); the high dose should at least be a tenfold increment of the biological dose. The route of application is selected by the intended route of treatment in humans; groups with additional routes of application may be included to increase exposure by achieving high initial concentrations. Pharmacokinetic monitoring of the achieved drug concentrations is essential, at

least one time point near the end of the study, with selection of adequate sampling time points based on available pharmacokinetic information. It may be necessary to treat the animals more than once daily, to achieve sufficient exposure. At the end of this study (24 h after the last drug dosing), the animals are weighed (final body weight), and the organs of the reproductive tract (see below) are dissected out, weighed, and processed for histology. In case of paired organs, the second organ specimen may be used for biochemical analysis (hormone contents) or molecular biology investigation such as in situ hybridization and gene expression pattern (genomics).

For hormone determinations, tissues are stored frozen at −20°C. For analysis of gene expression, specific conditions apply of rapid refrigeration and storage at −80°C; tissue handling should be done by specialized investigators who need to be present during the procedure of sacrificing the animals. Due to the complexity of such investigations, inclusion of further satellite groups may be advisable or mandatory.

14.3.1.1 Male Reproductive System

Reproductive organ weight and histology: dissect out and determine weight of the testes, seminal vesicles, ventral prostate, and levator ani muscle (optional for anabolic activity). Preserve for histology one testis, seminal vesicles, and ventral prostate. Optional investigation: store one testis at −20°C for determination of gonadal steroid content, perform ex vivo stimulation test with one testis by incubation for 3 h with human chorionic gonadotropin (hCG), isolate Leydig cells by collagenase, and perform stimulation tests on the Leydig cell preparation (LHCG-receptors, testosterone production).

Hypothalamus

Take hypothalamic tissue fragment at autopsy (clearly visible at base of brain, excise with curved scissors, shock freeze immediately, and store at −20°C) and record weight of fragment. For control tissue, take sample of brain cortex (about same size and weight). An alternative way of handling sample is to homogenize tissue samples immediately in cold saline-containing bacitracin or another inhibitor of peptidase activity and store homogenate at −20°C. Tissue content of luteinizing hormone-releasing hormone (LHRH gonadorelin) is determined by specific radioimmunoassay (RIA).

Anterior Pituitary

Anterior and posterior pituitary are found together at the base of the skull, once the brain has been removed. The neurohypophyseal part (posterior pituitary) is discarded, and the anterior pituitary is bisected to provide one-half for histology and the other half for hormone tissue content (median sagittal cut through the anterior pituitary). Store pituitary tissue at −20°C for measurement of hormones.

In principle, all hormones can be determined from pituitary homogenates. Specific RIA methods are available from several suppliers (see "Internet Resources"), and hormone assays may also be performed using reagents of the National Pituitary Program, USA. For the reproductive system of rats, species-specific reagents and assays are available, as well as some cross-reacting RIA methods based on hormones of others species. Make sure that in the analytical department, there is some prior experience with rat hormone determination, to avoid problems of assays designed for other species. Contents of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are to be determined; prolactin (PRL) and growth hormone (GH) are optional depending on the known pharmacology profile of the test compound. These hormones may change due to target organ effects of the test compound, in particular due to estrogenic effects or to stimulation of estrogen secretion.

Testes, Prostate, and Epididymis

Determine testis weight and histology. There are several methods for obtained additional information about the mechanism of observed changes in androgen biosynthesis.

The tissue content of the testis can be analyzed by several methods for androgens, for example, high-performance liquid chromatography (HPLC), specific RIA or ELISA for testosterone and progesterone, and HLC analysis of testosterone precursors in the biosynthetic pathway (Sanderson 2006).

MODIFICATIONS OF THE METHOD

There are a number of interesting options to include investigation of biological samples of the male gonadal system from a targeted repeated-dose study. Receptors for FSH and LH in the testis may be determined for specific investigations targeted at Sertoli cell and Leydig cell function. The risk assessment

information provided by this determination is useful in mechanistic studies. Incubation of the testis ex vivo with human chorionic gonadotropin (hCG) is of considerable importance, and biosynthesis of androgens may be assessed by measurement of the incubation media.

The ventral prostate may be dissected in rats in order to obtain tissue for investigation of nuclear receptors (Gaston et al. 2002); similar investigation may be carried out with the epididymides (Robaire and Hamzeh 2011). The receptor for luteinizing hormone and chorionic gonadotropin (LHCG-R) has been studied extensively and may be measured in gonadal tissue (Christin-Maitre and Bouchard 1996; Hakola et al. 1997) in the context of evaluating biosimilar preparations of human recombinant luteinizing hormone and derivatives. Interestingly, non-peptide ligands of the LHCG-receptor have also been explored (Heitman et al. 2009).

Specific relevance for the assessment of changes observed in the pituitary glands of rats in long-term toxicology studies (Sandusky et al. 1988; Jameson et al. 1992; Brown et al. 1993) is attributed to measurement of pituitary hormone contents after repeated-dose treatment. Even though the formation of pituitary tumors in rat toxicology is of limited clinical relevance when epidemiology studies are compared (Tucker 1999; Gopinath 1999), questions for translational relevance may be investigated.

CRITICAL ASSESSMENT OF THE METHOD

The application of methods to the assessment of endocrine safety pharmacology depends on trends in research and development. Hypothalamic hormones, their agonists, and antagonists have been an active area of clinical development for considerable time, the LHRH antagonists had safety problems of histamine release, the desensitization of gonadotropin secretion by continuous infusion and controlled release required a number of mechanistic studies, and direct actions on the ovary and testis were explored to understand and differentiate mechanisms of action in oncology. Endocrine safety pharmacology of the new gonadotropin preparations is an ongoing task; there are biosimilar products and closely related derivatives to be characterized. In the field of new gonadal steroid derivatives, there are numerous new developments which may require mechanistic studies. The endocrine profile is a valuable step toward clinical studies because the extensively characterized interaction with nuclear receptors needs to be supplemented by evidence for the biological system effects (Riggs and Hartmann 2003). There are striking similarities in the methods for evaluation of environmental and industrial chemicals (EPA program) to characterize the toxic effects of endocrine disruptors (Whitehead and Rice 2006) and explore their level of risk at concentrations found in the environment (endocrine disruptors with hormonal activity).

14.3.1.2 Testis Incubation and Androgen Biosynthesis

PURPOSE AND RATIONALE

Androgen biosynthesis (secretory capacity for androgen precursors and testosterone) is assessed by incubation of the testis in vitro with hCG 250 mU for 3 h.

PROCEDURE

The testes of each rat were decapsulated and gently washed in tissue culture medium (TCM) 199 (Sigma biochemicals #M 2154) previously gassed with carbogen (95% O_2, 5% CO_2). Each decapsulated testis was transferred into a 20-ml glass vial containing TCM 199 at +35°C and preincubated for a minimum of 10 min in a temperature-controlled water bath (+35°C) with shaking at 80 cycles per minute. After 10 min, the incubation medium was removed and replaced with 6 ml of pregassed TCM 199 at +35°C containing 250 mU hCG (Sigma biochemicals CG5, 5,000 IU/vial). Throughout the incubations, each vial was continuously gassed via a plastic tube, with carbogen (3 L per min supplied to 8 vials). After 3 h, the incubation was terminated by removing the vials from the incubator, decanting the incubation medium into refrigerated test tubes, and transferring the testis tissue to separate vials. The incubation medium and testis tissue were stored frozen at −20°C for subsequent steroid hormone assays.

EVALUATION

The steroids in the incubation medium and in the tissue are determined (testosterone, progesterone, 17-OH-progesterone, delta-4-androstenedione). Steroids measured in medium and tissue may be testosterone (testo), progesterone (prog), 17alpha-OH-progesterone (17a-OH-prog), delta-4-androstenedione (4-A-dione),

and estradiol-17beta (E2). Testosterone and progesterone in unextracted media and ether extracts of testicular homogenates can be determined by radioimmunoassays (RIA). The steroids 17a-OH-progesterone, 4-alpha-androstene-dione, and E2 can be measured by specific 125-I-RIA methods using commercially available reagents and procedures suitable for steroid hormones without prior extraction ("Internet Resources").

Group means are calculated, and the significance of differences between groups is assessed by analysis of variance and appropriate tests of significance.

CRITICAL ASSESSMENT OF THE METHOD

Testis incubation is a valuable and flexible approach to risk assessment, which can be applied ex vivo, in repeated- dose studies for clarification of mechanisms of action, and as part of subacute toxicology studies. The immediate processing of the decapsulated testis is an advantage when compared with the assays based on an enzymatic dispersion of Leydig cells.

14.3.1.3 Testosterone Secretion In Vivo

The stimulation test can be performed as a single-dose test in rats during the treatment period, preferably 3–4 days before the end of the study, or as a single-dose test in separate groups of animals. Stimulation of testosterone in rats can be indirect via the release of luteinizing hormone by injection of LHRH (gonadorelin) or an LHRH agonist (e.g., buserelin at an equipotent dose). This stimulation test can be combined for the measurement of FSH, LH, and testosterone after a single dose of LHRH injected subcutaneously in conscious rats, or by infusion of LHRH for in rats during anesthesia. Another possibility is to stimulate testosterone secretion directly in conscious rats by the injection of hCG. These stimulation tests are similar to the procedures used in clinical pharmacology.

CRITICAL ASSESSMENT OF THE METHOD

The ex vivo assessment of steroidogenic capacity after repeated-dose treatment is much preferred when compared with the options of in vitro incubation of Sertoli cells to assess FSH-related effects (Wang 1988; Reichert and Dattatreyamurty 1989; Sairam and Krishnamurthy 2001; Heckert and Griswold 2002; Moffit et al. 2007; Hermann and Heckert 2007) or Leydig cells (Steinberger and Klinefelter 1993).

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14.3.1.4 Female Reproductive System

For reproductive organ weight and histology, dissect out and determine weight of the ovaries and uterus, and take tissue samples of the mammary gland (optional). For any specific investigation of cell proliferation (biomarkers), satellite groups are added. This is especially important for investigation of the mammary gland.

Preparation of hypothalamic and pituitary tissue and storage for assay is identical with the procedures for male animals.

Ovaries

There are all many possibilities for a detailed investigation of ovarian function (Findlay 1994). The problem for safety pharmacology is the complexity of ovarian function and the species-specific details which often preclude translational predictions for clinical pharmacology. Many aspects are covered in reproduction toxicology (Neubert et al. 1999), however, without reference to changes in nuclear receptor function or steroid biosynthesis. For details of classical bioassays, consult the chapter on endocrinology (Vogel 2008c). Binding capacity of gonadotropin receptors can be added to the morphological investigation of follicular function and corpus luteum formation, with limited gain of information. In the context of endocrine safety studies, the results of segments I–III on reproduction and fertility in the regulatory toxicology protocol should be considered before deciding on any specific investigation of rat ovarian function, including vaginal cytology. Interpretation of these findings is difficult, and relevance for the clinical situation is limited.

Mammary Gland

For the evolution of histological findings, measurement of pituitary prolactin concentrations should be considered as well as inclusion of proliferation biomarkers (Ki67).

EVALUATION

For all quantitative parameters, group means are calculated, and the significance of differences is assessed by at the appropriate statistical methods. Histology may be reported either in descriptive terms or evaluated by a semiquantitative methods (histomorphometry), to provide quantitative information suitable for statistical analysis. Specific add-on methods in molecular biology of in situ hybridization and gene expression need to be considered.

MODIFICATIONS OF THE METHOD

The investigation may be restricted to female or male animals of reproductive age, targeted to prepubertal rats or to specific age groups (Sect. 14.7.2). The important component is sufficient duration of treatment of at least 2 weeks, preferably 4 weeks. Histology of the reproductive tract is essential; vaginal cytology is time consuming but may contribute. Application of ex vivo investigation (cell proliferation, in situ for hybridization, spectrum of gene expression) adds to the complexity and should be considered when the primary investigation of histology warrants detailed follow-up.

CRITICAL ASSESSMENT OF THE METHOD

The repeated-dose study of at least 14 days is an essential and recommended element in safety pharmacology, with flexible inclusion of biomarkers of endocrine function (e.g., gonadotropin receptors, nuclear receptors, steroidogenic enzymes, and proliferation markers) if the initial findings warrant follow-up by an animal study. Many endocrine effects require exposure for 14–28 days to become detectable. For any translational prediction, it is helpful to have the confirmation of a distinct weight change or a histological change in addition to the observed change of biomarkers.

Suitable modifications of this method are required for all unexpected findings concerning reproductive function in the regulatory toxicology study and in reproduction toxicology. Testing of a new compound with classical bioassays is nowadays very unusual, due to the availability of many short-term in vitro tests for receptor affinities and receptor signaling. It is therefore recommended to look for biological expression of any hormone activity predicted from early toxicology, receptor screening, and structure activity information. Frequently, unexpected effects on the reproductive system are observed in the toxicology studies in rats and dogs, requiring an explanation in relation to the predicted therapeutic dose range and supraphysiological dose selection, to confirm selection of an appropriate dose range in regulatory toxicology and to include targeted investigation in the early clinical pharmacology program.

It is advisable to investigate the reason and mechanism for the observed changes (with supraphysiological dosing), enabling prediction of the

relevance for the proposed clinical indication. There are certain areas where the prediction is very limited, for instance in relation to human reproductive function when changes in the estrous cycle of rats of observed, due to interference with luteal function. Any observation of dose-related endocrine interference is valuable for the risk assessment, concerning clinical pharmacology and future indications which are presently not considered.

The exploration of ovarian-testicular function is sometimes done in prepubertal and in young rats (Davis et al. 2001; Marty et al. 2001). Monitoring of the estrous cycle by vaginal cytology is important but rarely included in a standard protocol, as well as steroid hormone contents in relation to the estrous cycle. In all mechanistic studies, all rats should be sacrificed at the same stage of the estrous cycle. This is impractical in routine toxicology studies. Weight of the uterus and ovaries frequently indicates endocrine disruption at the pituitary or gonadal level (Owens and Ashby 2002).

Testing in animals before puberty (immature rats) often reveals pituitary-gonadal activities more readily than testing in adult animals (Kim et al. 2002). The use of in vitro methods (receptor binding, cell lines) is still burdened by many problems of interpretation of the results. The biological relevance of receptor data needs to be confirmed in each case by bioassay (Rogers and Denison 2000; Beresford et al. 2000; Fang et al. 2000). These methods are an advantage only when compounds are selected at an early stage, not for safety pharmacology and risk assessment of advanced compounds. Histology of the uterus is very important; it reflects the classical bioassay findings for estrogenic and progestational activity. Ex vivo studies with short-term incubation of ovaries are conceivable but have not been frequently investigated. Their purpose would be information on steroid biosynthesis, at specific times of the estrous cycle.

In female rats, measurement of steroid hormones is less useful due to the precise reflection by histology (publication) and the rapid fluctuations during their estrous cycle. Pituitary hormone contents are more reliable and informative. At autopsy, the neurohypophyseal part (posterior pituitary) is discarded, and the anterior pituitary is bisected to provide one-half for histology and the other half for measurement of hormone tissue content, which changes often in a dose-related manner, whereas it is difficult to quantitate the histological changes in the pituitary gland by histomorphology. LHRH (GnRH) stimulation tests are recommended near the end of each study, by a subcutaneous injection of 0.1 μg/kg measuring the LH and FSH concentrations 60 min later. These tests can also be performed once per week during the study without compromising the end result.

Basal LH and FSH concentrations may be measured 2 h after medication with the test drug or 24 h after medication. Changes are often more readily detected shortly after medication, and pharmacokinetics of the test drug should be the basis of decision for selecting the time point. In general, measurement of LH is the more sensitive parameter; hypothalamic LHRH content is rarely useful for diagnosis. Pituitary prolactin content 24 h after last medication and mammary gland histology need to be included for specific classes of drugs; the prolactin stimulation test can be performed by oral administration of monoiodotyrosine (MIT) 1 mg/kg with serum prolactin determination 20–30 min later.

In male rats, weight changes of the testes and androgen-dependent organs (seminal vesicles, ventral prostate, levator ani muscle) readily detect interference with androgen biosynthesis and secretion (Leydig cell function). The effect on spermatogenesis and Sertoli cell function is readily detected by histology but difficult to quantitate. Before going to detailed histomorphology, effects on pituitary hormones should be evaluated by measuring the tissue content of LH, FSH and prolactin (as in females), the serum concentration of testosterone, and the testosterone content in testis tissue. It is advisable to use one testis for steroid hormone analysis and the contralateral testis for histology. When there are indications for an effect on androgen biosynthesis, the testis can be studied ex vivo by incubation with human chorionic gonadotropin (pattern of androgen biosynthesis), including tissue contents at the end of the incubation period. Leydig cells isolated by collagen dispersion may also be able to perform similar stimulation tests.

In order to assess pituitary function, inclusion of the LHRH stimulation test for LH and FSH is recommended for female rats, and the monoiodotyrosine (MIT) stimulation test for prolactin secretion is recommended at least for the female animals. Pituitary content of LH, FSH, and prolactin should always be determined at autopsy (half of the pituitary for hormone assays and the other half for histology).

In our experience, many examinations described here for the rat can also be performed in dog toxicology studies, the major limitation however being the small number of animals per group in the regulatory dog studies.

Any detailed examination on intrauterine development, sex differentiation, and pubertal development is beyond the scope of this discussion. Highly specialized methods need to be applied, and there is usually no indication for such effects from the early regulatory toxicology studies (28 days in rats and dogs). The special aspects of toxicological findings with xenobiotics have been reviewed for the female reproductive tract by Creasy (1999) and for the male reproductive tract by Newbold (1999). There is now an extensive body of evidence for the interference of industrial chemicals and xenobiotics with gonadal steroid biosynthesis (Whitehead and Rice 2006) as well as studies on classical reference compounds such as androgens, antiandrogens, estrogens, and antiestrogens.

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14.3.1.5 FSH Receptor Binding and Effect on FSH Receptors

PURPOSE AND RATIONALE

This evaluation can be performed as a single-dose in vitro test using, for example, cell membranes containing FSH receptor or as an ex vivo test measuring the concentration of FSH receptors in organ tissue of treated animals, after a suitable treatment period of at least 7 days. The application of ex vivo testing for gonadal tissue is an interesting extension of repeated-dose studies where changes in the target tissue need to be quantitated. The method is applicable to studies of ovarian function.

PROCEDURE

Membrane preparations from bovine testes are used according to the methods of Cheng (1975) and Andersen et al. (1983). Fresh bovine testes or testes from rats weighing 220–280 g are decapsulated and rinsed with cold 0.025-M Tris-HCl buffer at pH 7.2, containing 0.3-M sucrose, and then minced and homogenized with a Polytron homogenizer at maximum speed for 30 s at a concentration of 5-ml buffer per g of tissue. The homogenate is first filtered through four layers, and the filtrate is again filtered through eight layers of cheesecloth. The filtrate is then centrifuged at 12,000 g for 30 min at 4°C. The pellet is discarded, and the supernatant is further centrifuged at 100,000g for 1 h at 4°C. The supernatant is discarded and the pellet resuspended in cold 0.025-M Tris-HCl buffer at pH 7.2, containing 10-mM $MgCl_2$, at a concentration of 1-ml buffer per g of the original weight of the testis. The isolated membranes are stored at −70°C in aliquots of 10 ml per vial until use.

For assays, 12/75-mm glass disposable tubes are used. To each tube, 0.2 ml of 0.025-M Tris-HCl buffer at pH 7.2, containing 10-mM $MgCl_2$ and 0.1% BSA, 0.1 ml of standard FSH or unknown samples in the same buffer, 0.1 ml of ^{125}I-hFSH tracer labeled by the lactoperoxidase method (50,000 cpm, approximately 2 ng), and finally 0.1 ml of plasma membrane receptors of appropriate dilution (approximately 1–2 mg/ml) are added to reach a final volume of 500 µl per tube. The tubes are then shaken vigorously and

incubated at room temperature for 20 h. Following incubation, the reaction is stopped by adding 3.0 ml of cold 0.025-M Tris-HCl buffer containing 0.1% BSA. After centrifugation at 4,000 rpm for 30 min, the supernatant is drained. The pellet remaining at the bottom of the tube is counted in an automatic gamma counter.

EVALUATION

Specific binding to the testicular receptor (pellet) and nonspecific binding (nondisplaceable by 1,000-fold excess of unlabeled hFSH) are determined. The specific binding of 125I-hFSH in the presence of a range of standard hFSH concentrations is used for calculating a standard curve, and the sample concentrations are calculated from the FSH receptor standard curve, using suitable computer programs.

MODIFICATIONS OF THE METHOD

For the use in safety pharmacology, selection of a specific method for determination of the FSH receptor concentration is not important; however, biological validation of the method is very important. In reference experiments, the effect of a preceding treatment on the concentration of FSH receptors should be detected, for example, reduction in receptor concentration after inhibition of gonadotropin secretion.

CRITICAL ASSESSMENT OF THE METHOD

Significant differences between biological activity and receptor-binding activity of FSH preparations have been found by Marana et al. (1979), Zaidi et al. (1981), Foulds and Robertson (1983), and Burgon et al. (1993). This is attributed to the assay principle of measuring binding activity, but not subsequent intracellular signaling. Several receptor-binding assay procedures have been described, for example, Cheng (1975), Andersen et al. (1983) using bovine testes, and Reichert (1976) using rat testes tubule tissue. Interpretation of the relevance of toxicology findings in the pituitary gland (Tucker 1999), testis (Sikka 1999), comparative carcinogenesis (Gopinath 1999), and relevance for regulatory reproduction toxicology (Neubert et al. 1999) is often plausible due to defects and adaptive changes found in the 4-week treatment protocol; in some cases, longer treatment periods may be needed as well as inclusion of assessment of reversibility by extending the studies.

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Internet Resources, Reagents, and Methods

http://www.bachem.com/research-products/catalog-products
Mouse diabetes, http://www.bio-rad.com/prd/en/US/LSR/PDP/LTBW3O4VY/
Rat diabetes, http://www.bio-rad.com/prd/en/US/LSR/PDP/LTBW52KG4/
http://www.rapidtest.com/index.php?i=Steroid-ELISA-kits%26id=68%26cat=14
http://www.rapidtest.com/products-elisakits.php?product=ELISA-Kits%26cat=2

14.4 Hypothalamic-Pituitary-Adrenal System

Safety pharmacology of the adrenal glands is related to effects on the secretion and synthesis of glucocorticoids (Harvey 1996; Harvey et al. 2007; Harvey and Sutcliffe 2010) and mineralocorticoids (Funder 2009; 2010). In the rat toxicology studies, effects on the adrenal medulla are rare, and there is an incidence of spontaneous pheochromocytoma (Tucker 1996) not found in humans. The morphological and histological toxic reactions of the adrenal glands have been characterized (Hinson and Raven 1996, 1999; Greaves 2007a; Harvey et al. 2007; Jones 2009). The primary task in safety pharmacology is to assess whether a drug candidate can induce changes in the biosynthesis and secretion of adrenal steroids, for example, by noninvasive monitoring of adrenal steroid excretion or by dynamic tests of ACTH responsiveness (ACTH challenge).

A significant number of drugs and industrial chemicals which have toxic effects on the adrenocortical function have been evaluated in the context of programs on endocrine disruptors, for example, in several steps of the OECD 407 method development (Lahiri and Sircar 1991; Hinson and Raven 2006) and in many related in vitro studies (Molina-Molina et al. 2006; Sanderson 2008; Parmar and Rainey 2008; Parmar et al. 2008; Ziolkowska et al. 2006; Wang et al. 2012).

14.4.1 Introduction

The methods described here are applied for supplemental safety pharmacology studies (ICH S7A), if the test substance has shown indications of effects on the hypothalamic-pituitary-adrenal system in the preceding pharmacology studies. This includes effects in receptor profiling and enzyme profiling directed at, for example, the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and characteristic steroidogenic enzymes (Wilkinson et al. 2008). The biological evaluation may also be needed as a final step of risk evaluation. There are many toxicological observations related to the unexpected interference of drugs with adrenocortical function (Ribelin 1984; Mann 1996). There are protocols for clinical assessment (Weiss and Patel 2002; Babic et al. 2011) related to adrenocortical activation by compounds with direct action on steroid biosynthesis and to suppression of adrenal steroid secretion with compensatory hypothalamic-pituitary feedback effects (Harvey and Sutcliffe 2010) masking the adrenal cortical atrophy. Several reference compounds are available for comparison and for clinical reference, for example, ketoconazol, etomidate, trilostane, and the antiglucocorticoid/antiprogestin mifepristone (Potts et al. 1971; Potts and Jüppner 2008; Albertson et al. 1994; Loose et al. 1983; Lambert et al. 1986; Weber et al. 1993; Thomas and Thomas 1999), including recent studies on industrial chemicals, for example, fungicides (Molina-Molina et al. 2006) assessed by steroid receptor profiling. Receptor profiling of NCEs is valuable in the early selection of lead compounds and profiling of candidates (Ojasoo et al. 1988; Jiang et al. 2010). The relevance of adaptive changes in the S7A d guideline has been reviewed (Valentin et al. 2005), with reference to assessing the capability of models to predict adverse events in humans. Based on recent clinical experience, this may be considered in the context of the need for preclinical evaluation of aldosterone secretion, referring to unexpected effects on inducing hypertension and cardiovascular complications in the clinical evaluation of the CEPT inhibitor, torcetrapib (Hu et al. 2009; Funder 2009; Johns et al. 2012). The related mechanistic studies (Funder and Mihailidou 2009; Funder 2010) on the effects of the compound on aldosterone secretion raised questions and provided explanations, also giving directions for the future safety evaluation of other CETP inhibitors.

The approach to a stepwise investigation of adrenal toxicology has been reviewed with reference to the sequential approach from in vitro studies to final evaluation of dose-related assessment of biological relevance in targeted animal studies (Harvey 1996; Hinson an Raven 1999; Harvey et al. 2007). The in vitro studies are generally performed with several increasing doses to investigate concentration-effect relationships (Colby 1994; Colby 1996; Colby and Longhurst 1992). The preliminary information from these in vitro studies is then compared with the biological information available from in vivo studies, to assess the need for additional in vivo animal studies based on single-dose or repeated-dose administration of the test compound. The need for noninvasive investigation of adrenocortical function including corticosterone and aldosterone excretion in rats by noninvasive methods is obvious from the recent experience with CEPT inhibitors (Forrest et al. 2008). The stepwise evaluation of hormonal parameters in the OECD 407 protocols for endocrine disruptors in a 28-day treatment protocol is helpful to appreciate the difficulties of including several parameters of hormone activity, in particular selection of time points for measurement, sampling procedure for blood samples, and selection of reference compounds for validation of reliable detection of changes (Andrews et al. 2001; Hinson and Raven 2006; Sellers et al. 2007; Ullerås et al. 2008, Ziolkowska et al. 2006). Unfortunately, noninvasive methods for adrenal steroid excretion were not assessed in the OECD-EPA studies. It should be considered here that the objectives of assessing environmental contaminants and industrial chemicals are different from those of safety pharmacology procedures, directed at screening a large number of compounds rather than going into exploration of detail.

In the endocrine safety protocol, the interaction of multiple endocrine systems in test animals is addressed. As discussed by Harvey (1996) and by Harvey and Everett (2003), effects on adrenocortical function are frequently found in toxicology studies, sometimes related to enzyme induction and effects on steroid biosynthesis (Loose et al. 1983; Weber et al. 1993; Nebert and Russell 2002; Rossol et al. 2001). Test procedures in animals are required when there is a reason for concern. Frequently, the effects observed are due to stress of toxicology procedure, rather than specific interaction with the target organ, and may involve effects on catecholamine release from the adrenal medulla (Tucker 1996). Much new evidence has been accumulated from the testing of industrial chemicals with effects on adrenal steroid biosynthesis (Cockburn and leist 1999; Harvey and Johnson 2002; Sanderson et al. 2002; Harvey and Everett 2003; Hinson and Raven 2006; Higley et al. 2010).

14.4.2 Adrenal Steroid Excretion in Rats in a Repeated Dose Study

PURPOSE AND RATIONALE

This investigation is performed in adult male and female rats. It may be applied in the context of a toxicology study as an add-on investigation or performed as a separate study to detect changes in adrenal function during treatment (satellite animals, mechanistic study). Assessment of the change in adrenal function is performed in a minimal invasive procedure, by measuring the excretion of adrenal steroid hormones in the urine during the night period. Successful application of the method critically depends on suitable housing conditions for the animals, and preliminary training and adaptation of the facility is required. The animal house should have a controlled light-dark cycle and well-controlled air-conditioning. Animals need to be adapted to metabolism cages for collection of urine (prestudy handling). An option is adaptation during the study. This can be achieved by transferring the animals twice per week for one night (e.g., 18:00–06:00) to metabolism cages, including collection of urine for control of volume, osmolarity, electrolyte concentrations, and exploratory hormone determination of corticosterone and aldosterone, the results being evaluated after correction for creatinine excretion ("creatinine correction"). This procedure allows for control of the consistent effect of handling on the reproducibility of results and for detection of a time-action profile in changes of adrenocortical function. It is important to use six rats per group, assigning two rats to each metabolism cage. The social interaction of rats is important for meaningful results. This is a general requirement for the animal facility: absence of noise and any entry of personnel during the night period. Moreover, reproducible results are obtained when rats have social contact, whereas isolation of single rats in metabolism cages produces artifacts. This consideration is also valid for studies in dogs, where the procedure of

staying in a metabolism cage is well tolerated in the presence of other dogs in the same room, whereas results become erratic during isolation of the dogs.

The final urine collection in the noninvasive rat adrenal procedure needs to be done 3–4 days before the end of the treatment period, under conditions of established adaptation. In this study, treatment of the animals needs to be done in the afternoon, before starting the collection procedure, and for a consistent protocol, it is advisable to treat the animals at the same time of the afternoon throughout the study. Selection of this procedure is preferable to obtain consistent effects even for test drugs with a short duration of action.

PROCEDURE

Male and female rats (preferably of 200 g initial body weight) are kept in a light- and temperature-controlled animal house, under standard conditions, on a pelleted rat food, with free access to drinking water. Starting 2 days after the beginning of treatment, the animals are transferred to metabolism cages for the night period (two rats per cage to avoid isolation stress). Throughout the study, treatment is administered in the afternoon. Urine is collected during the night, osmolarity and electrolytes are determined, and one volume is dispensed immediately and stored deep-frozen for the analysis of adrenal steroid hormones and creatinine.

In a toxicology study, groups of animals are randomly assigned to one control group and three treatment groups (three increasing dose levels). Treatment is administered in the morning, and animals are transferred to their metabolism cages in the evening. This procedure does not interfere with the standard protocol of regulatory studies; collection of urine samples is an add-on procedure which pertains to all animals of the study. Urine collection should be done sparingly to reduce any effects on the animals, for example, one night each during the third and fourth week of the study. Prestudy adaptation of the animals to metabolism cages can be considered to make them acquainted with the procedure and reduce stress effects. The last urine sampling should be done several days before final autopsy, such that effects on histology and clinical chemistry at the end of the study are avoided.

For evaluation and satellite groups, treatment should be given in the evening, before the onset of the dark period and before transfer to urine sampling cages. This ensures that any effects on adrenal steroid secretion of limited duration and at lower dose levels are detected in the urine procedure. For each cage and collection night, the urine volume of two rats per cage collected during the night is recorded.

At the end of the treatment period, the animals are killed by decapitation, preferably 24 h after the last treatment injection. Body weight and organ weights are recorded, and organs of interest are dissected out at autopsy for histological examination and/or measurement of hormone contents.

In male animals, weight of the adrenals, testes, seminal vesicles and ventral prostate is recorded (because the effects on steroid biosynthesis frequently involve both the adrenal and gonadal system). In female animals, weight of the adrenals, ovaries, and uterus is recorded. As an added investigation, the hypothalamus of the animals may be dissected out and shock frozen immediately for assay of corticotropin-releasing hormone (CRH), and the adenohypophysis (pituitary) is dissected out for assay of corticotropin content (ACTH) by radioimmunoassay. The pituitary tissue is stored frozen at $-20°C$ until the assay of hormone content can be performed.

During a treatment period of 7 days or up 28 days (toxicology study), the excretion of corticosterone and of aldosterone during the night period is monitored on the last 3 days of treatment or on 2 consecutive days of each week (toxicology study). This approach ensures a check on consistency of changes in adrenal steroid excretion.

EVALUATION

The following parameters are evaluated at the end of the study period by calculation of the group means and standard deviation, applying suitable methods for analysis of variance and tests for significance of differences between groups. In most instances, the significance of differences for treatment groups versus control groups is calculated at the 95% level by analysis of variance and a distribution-free rank test. Here is a list of the important parameters which are to be calculated in the evaluation of studies on adrenocortical function:

- Excretion of corticosterone, in the urine of at least six rats of control and treatment groups (pooled urine of two rats per metabolism cage)
- Excretion of aldosterone in the same samples
- Weight of adrenal glands at autopsy

- Tissue content of corticosterone and aldosterone, expressed as total content per gland (ng/adrenal) and as relative concentration (ng/g adrenal tissue)

Supplementary information about the reproductive system is obtained in the same study and should generally be obtained to investigate the possibility of interference by the test substance with the secretion and synthesis of steroid hormones of the adrenals and of the male and female gonads.

- Male rats: weight of testes, seminal vesicles, and ventral prostate
- Female rats: weight of the ovaries and uterus

Optional parameters of hypothalamic-pituitary activation or feedback effects are the hypothalamic content of CRH and the anterior pituitary content of ACTH (group means and standard deviation). Final data may be calculated as hormone content per mg or gram of organ tissue (pituitary hormone contents) or as hormone content per tissue equivalent (hypothalamic fragments).

MODIFICATIONS OF THE METHOD

It is recommended to perform this extensive investigation in rats, for comparison of the findings with the related regulatory toxicology study, alternatively in satellite groups of the regulatory toxicology (e.g., controls and high dose). Under special conditions, a similar methodology can be applied to the dog (Stolp et al. 1983; Rijnberk et al. 1988; Feldman et al. 1996; Colagiovanni and Meyer 2008). A modification for veterinary medicine concerning the 24-h determination of aldosterone excretion in dogs with creatinine correction has been developed (Gardner et al. 2007) in the context of diagnostics in cardiology; 24-h diagnostic assessment of the cortisol/creatinine ratio in canine urine has also been validated (Tidholm et al. 2005). This is important because the principal adrenal steroid in rats is corticosterone, whereas in the dog cortisol is excreted in the urine. Collection of urine samples during the night under minimum invasive conditions (in the context of pharmacokinetic studies) as well as 24-h collection has been successfully performed in dogs.

The core of this method is the assessment of adrenal steroid excretion during minimum invasive conditions, during repeated-dose daily administration of the test substance. Supplementary investigation may be an ACTH stimulation test (Feldman et al. 1996).

Modifications of the method are the duration of treatment (not less than 7 days) and timing of treatment injections (conventional regimen in the morning, or injections late in the evening shortly before transfers to metabolism cages for starting the urine collection period).

Concerning analytical methods for cortisol, corticosterone, and aldosterone in serum and urine or tissue samples, it is recommended to search the Internet at the time of planning the study for commercial sources of assays and perform the analytical validation for multiple sample matrices (serum, plasma, urine, extraction from feces, tissue extracts) before including these methods in the final study design (prestudy validation procedure).

Suppliers often indicate that their methods are applicable in a variety of animal species; there are however considerable differences in steroid metabolism and in the percentage of steroid that is detected by the analytical method envisaged. There may also be strain differences (Sarrieau and Mormède 1998) and specific details of the animal facility (Eriksson et al. 2004) which need to be considered before selecting a rat strain for a detailed investigation which is as different from the strain used for the regulatory toxicology studies of the institution.

14.4.2.1 Adrenal Steroid Content

Determination of adrenal gland steroid content may be included but is of limited value. Determination of serum concentrations of corticosterone and aldosterone may be included during up to week 3 for studies of 4 weeks duration, but needs to be performed under well-controlled conditions which reduce stress to a minimum.

14.4.2.2 Serum Corticosterone in Rats

Any sampling of serum corticosterone in rats without anesthesia will lead to highly variable and arbitrary results due to stress effects, even if performed at a standard time of the day. For this reason, either anesthesia or premedication with dexamethasone has been developed and may be applicable in a limited number of animals, for example, control animals and high-dose group for orientation, albeit with limited reliability of result. Inclusion of monitoring in serum is a matter of handling the animals, not one of analytical methods. There are reliable

methods for adrenal steroids including aldosterone (even though concentrations of aldosterone are very low). For diagnostic evaluation of serum corticosterone in groups of any months, preferably, a stimulation test with ACTH after low-dose dexamethasone blockage of spontaneous secretion may be included. Rats are injected with 0.5 mg/kg of dexamethasone 18 h before the stimulation test. The test is then performed by an injection of synthetic ACTH (1–24), which is readily available as a clinical diagnostic tool. The clinical test procedures have been reviewed (Weiss and Patel 2002). Extended investigations are the assessment of effects on other steroid-secreting organs (gonadal system) and the assessment of hypothalamic-pituitary changes at the end of the treatment period. Determination of pituitary ACTH content may be particularly useful in case of inhibitory effects on adrenal steroid biosynthesis.

14.4.2.3 Serum Cortisol in Dogs

Blood sampling for cortisol: a dexamethasone suppression tests for the diagnostic evaluation of adrenocortical function in dogs with hypercorticism or adrenal tumors has been established (Feldman et al. 1996) and may be included in the adrenal profile study for dogs.

CRITICAL ASSESSMENT OF THE METHOD

Changes in the secretion of adrenal steroids are often induced by stress effects, which need to be differentiated from drug effects on the adrenal steroid secretion (Harvey and Everett 2003). This is the reason for selecting the minimum invasive procedure of monitoring adrenal steroid excretion in urine, which avoids blood sampling and reduces the stress on animals by adequate adaptation periods for the nighttime sampling procedure.

It is always important to compare the effect of well-established drugs which affect adrenal cortical steroid secretion and synthesis (comparator drugs). The best reference compound for adrenal steroid excretion is synthetic corticotropin-24 (Synacthen), which readily induces adrenal hyperplasia at high doses or when given by sustained subcutaneous infusion (osmotic minipumps). There are many synthetics steroids with some adrenocortical activity. The problem as with other hormonal evaluations is the pronounced circadian rhythm of corticosterone secretion and excretion in the urine (Miki and Sudo 1996; Hilfenhaus and Herting 1980). To avoid these difficulties of sampling under stress conditions, in a system constantly changing and responding immediately to any stress, the night period is the most adequate selection of minimum stress and interference especially in well-controlled animal houses.

The equivalent of cortisol secreted by monkeys can also be detected by a noninvasive procedure of urine sampling (Pal 1979); many studies of this kind are available for humans. Blood sampling needs to be done during specific times of the day; for advance stimulation tests, endocrine essays have been developing based on a temporary suppression of the pituitary adrenal response by pretreatment with dexamethasone. Such tests may be included during a study of 28 days' duration, but should be done at least 7 days before the end of the study in order not to interfere with the final hormonal evaluation. Stimulation tests with synthetic corticotropin may be built into the study. For the evaluation of stimulatory drug effects, there is a noninvasive procedure for corticosterone secretion which could at the same time be applied to electrolyte balance studies (Haack et al. 1978). During the night period (14 or 16 h), the animals are placed in metabolism cages for urine collection; the amount of corticosterone and aldosterone excreted during this period is determined in the urine samples, together with the electrolyte concentration. The principle is derived from studies on electrolyte excretion after treatment with diuretics (Hilfenhaus 1977).

MODIFICATIONS OF THE METHOD

For the noninvasive exploration of adrenal steroid secretion by monitoring the excretion in fecal samples, numerous studies have been reported in pharmacology and zoology for a number of animal species (Kley et al. 1976; Ganswindt et al. 2003; Carlsson et al. 2009; Miki and Sudo 1996).

Human studies on the stimulation of adrenal steroid excretion by ACTH administration were performed (Vecsei et al. 1982; Gomez-Sanchez et al. 1988) including the evaluation of stressful procedures in human physiology (Muñoz et al. 2010). Similar procedure could be adapted for the rat to assess secretory capacity after the repeated-dose treatment (Siswanto et al. 2008).

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14.4.3 Corticosterone Secretion in Dexamethasone Blocked Rats

PURPOSE AND RATIONALE

Corticotropin activity can be measured by the increase of corticosterone in venous blood of hypophysectomized or dexamethasone-blocked rats, as a single-dose test when receptor affinity for ACTH receptor is found with a test compound. The test can be used to measure time-response curves of corticotropin preparations and compounds with adrenal-stimulating activity (Vogel 1965, 1969). The use of hypophysectomized rats for evaluation of the pituitary response to compounds with corticotropin activity has been entirely replaced by the dexamethasone-blocked model.

In the context of repeated-dose studies for endocrine safety evaluation, a modification of this test is built into the procedure, as described above (Sect. 14.4.2).

PROCEDURE

For the ACTH assay, male Sprague-Dawley rats weighing 150–200 are injected subcutaneously 24 and 1 h prior to subcutaneous injection of the ACTH preparation or the standard with 5 mg/kg dexamethasone in oily solution. Eight rats are used for each dose of test preparation or standard. Various time intervals after ACTH injection, the rats are anesthetized with 60 mg/kg pentobarbital i.p., and blood is withdrawn by cardiac puncture. One-ml plasma is diluted with 2-ml distilled water and extracted (washed) with 5-ml petrolether to remove the lipids. The petrolether is discarded. Two ml of the water layer are extracted twice with 5-ml methylene chloride by vigorous shaking for 15 min. The methylene chloride phase is separated by centrifugation. Both methylene chloride extracts are unified and shaken with 1 ml ice-cold 0.1 N NaOH. The water phase is immediately removed, and the methylene chloride extracts dried by addition of dry sodium sulfate. An aliquot of 5 ml of the methylene chloride extract is mixed with 5 ml of the fluorescence reagent (seven parts concentrated sulfuric acid, three parts 96% ethanol, v/v). After vigorous shaking, the methylene chloride phase is removed, and fluorescence is measured with primary filters of 436 mm and secondary filters of 530–545 mm. For calibration, concentrations of 0, 20, 50, 100, and 250 mg/ml corticosterone are treated identically and measured in each assay. Synthetic ACTH (1–24) is used as the reference standard; this compound has full biological activity (Schuler et al. 1963) when compared with natural ACTH (1–39).

The classical analytical method of methylene chloride extraction is now entirely obsolete; there are direct assays for corticosterone and aldosterone in rat plasma. Similar analytical methods are applicable for adrenal steroid excretion in urine (Kabra 1988; Samtani and Jusko 2007; Carvalho et al. 2008; Kushnir et al. 2011). Selection of simple and reliable assay methods (RIA, ELISA) is a logistic decision. An Internet search will indicate suppliers. For the complex analytical tasks, one of the methods indicated above with clinical reference experience and validation of specificity is to be preferred.

EVALUATION

Using three doses of test compound and standard, activity ratios with confidence limits can be determined after each time interval with the 3 + 3-point assay giving evidence for the duration of action (Vogel 1965, 1969).

MODIFICATIONS OF THE METHOD

In contemporary methods, the assay of corticosterone is performed by immunoassay in unextracted serum samples or in serum and tissue extracts by HPLC. In the classical methods, fluorometry was applied. Pekkarinen (1965) used fluorometric corticosteroid

determinations in guinea pigs resulting in highly deviating activity ratios of synthetic and commercial corticotropins as compared with the international working standard of ACTH. The option now is to select a low-dose dexamethasone test or high-dose dexamethasone test consistently, depending on animal species and experience. Analytical methods depend to some extent on the number of samples to be analyzed and the expected workload.

CRITICAL ASSESSMENT OF THE METHOD

It is recommended to include these dynamic function tests during the early investigation of adrenocortical function in satellite groups of rats as well as in repeated-dose studies in dogs (Feldman et al. 1996). Pretreatment with dexamethasone is essential for reproducible results, because stressful procedure interferes with the secretion of corticosterone/cortisol during the sampling procedure, even if animals are handled carefully in a separate room of the animal facility. This is particularly the case with dogs. Due to the analytical methods now available (RIA, ELISA, IRMA, HPLC), the response of the pituitary gland and any feedback effects after repeated-dose treatment (3–7 days preferred) are readily detected.

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14.4.4 Corticosteroid Release from Adrenal Cell Suspensions In Vitro

PURPOSE AND RATIONALE

The principle of this method is to assess the direct effect on adrenal cells, either by incubation of dispersed adrenal cells with ACTH preparations or with test compounds in the presence of ACTH or angiotensin II. Ideally, a homogeneous preparation of adrenocortical cells is obtained, variability is eliminated, and the number of animals required is much reduced. An in vitro assay of corticotropin was described by Saffran and Schally (1955a). This test has been modified by Van der Vies (1957) and used by several authors (Staehelin et al. 1965; Vogel 1969). Subsequently, adrenal cell lines have replaced the method when large numbers of compounds need to be evaluated. The previous analytical method using adrenal cell suspensions as an assay for corticotropin preparations is a cumbersome procedure which was replaced by RIA and ELISA methods.

PROCEDURE

There are many modifications of this method, based on incubation of small fragments of adrenal glands, or on the use of dispersed adrenal cells. Cells are incubated in a physiological buffer. The final solution is gassed with

a mixture of 95% O_2 and 5% CO_2 for 10 min. As with similar methods—for example, for pituitary cells or dispersed Leydig cells—there is a preincubation period, followed by the test incubation with corticotropin or test compound. The vessels are incubated and gassed with a mixture of 95% O_2 and 5% CO_2 at 38°C under continuous shaking for 2 h. The initial analytical procedure for corticosterone has been replaced by measuring aliquots in extracts of incubation medium by HPLC or directly in the media by specific immunoassays.

EVALUATION

The concentrations in the incubation flasks are compared (duplicates or triplicates); potency ratios with confidence limits may be calculated from a 2 + 2 design or from dose-response curves.

MODIFICATIONS OF THE METHOD

Corticosterone is now conveniently determined by RIA or HPLC, and dispersed adrenal cells are incubated instead of adrenal tissue fragments.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable to detect changes in steroid biosynthesis induced by test compounds. In endocrine safety evaluation, it is recommended to apply measurement of corticosterone and adrenal steroid in metabolites in the tissue as an ex vivo investigation to test material from animals previously treated in a repeated-dose protocol.

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14.4.5 Adrenal Cell Culture, Human Cell Lines

There has been an impressive progress in the use of adrenal cell lines for the assessment of effects on steroid biosynthesis, in particular due to the programs on industrial chemicals and environmental contaminants (endocrine disruptors).

The intrinsic problem of using in vitro systems for endocrine safety pharmacology is the complexity of such systems with regard to biological interpretation. When a two-stage approach is selected of using an adrenal cell line with subsequent bioassay in rats, the evidence for designing early clinical trials becomes more reliable. Incubation of dispersed adrenal cells was the initial step leading to the development of human adrenal cell lines. The cell lines were derived from human tumors, and they enable a detailed analysis of effects on steroidogenesis. There are several examples for the importance in safety pharmacology, particularly relevant is the observation that torcetrapib showed an off-target effect on secretion of aldosterone in H295R adrenocortical cells and related test systems (Forrest et al. 2008; Capponi et al. 2008; Hu et al. 2009; Stein et al. 2009; Clerc et al. 2010).

Short-term assays have been developed with rat and bovine adrenal cells. Adrenocortical cells (BAC) were prepared by treatment of adrenocortical slices with trypsin, cells were cultured in a defined media, and treatment was started at the end of the second day of culture. LeRoy et al. (2000) investigated the time course of the response to ACTH, angiotensin II (AT2), and other proteins and examined the effects of peptides on the expression of specific genes. This approach is useful in research and for early assessment of drug candidates. In a similar manner, Ziolkowska et al. have used rat adrenocortical cells in vitro to study the effect of several compounds on secretory and proliferative activity. The parameter was basal

corticosterone production from cultured adrenocortical cells after 24-h exposure to the test compounds. The conclusion of the study was that proliferative activity of cultured adrenocortical cells exhibits great variability which depends on the dose and duration of exposure of the studied cells. The practical use in safety pharmacology is therefore limited.

An extensive program has been conducted with the human adrenocortical carcinoma cell line H295R in the context of steroidogenic response elicited by endocrine disruptors. Samandari et al. (2007) stated that the NCI-H295A and NCI-H295R cells progeny originates from tumors which produce predominantly androgens and a small amount of mineralocorticoids and glucocorticoids. It was found that the two cell lines differ markedly in their steroid profile and expression pattern of important genes involved in steroidogenesis. One cell line produces more mineralocorticoids, whereas the other line produced more androgens. Both cell lines were found to express the ACTH receptor at lower levels consistent with low stimulation of cells by ACTH. The angiotensin I receptor (AT1R) was expressed in both cell lines, and angiotensin II stimulated steroidogenesis in H295A but not in H295R cells. The studies were initially targeted at clinical questions related to disorders of adrenocortical function. Two cell lines were characterized with regard to predominant mineralocorticoid biosynthesis in one assay and androgen biosynthesis in the other system. The application of these cell lines to studies of preclinical safety pharmacology is limited, whereas these lines have their specific relevance and have been extensively used in environmental toxicology.

Ullerås et al. (2008) studied the secretion of cortisol and aldosterone in relation to disruption of adrenal function by chemicals. They screened a large selection of chemicals in this model, using specific ELISA methods for hormone production in vitro. An added test was angiotensin II-stimulated hormone secretion. The inclusion of several known toxicants was helpful to characterize profiles for aminoglutetimide, ketoconazole, and etomidate. The test system was found to be reliable and responding in a reproducible manner to established toxicants. An application for safety assessment of drug candidates is supported by these findings, for instance by selecting well-characterized toxicants as reference compounds for safety pharmacology satellite studies (Hecker et al. 2006).

Details of using the adrenocortical cell lines have been reviewed by Hinson and Raven 2006. Higley et al. (2010) performed a detailed assessment of chemical effects on aromatase activity in the H295R cell line. The reference assays are commonly used aromatase assays, including a human microsomal assay established in the EPA-EDSP program. The H295R cells were exposed to a number of known toxicants, and the response of direct, indirect, and combined effects of aromatase activity was measured. Exposure to letrozole, ketoconazole, and aminoglutethimide resulted in greater indirect aromatase activity after a 48-h exposure. The overall conclusion was that a combination of direct and indirect aromatase measurements is needed in order to predict the effects of industrial chemicals on estrogen production and the mechanism of action by aromatase activation. It was shown that direct aromatase measurements in vitro need to be interpreted with caution and a subsequent cell-based assay is required for application in safety pharmacology.

The method of evaluation in the H295R adrenocortical cell lines has also been applied to effects on mineralocorticoid secretion (aldosterone), for example, in studies on CEPT inhibitors (Capponi et al. 2008).

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14.4.6 ACTH Receptor Affinity

The corticotropin receptors have been a matter of great interest concerning the regulation of adrenocortical function, and more recently, an array of functions related to energy balance, metabolism, immunology, and pigmentation which are explored in the context of melanocortin receptors, of which the ACTH receptor is now designated one of the melanocortin receptors, MC2R (Fong et al. 2012).

PURPOSE AND RATIONALE

For all new compounds which have some structural similarity or relation to the corticoid scaffold. There are now many such test systems which measure the initial membrane binding to the ACTH receptor and to define the initial steps of hormone-related activation. However, the binding affinity does not necessarily imply biological activation, for example, of adrenal cortical cells. Therefore, such tests always need to be followed up by an in vitro testing on adrenal glands or dispersed adrenal cells.

Corticotropin (ACTH) receptors have been used as the test preparations for comparison of the binding affinities of adrenocorticotropin peptides using a cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line (Kapas et al. 1996). Such assays measure the initial membrane binding but not strictly the biological activation, for example, of adrenal cells or melanocytes.

PROCEDURE

HeLa cells are seeded into 12-well culture plates at a density of 106 cells/well. On the second day of culture, the cells are washed as follows: 2× in 1 ml of ice-cold 0.9% NaCl, 1× in 1 ml of ice-cold glycine (50-mM glycine, 100-mM NaCl, pH 3.0) for 5 min, and 2× in 0.5 ml of ice-cold 0.9% NaCl. Cells are then incubated for 60 min at 20°C with increasing concentrations of nonradioactive ACTH or various ACTH analogues and the reactions initiated on the addition of [125I-iodotyrosyl23]ACTH[1–39] (2,000 Ci/mmol; final concentration 0.1 pmol/l) in DMEM. At the end of the incubation, the medium is removed and the cells washed three times with 0.9% NaCl and then dissolved in 0.5-M NaOH/0.4% sodium deoxycholate. Each point is determined in triplicate. Specific binding is determined as the difference of total radioactivity bound minus nonspecific binding radioactivity determined in the presence of an excess of 10–5 M nonradioactive ACTH.

EVALUATION

Binding parameters are calculated from computer programs, one example being the ligand program of Munson and Rodbard. Estimates of binding affinity are then compared with the binding affinity of natural corticotropin, corticotropin analogues, and more importantly with the drug concentrations of the compound to be evaluated reached in the toxicology studies or similar pharmacokinetic studies (level of exposure).

MODIFICATIONS OF THE METHOD

There are many different modifications of the method; early technology used membrane preparations and modern methods use solubilized receptors and receptor constructs which enable fast signal detection. In the early studies, ACTH receptors of rats were measured

by Buckley and Ramachandran (1981); Grunfeld et al. (1985) characterized the ACTH receptors in rat adipocytes. Subsequently, the ACTH receptor on human adrenocortical cells was measured (Catalano et al. 1986). Expression of the ACTH receptor mRNA was studied in mouse and human adrenocortical cell lines (Mountjoy et al. 1994). Extensive mechanistic studies were performed on bovine adrenal cell cultures, including the time course of the response after adding test compounds (LeRoy et al. 2000). Functional expression of the human ACTH receptor gene has been explored by molecular biology methods (Penhoat et al. 2000).

Penhoat et al. (1993) reported the identification and characterization of corticotropin receptors in bovine and human adrenals, using cultured bovine adrenal fasciculata reticular cells and crude plasma membrane fractions. Lebrethon et al. (1994) and Penhoat et al. (1991, 1995) studied the regulation of ACTH receptor mRNA and binding sites by ACTH and angiotensin II in cultured human and bovine adrenal fasciculata cells. Picard-Hagen et al. (1997) found that glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells, as an equivalent of receptor regulation by circulating glucocorticoid concentrations.

Naville et al. (1996, 1997) developed a stable expression model in order to characterize the human ACTH receptor by binding studies and functional coupling to adenylate cyclase.

Schiöth et al. (1996) described the pharmacological distinction of the ACTH receptor from other melanocortin receptors in the mouse adrenocortical cell line Y1. Melanocortin receptors do not have a binding epitope for ACTH beyond the sequence of alpha-MSH (Schiöth et al. 1997). The human ACTH receptor (MC2R) is now investigated as part of the wide spectrum of melanocortin receptors in several animal species, which may be used in environmental toxicology, for example, for investigation of endocrine disruptors as well as for phylogenetic comparisons (Schiöth et al. 2005; Ling and Schiöth 2005; Haitina et al. 2005; Kobayashi et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

This test is to be performed if the specific pharmacology of the compound indicates the potential for interaction with the ACTH receptor. It is used to prove that the compound achieves highly specific receptor targeting (Behrens and Ramachandran 1981; Oelofsen and Ramachandran 1983) or modulates the receptor density by changing peripheral glucocorticoid concentrations. For specific compounds, the extent of safety pharmacology studies to be performed with the product is reduced (Stocco and Clark 1996; Abdel-Malek 2001; Vaisse et al. 2000).

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14.4.7 ACTH Secretion and Tissue Content

The secretion of ACTH is stress related, and measuring levels in unanesthetized animals does not provide valid estimates due to wide fluctuations. For that reason, the dexamethasone pretreatment is recommended in rats as well as in dogs to establish dynamic function tests of ACTH responsiveness, whereas measurement of basal serum ACTH concentrations is impractical. The pituitary tissue content of ACTH can be measured readily; this should however be done only in the context of a search for feedback effects, for example, of adrenal inhibition (reduced glucocorticoid secretion due to inhibition of steroid biosynthesis). Measuring the ACTH receptor in tissues (MC2R) may be more appropriate, in particular for mechanistic studies.

PURPOSE AND RATIONALE

Numerous assay procedures have been developed for the measurement of serum ACTH concentrations in clinical diagnostics (e.g., in tumor-related secretion, for ectopic hormone production, Krieger 1983; Genazzani 1975; Ganong 1974). This principle can also be applied (in a limited way) to safety pharmacology studies, when a reduction in glucocorticoids secretion leads to upregulation of ACTH secretion, by removing the inhibitory feedback. This is the primary diagnostic application in repeated-dose studies with test compounds which may interfere with adrenal steroid biosynthesis.

PROCEDURE

Several methods have been described, starting with radioimmunoassay (RIA) procedures (Rees 1971; Kao 1979; Krieger 1975) and proceeding to immunoradiometric (IRMA) methods (Hodgkinson 1984; White 1987; Zahradnik 1989; Raff 1989; Gibson 1989; Fukata 1989).

EVALUATION

Hormone concentrations in serum extracts or in unextracted serum and ACTH concentrations in dilutions of pituitary homogenate are calculated from standard curves using an appropriate reference standard for pituitary corticotropin (for the biologically active sequence the ACTH analogue ACTH 1–24 may be used). Sample data are calculated as ng ACTH/ml serum, as ACTH per pituitary gland or per pituitary weight (mg).

MODIFICATIONS OF THE METHOD

For analytical determination of ACTH, a method should be selected that can be applied to unextracted serum, provided that ACTH degradation is prevented by adding an enzyme inhibitor and the time of blood collection.

CRITICAL ASSESSMENT OF THE METHOD

A very critical item has been a tendency of ACTH to fluctuate widely and rapidly under conditions of stress (Meeran 1993; Lambert 1985; Goverde 1989). This is especially a problem in studies with dogs, where the

experiments need to be performed under stress-free conditions, and even then there will be a difficulty of rapidly fluctuating concentrations.

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14.4.8 Adrenal Steroid Activity

In the endocrine safety pharmacology of test compounds affecting nuclear receptors for glucocorticoids and mineralocorticoids (GR and MR), the levels to be examined after treatment of mice and rats are accessible for investigation; the information concerning risk assessment is discussed below.

PROCEDURE

Adult male and female rats are treated with the test compounds for 7–28 days, and levels of action are identified by measuring hypothalamic CRH content, ACTH secretion and synthesis, and the effect on adrenal steroid excretion, preferably at two dose levels. Test compounds are those which have been identified due to their interaction with nuclear steroid receptors and by structure-activity considerations. CRH activity, for example, hypothalamic CRF/CRH content, may be measured; the information obtained by this measurement is limited; and CRH content is controlled by the feedback action of pituitary hormone release and adrenal steroid secretion. There is always a clear response to glucocorticoids and inhibitors of adrenal steroid synthesis.

ACTH secretion and synthesis, namely, the tissue ACTH content, may be modified by compounds affecting corticotropin secretion, in particular by inhibition of glucocorticoid secretion. This information is valuable, because measurement of ACTH secretion reflects the level of secretion of glucocorticoids over several days of treatment.

Affinity for the glucocorticoid and mineralocorticoid receptors is usually known from early pharmacological profiling of the test compounds (Wilkinson et al. 2008; Houck and Kavlock 2008; Zwermann et al. 2009; Sedlak et al. 2011). For an assessment of the relevance of receptor signaling, studies in human adrenocortical cell lines are recommended (Parmar and Rainey 2008; Sanderson 2008; Pippal and Fuller 2008; Wang et al. 2012).

Clearly, compounds interacting with adrenal steroid biosynthesis by effects on enzyme and receptors will directly affect the target organ functions dependent on adrenal steroids. These activities may be detected by single-dose tests in the classical in vitro and in vivo bioassays for adrenocortical activity. The time course of effects is of interest and needs to be explored in relation to the nuclear receptor activation (GR, MR).

It is assumed here that any indications for receptor-mediated effects found in early pharmacological profiling will support the decision to perform an endocrine safety evaluation in satellite groups (Sullivan and Kinter 1995). The direct assessment of steroid activity by nuclear receptor assay is not discussed here. At the biochemical level of investigation, signaling mechanisms have often also been investigated (Wittliff and Raffelsberger 1995). The purpose of the endocrine safety pharmacology is to assess the relevance of these in vitro findings and their manifestation of biological effects in the intact animal test, by repeated-dose administration.

PURPOSE AND RATIONALE

There are numerous classical bioassays as for the glucocorticoid activity and mineralocorticoid activity of steroidal and nonsteroidal compounds. For the scope of endocrine safety pharmacology, however, it may be assumed that in the pharmacology procedures used for this selection of these compounds, modern methods were applied such as receptor screening and screening for enzyme activities (see reviews by Barnes and Adcock (1993), Berger et al. (1992), Jensen (1996), Lieberman (2001)).

CRITICAL ASSESSMENT OF THE METHOD

Direct tests for adrenal steroid activity are at the entry level of endocrine safety pharmacology; such activities are usually expressed by a feedback regulation at the pituitary level, changing the ACTH response, or by one of the multiple biological effects of the adrenal steroid hormones ranging from anti-inflammatory to immunosuppressive activity (Schimmer and Parker 1995).

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14.5 Hypothalamic-Pituitary-Thyroid Function

In rodent toxicology studies, effects on the thyroid gland are frequently found in histopathology and often require an extensive retrospective evaluation. The inclusion of measurement of thyroid stimulating hormone (thyrotropin, TSH) and thyroid hormones (in particular free thyroxine) at an early stage is helpful to provide mechanistic guidance for explanation of the effects, which are often observed in rodents but not in humans. The toxicological findings in rodents, other laboratory animals, and domestic animals have been reviewed with regard to the toxicants and chemical mechanisms involved (Capen 1992, 1994), mechanistic studies and toxic endpoints (Capen 1997; Capen 1998), and specific details of the histopathological findings and their interpretation (Capen 2001).

A detailed overview of the toxicological and relevance including findings of thyroid and parathyroid glands was published (Capen et al. 2002). An extensive review of the histopathology of the thyroid and other endocrine glands was provided by Greaves (2007a). The clinical spectrum of drugs affecting thyroid function includes thyroid hormones, inhibitors of thyroid hormone synthesis (antithyroid drugs), and drugs interacting with the thyroid hormone receptor isoforms being developed for several indications in metabolic diseases (Farwell and Braverman 2006; Dong and Greenspan 2009). In regulatory toxicology studies, in particular 6-month, 12 months and carcinogenicity studies, adverse findings affecting thyroid system are frequently found and inclusion of satellite groups for analysis of TSH, T3 and T4 is advisable, as well as the inclusion of thyroid-related peptides calcitonin and parathormone in particular cases (Capen et al. 2002).

14.5.1 Pituitary Thyroid Evaluation in Rats in a Repeated Dose Study

PURPOSE AND RATIONALE

Inhibitory effects on pituitary-thyroid function are readily detected in toxicology studies by changes in thyroid weight and histology of the thyroid and pituitary gland. Their interpretation regarding relevance for human is difficult and limited, because in many cases, there are underlying mechanisms such as enzyme induction which are not found in clinical studies (Capen 1998, 1999) and in clinical epidemiology. When effects on the thyroid are suspected, the inclusion of serum concentrations of TSH, T3, and T4 is recommended, and duration of treatment should be 28 days for reliable detection of changes. A treatment period of 15 days in intact male rats has been recommended based on testing of endocrine active compounds (EAC), comparing daily treatment by a intraperitoneal injection or by gavage (O'Connor et al. 1999, 2000, 2002b). Hormones measured were TSH, T3, and T4. The endocrine safety evaluation may use the 28-day toxicology study for orientation, with findings in pituitary histology and serum TSH as the relevant and reliable source of information. These parameters can be readily built into the study design, based on blood sampling 1 or 2 days before end of the treatment period (or terminal autopsy) and determination of the pituitary TSH and prolactin content (using one-half pituitary for the hormone assay). An increase in the serum concentration of TSH is the most frequent a relevant finding; a stimulation test can be included for the secretion of TSH and prolactin, by injection of thyroid-stimulating hormone (TRH). Changes in biosynthesis of thyroid hormones due to enzyme inhibition are usually detected by the rise in TSH. Measurements of free T3 and free T4 do not contribute to diagnostic relevance in rats. The uptake of radioiodine (131-I) into the thyroid of treated animals is a very useful additional test.

PROCEDURE

Separate groups of 5–10 male and female Sprague-Dawley rats weighing 100 g are used (juvenile rats). For some compounds, rats of 200-g body weight may be required (adult rats). They are treated daily over a period of 6–12 days with the test compound by the intended route (usually orally by gavage or by subcutaneous injections, sometimes by exposure to test compounds in the feed). For toxicological studies, a treatment period of 4 weeks is preferable. A similar protocol is applied in chronic toxicity studies in rats and dogs. On the day after the last application (alternatively 2 h after the last dosing), the animals are sacrificed and weighed and the following parameters determined: pituitary weight and thyroid weight, pituitary content of thyrotropin (TSH) and prolactin (PRL), TRH content in hypothalamic tissue (optional), and serum concentrations of TSH, T3, and T4. Determination of free thyroid hormones does not contribute to diagnostic evidence in rats and is not recommended. Samples should always be taken at the same time of the day, preferably in the morning, here to avoid artifacts by diurnal fluctuation of thyroid hormones (Weeke 1973; Brabant et al. 1990).

There are nowadays numerous possibilities for additional exploration, using ex vivo or tissue samples. The classical approach is histology of the anterior pituitary gland (Tucker 1999), and this can be extended to histomorphometry, in situ hybridization for enzymes, and gene expression profiling. In general, pituitary hormone contents are sufficiently informative (pituitary TSH content and serum TSH concentration). These hormones reflect the effects on receptors and enzyme systems, including the action of hepatic enzyme-inducing drugs on thyroid hormones and related histological findings of the thyroid gland (Curran and DeGroot 1991).

EVALUATION

For each parameter, means, and standard error calculated, an analysis of variance is performed, and the appropriate tests of significance are applied. The mean values of each parameter of the treated groups are compared with the values of the vehicle control group. It is important to compare the results with reference to their use for the rat strain commonly used in the laboratory. For many studies, it may be advisable to include groups treated with reference compounds of established endocrine activity.

There are many published toxicology studies with xenobiotics, which may serve as examples and facilitate the selection of reference compounds for defined mechanisms of action (Capen and Martin 1989).

MODIFICATIONS OF THE METHOD

The main modifications are selection of the age of the animals, duration of treatment, and time of autopsy related to the last drug application.

CRITICAL ASSESSMENT OF THE METHOD

Several reference compounds have been explored in the OECD endocrine disrupters program, phenobarbital and propylthiouracil being the most frequently studied (O'Connor et al. 2002; Mellert et al. 2003; Cooke et al. 2004; Cho et al. 2003). The interpretation of positive findings in the rat concerning inhibition of thyroid function which has been frequently found presents considerable difficulty, and there are many cases where such findings in rats have been shown to be of no relevance for the human (Akhtar et al. 1996; Waritz et al. 1996; Capen 1998; Poirier et al. 1999). Many drugs that act on thyroid function in rats share a mechanism of action based the induction of microsomal enzymes, which in turn may enhance the biliary excretion of thyroid hormones (Vansell and Klaassen 2001).

The response of rats of different strains with regard to the TSH response when treated with thyroid inhibitors appears to be in a similar range (Fail et al. 1999); the commonly used Sprague-Dawley and Wistar rats also share a similar response of thyroid hormone inhibition as found with propylthiouracil. The mechanism of thyroid inhibition by interference with iodine uptake is frequently found, the reference component being perchlorate (Kyung et al. 2002). A separate mechanistic study is required to clarify this question.

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14.5.2 TRH Radioimmunoassay

This is essentially a research method which was of interest during the development of TRH and TRH analogues in the context of proposed applications in neurological diseases. It may be included in the thyroid endocrine profile.

PURPOSE AND RATIONALE

In repeated-dose studies, hypothalamic tissue is readily available at autopsy of rats and can be used for the determination of hypothalamic hormone contents. It is important to take samples rapidly and stop enzyme inactivation in the tissue immediately (Griffiths et al. 1975; Jeffcoate and White 1975; Eskay et al. 1976) by the addition of suitable enzyme inhibitors. The hypothalamic TRH content has been measured by numerous groups (Mitsuma et al. 1976; Montoya et al. 1975; Pease et al. 1980; Utsumi et al. 1975).

PROCEDURE

The TRH radioimmunoassay was developed as a double antibody assay, with modifications mainly depending on the antiserum used and on the conditions of incubation. Addition of an enzyme inhibitor to the assay tubes is essential to avoid degradation of TRH in samples and of radioiodinated TRH.

EVALUATION

As with other radioimmunoassays, sample concentrations are calculated from a standard curve of synthetic TRH. Using various doses of standard and test preparation, dose-response curves can be established, allowing calculation of sample concentrations or potency ratios for tissue extracts tested at several concentrations.

MODIFICATIONS OF THE METHOD

Modifications of this assay have been applied to the measurement in serum, in hypothalamic tissue homogenates and extracts, and in human urine.

CRITICAL ASSESSMENT OF THE METHOD

The method is of interest because the results can be correlated with measurements of pituitary TSH and prolactin secretion and tissue content of these hormones. In a number of studies, it has been shown that changes in the secretion of thyroid hormone in the treated animals as well as administration of hormonally active compounds can change the hypothalamic TRH content in characteristic manner (Emerson and Utiger 1975; Schwinn et al. 1976; Simpkins et al. 1976).

Synthetic TRH is used as a diagnostic tool in animals and in humans. There is a response of the secretion of TSH as well as of prolactin (van Landehem AA and van de Wiel DF 1978; Andreassen et al. 1979). Pituitary cells in culture respond readily to the edition of TRH with the secretion both of TSH and prolactin (Queen et al. 1975; Haug and Gautvik 1976; Tal et al. 1978; Snyder et al. 1978).

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14.5.3 TSH Immunoassay and TSH Receptor Assay

Traditionally, TSH activity was determined by bioassays in mice based on the release of ^{131}I by TSH injection. The concentrations of TSH in serum and tissue are now measured by RIA and ELISA methods. There is a close correlation of biological activity and results of immunoassays. Reference standards are available for rat TSH, dog TSH, and human TSH (for current diagnostics, an Internet search is recommended). Immunometric measurements of TSH are reported (Utiger 1979; Spencer 1994; Meinhold et al. 1994), there are commercially available rat TSH assays, and the wide spectrum of analytical methods for clinical purposes has been reviewed (Spencer 2010), including TSH receptor-stimulating antibodies which characteristic for clinical thyrotoxicosis. Large-scale synthesis of recombinant human thyrotropin has been reported (Cole et al. 1993; Hussain et al. 1996). The role of the thyrotropin receptor has been reviewed by Vassart and Dumont (1992). Castagiola et al. (1992) described

a binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. A brain-derived TSH receptor has been cloned and expressed (Bockmann et al. 1997). Binding characteristics of antibodies to the TSH receptor were described by Oda et al. (1998). The diagnostic procedures used in clinical assessment of thyroid function have been reviewed (Spencer 1994; Spencer 2010).

[Internet resource Thyroid Manager: This Internet site provides a clinical overview of the analytical difficulty in application of methods for assessment of thyroid function and a review of hypothalamic-pituitary-thyroid physiology (Mariotti 2002)].

Application of the TSH receptor assay is recommended for assessment of the functional state of the pituitary gland after repeated-dose treatment, and serum TSH radioimmunoassay is important for the detection of inhibition of thyroid hormone synthesis by test compounds.

The difficulty in using TSH receptor assay is essentially that the initial step of binding of the hormone to the receptor is determined (receptor affinity), which does not change in proportion to the biological changes subsequently induced by TSH release. Receptor assay is therefore of limited relevance. There are functional assays which reflect activation of the receptor (Vitti et al. 1983; Perret et al. 1990).

PURPOSE AND RATIONALE

Determination of serum TSH concentrations is important for assessment of pituitary functional state (long-term elevation of TSH may result in goiter formation in rodents). The TSH RIA methods have replaced other methods for the quantitative assessment of changes in pituitary responsiveness. Test compounds which enhance the secretion of thyroid hormones lead to a suppression of the serum TSH concentrations, a clinical example being hyperthyroidism (Connors and Hedge 1981; Pekary et al. 1980). On the other hand, a reduction in circulating concentrations of thyroid hormones (T3 and T4) will impair feedback control of the pituitary gland, and the serum concentration of TSH may rise in an exponential manner.

There are TSH assays for research and clinical application with different sensitivity and limit of detection (Utiger 1979; Spencer 2010). In the endocrine safety protocol in rats (also applicable to dogs); the TRH test injection is a dynamic test for assessment of the serum TSH response and is useful for assessment of changes in thyroid hormone responsiveness, indicating modification of the pituitary TSH and prolactin response.

CRITICAL ASSESSMENT OF THE METHOD

Measuring serum TSH concentrations after 7–28 days of treatment is an important component of assessment in thyroid safety pharmacology. It is recommended to include these both in regulatory studies and satellite studies whenever there are any indications from receptor profiling or from in vitro assays on changes in thyroid peroxidase activity. Thyroid peroxidase is inhibited by the thioamide drugs, such as propylthiouracil and methimazole, and its inhibition in rats will result in goiter formation (Capen 2001).

Determination of TSH receptor affinity and related changes in receptor signaling are research methods which do not contribute to thyroid safety pharmacology.

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14.5.4 Iodine Uptake and Release in Rats

PURPOSE AND RATIONALE

This method may be used in the context of repeated-dose treatment, on satellite groups of rats which show changes in thyroid uptake of labeled iodine (^{131}I). It is essentially a confirmation of the effect of increased TSH secretion; it may be sufficient to determine radioiodine uptake in controls and in the high-dose group.

A modification of this method was previously used for evaluation of thyrostatic drugs and responds to inhibition of the iodine transport mechanism in the thyroid gland.

For conventional drug evaluation, all rats were pretreated with the same dose of radioiodine (preloading). The release of 131-I from the preloaded thyroid in rats is inhibited by treatment with thyroxin (Wolff 1951), and the degree of inhibition is related to the dose administered (Perry 1951). This phenomenon was used to compare thyroid hormone derivatives with the standard thyroxine. It is superseded by chemical assay of thyroid hormones by RIA and HPLC and by measuring feedback inhibition directly via the decrease in serum TSH following administration of high doses of triiodothyronine and thyroxine.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g are fed in a commercial laboratory with low concentrations of thyroid hormones, containing constant amount of unlabeled iodine. The animals are treated for at least 7 days with the test compound to be evaluated, at a pharmacologically active dose determined by previous biological studies. On the morning of the test day, rats are injected with a test dose of ^{131}I (intravenously or intraperitoneally), and that concentration of radioactivity in the thyroid glands is measured after 1–4 h. The blood concentration of ^{131}I at these time points is measured, and the tissue to blood ratios are calculated for individual animals.

EVALUATION

Statistical evaluation depends on the experimental design for such studies with prelabeling of the thyroid gland and release by thyroid-stimulating drugs or single-dose uptake of radioiodine into the pituitary of pretreated rats, for example, after several days of a thyrostatic drug.

From the dose-response curves, potency ratios and confidence limits are calculated. This approach may be selected to measure short-term uptake of ^{131}I as a parameter of thyroid peroxidase inhibition by antithyroid drugs.

MODIFICATIONS OF THE METHOD

The main modification for practical use in endocrine safety pharmacology is the application of this method to satellite groups of animals in a repeated-dose study. Most pelleted animal feeds nowadays contain a standard amount of iodine (it may be useful to check the specification). Therefore, the addition of potassium iodine to the drinking water is not required (as previously specified). In the satellite groups, the animals are injected with a standard dose of radioiodine after the last treatment, and the dose of radioactivity accumulated in the thyroid glands after a standard time interval (e.g., 2 h) is determined and evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Determination of 131-I in the thyroid of rats at the end of a treatment period is a useful additional test, which can be applied to satellite groups, preferably control

and high dose. This is a sensitive test which readily detects inhibition of thyroid hormone synthesis, for example, by propylthiouracil.

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14.5.5 Inhibition of Iodine Uptake into the Thyroid of Rats

This assay is applied to the study of drug candidates that inhibit thyroid peroxidase and may cause goiter in the preclinical studies due to decreased thyroid hormone synthesis, with subsequent compensatory increase in TSH secretion.

PURPOSE AND RATIONALE

Propylthiouracil (PTU) and a wide spectrum of drugs may inhibit thyroid hormone synthesis. Some of these drugs can be used for treatment of thyrotoxicosis. As a consequence of thyroid peroxidase inhibition, the iodine uptake and content of the thyroid is decreased. This phenomenon is dose-dependent and may occur at lower doses than those increasing thyroid weights in rats (McGinty and Bywater 1945). The historical parameter of measuring iodine content was replaced by measuring uptake and release of radioiodine (^{131}I).

PROCEDURE

Groups of male Wistar rats age 26–28 days, weighing 40–45 g, are placed in metabolism cages (two rates per cage). They are fed normal diet, and potassium iodide is added to the drinking water in order to minimize differences between thyroid status of the animals during treatment. Test compounds may be injected daily or added to the feed (mainly for testing of industrial chemicals. In this procedure, the test compounds and the reference standard are added in various concentrations to the diet over a period of 10 days). The amount of compound which each rat has ingested is calculated from the total food consumption over 10 days and expressed as milligram ingested daily per kilogram of body weight. After 10 days of treatment, the rats are sacrificed and the thyroids dissected free from adjacent tissue and capsule. The thyroid is weighed and iodine content determined (conventional procedure). In daily doses between 0.1 and 10.0 mg/kg, thiouracil decreases the iodine content of the thyroid dose-dependent. Definitely, higher doses are necessary to increase thyroid weight.

When the uptake of a standard dose of radioiodine is tested at the end of the treatment period, the radioactivity found in the thyroid gland is used as the parameter instead of the iodine content.

EVALUATION

Dose-response curves of test compounds are compared with those of the reference standard for calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Walker and Levy (1989) used implantable pellets of propylthiouracil to induce thyroid dysfunction in rats. In the modification based on uptake of labeled iodine, as standard dose of radioiodine, 131-I is injected in each animal, and the amount of radioactivity in the thyroid gland is determined in a gamma counter.

Release of labeled iodine may be stimulated by injection of TRH (TSH-releasing hormone, protirelin).

CRITICAL ASSESSMENT OF THE METHOD

This test is valuable as single-dose uptake test and may be performed (after 10 days of treatment) when changes in the serum concentrations of T3 and T4 of unknown relevance have been found, with no corresponding increase or decrease in the serum TSH concentrations. It may help to differentiate changes which occur or predominantly at the level of thyroid hormone synthesis.

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14.5.6 Thyroid Hormone Assays (T3 and T4)

Thyroid hormone assays are performed on serum samples, predominantly in rat studies to identify, for example, substances with goitrogenic activity.

PURPOSE AND RATIONALE

Measurement of thyroid hormone concentrations in the serum has become an important final step in the endocrine safety pharmacology procedures (Capen 1992, O'Connor et al. 2002). Basal concentrations are measured in serum samples in the morning before treatment (to avoid artifacts by the diurnal fluctuation of thyroid hormone concentrations). In contrast to the rise in TSH after a test injection of TRH (dynamic function test), there is no pronounced rise after TRH stimulation. However, in specific situations, the secretion of thyroid hormones may be stimulated by the injection of a biosynthetic TSH preparation.

PROCEDURE

Standard radioimmunoassay procedures are applied, nowadays mainly solid phase assays which can be rapidly performed and evaluated. There are also several enzyme-linked immunoassay (ELISA) procedures from commercial suppliers. It is recommended to perform an Internet check for the most appropriate method at the time of the study (for example, DSL 2005). Methods have been described for triiodothyronine (Nejad et al. 1975; Chopra et al. 1972a; Larsen 1972a, b) and for thyroxin (Chopra et al. 1971; Chopra 1972). The use of assays based on thyroxin-binding globulin (Chopra et al. 1972b) is no longer recommended and cannot be applied to the rat, because the rat does not have this binding protein. However, for the human, measurement of the thyroxine-binding globulin by radioimmunoassay has been successfully applied (Levy et al. 1971).

EVALUATION

The usual procedure for radioimmunoassay data is calculation by a computer program, from standard curves of the hormone to be measured. It is important to have a sufficient number of animals per group, to be able to perform analysis of variance and statistical evaluation by tests of significance.

CRITICAL ASSESSMENT OF THE METHOD

There are now many methods of RIA and ELISA design available for the rat and dog thyroid hormones. It is recommended that each laboratory performs an on-site validation for the methods to be selected for routine evaluation of endocrine safety pharmacology.

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14.5.7 Thyroid Function in Chronic Toxicology Studies

Effects on the thyroid gland are found frequently in rodent toxicology studies. The relevance of these findings can be assessed more clearly when measurement of thyroid-related hormones and enzymes is included. There are several toxicants which may be useful as a reference compounds. Long-term studies in environmental toxicology have contributed detailed information, and 28-day rat toxicology tests with thyroid endpoints have been established in the EPA-OECD research programs.

PURPOSE AND RATIONALE

The problems of evaluating thyroid function during high-dose drug exposure are well known for several classes of compounds. In endocrine safety pharmacology, this represents a recurrent difficulty which can be resolved by early inclusion of functional parameters into the long-term toxicology studies. To take preemptive action on possible effects disclosed by long-term toxicity studies, particularly carcinogenicity, it is advised to perform hormone measurements early in the development program, namely, during 6–12-month toxicity studies and carcinogenicity studies. To start mechanistic studies at a later date may be required of if special aspects of enzyme induction and changes in pharmacokinetics need to be investigated for drugs affecting thyroid function.

14.5.7.1 Mechanistic Studies

Test should include measurement of basal concentrations for TSH, total T3, and total T4—at several time points during the study. Measurement of the free fractions of T3 and T4 is in our experience not required and does not contribute to interpretation of the organ-related findings, for example, in thyroid histology. A TRH test may be included for the stimulation of TSH secretion by subcutaneous injection of TRH.

14.5.7.2 TRH Test

The TRH test is performed in a minimal invasive manner, 24 h after dosing on the next morning. The test dose is s.c. injection of 1 ug/kg body weight synthetic TRH followed by blood sampling in unanesthetized rats 30 min later. For mechanistic studies, it is also possible to use another specific TRH test blocking basal TSH by injection of levothyronine (L-T3) 18 h 1 ug/rat s.c. before the test. On the next morning, the TRH dose is injected i.v. followed by blood sampling at 15 min or (in anesthetized rats) after 15 and 30 min.

MODIFICATIONS OF THE METHOD

In carcinogenicity studies, such minimal invasive procedures (basal hormones, TRH test for stimulated TSH) can be included in small groups of animals. In other studies (chronic toxicity), pituitary hormone contents (autopsies 24 h after last dosing) of THS, PRL, and GH are a useful parameter (median sagittal sectioning of pituitary gland with half going to histology and half to hormone assay). Iodine uptake cannot be assessed in the toxicology studies and must remain a specific item satellite groups or for a mechanistic study. The thyroid content of iodoproteins can be assessed if thyroid glands (one-half as for pituitaries) are made available at autopsies, or from added groups included in the study.

CRITICAL ASSESSMENT OF THE METHOD

Inclusion of these investigations of endocrine safety pharmacology is a useful addition to chronic toxicology studies. The inclusion of thyroid safety pharmacology can be of considerable interest because there are frequent changes induced by drugs (examples are phenobarbital and benzodiazepines). Studies including thyroid safety pharmacology have also been performed for a number of environmental toxicants and xenobiotics. In a pilot study (O'Connor et al. 1999), phenobarbital and propylthiouracil (PTU) were investigated

in two short-term in vivo tests (5-day ovariectomized female rats and 15-day intake male Sprague-Dawley rats) and an in vitro yeast transactivation system. Thyroid endpoints were serum hormone concentrations, liver and thyroid weights, thyroid histology, UDP-glucuronyltransferase, and 5′- deiodinase activity. Several thyroid-related endpoints including serum hormone concentrations and histopathology of the thyroid gland were evaluated for their utility in detecting thyroid-modulating effects after 1, 2, and 4 weeks of treatment with PB or PTU. In the Sprague-Dawley rats, phenobarbital administration increased serum TSH and decreased T3 and T4 concentrations. In female Sprague-Dawley rats, PTU administration produced an increase in TSH concentrations and decrease in T3 and T4 concentrations, associated with distinct histopathology changes of the thyroid gland. In male Sprague-Dawley rats, PTU administration caused thyroid gland hypertrophy/hyperplasia and colloid depletion together with increased TSH and decreased serum T3 and T4. These studies with the toxicants confirmed that thyroid gland histopathology coupled with decreased serum T4 concentrations was the most useful criteria for identifying thyroid toxicants in studies of 1–4 weeks' duration. Suitable chronic treatment assays were discussed by Clode (2006). The female pubertal essay was recommended by the EPA for antithyroid activity and antiestrogenic effects. The OECD recommended an enhanced version of the 28-day rat toxicity tests. The characteristic changes in thyroid function observed in the 28-day test have been established using reference toxicants phenobarbital and propylthiouracil (PTU). An early extensive study was performed with an industrial chemical of the thiourea class. This study (Biegel et al. 1995) was a 90-day gavage study and included measurement of serum T3, T4 and TSH, hepatic UDP-glucuronyltransferase activity, and cell proliferation of thyroid and liver. There were characteristic effects elicited by the compound PTI (1-methyl-3-propylimidazole-2-thiol). Due to the toxic dose, there were substantial reductions in body weight gain. The primary target organs were the thyroid and liver. Significant alterations in T3, T4, and TSH levels were found together with increased cell proliferation and UDP-glucuronyltransferase activity. There were characteristic histopathological changes in cell proliferation. The effect of the PTI thiourea on thyroid peroxidase activity was also evaluated in vitro using porcine thyroid microsomes. Findings of the study indicate that PTI enhances the excretion of T4 via induction of glucuronyltransferase and inhibits thyroid hormone synthesis via a direct effect on thyroid peroxidase. Disruption of the hypothalamic-pituitary-thyroid axis results in sustained elevation of TSH and the corresponding thyroid hypertrophy and hyperplasia. In the assessment of drug effects on the thyroid, the rise in serum TSH can be used as a paradigm for thyroid toxicants with inhibitory effects on thyroid peroxidase.

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14.6 Safety Pharmacology of the Pancreas

14.6.1 General Considerations

Safety pharmacology of drugs and drug candidates affecting the pancreas is a rapidly expanding field. In the toxicologists' view, there are significant morphological changes of the exocrine pancreas and endocrine pancreas detected in regulatory toxicology studies, including the findings in carcinogenicity studies of 2 years' duration. The current pharmacologist's view is rather single minded, mainly addressing the antidiabetic class of compounds. Various effects on

the exocrine pancreas have been of limited importance. In the antidiabetic field, the main concern is compounds which induced damage to the beta cells, "diabetogenic compounds." The classical examples are alloxan and streptozotocin. There are also a number of potential environmental toxicants (endocrine disruptors), components which can induce damage to the endocrine pancreas upon long-term exposure. In the area of antidiabetic agents, there is considerable evidence from regulatory studies with sulfonylurea compounds, glinids, insulin analogues, incretin mimetics (GLP-1 analogues), and DPP-4 inhibitors. Recently, evaluated compounds have shown particular difficulties concerning risk evaluation of inducing pancreatitis and C-cell hyperplasia in the thyroid of rodents. Details of this effect have been studied, in particular, with relevance to exenatide and liraglutide where such changes have been a matter of debate and discussion concerning clinical relevance. There are a number of established and useful preclinical biomarkers in the assessment of drugs affecting the endocrine pancreas, their relevance being confirmed by application as clinical biomarkers. There are also established biomarkers for damage to the exocrine pancreas.

14.6.2 Antidiabetic Agents

Safety assessment of the pancreas is of particular relevance in the context of antidiabetic agents, of which there is now a wide range including the sulphonylurea compounds, human insulin and insulin analogues (Owens 2011; Swinnen et al. 2011), incretin mimetics for injection (GLP-1 analogues), and DPP-4 inhibitors (gliptins) for oral administration (Barnett 2011; Dicembrini et al. 2011; Forst and Pfützner 2012; Ghatak et al. 2010; Ghatak et al. 2011; Joy et al. 2005; Neumiller et al. 2010; Stephens and Bain 2007). A list of drugs with cytotoxic effects on the pancreas was compiled by Longnecker and Wilson (2002). A new class of antidiabetic is currently under investigation, SGLT2 inhibitors acting on sodium-glucose-cotransporters (e.g., dapagliflozin, Asano et al. 2004; Washburn 2009; Idris and Donnelly 2009; Abdul-Ghani et al. 2011; Kinne and Castaneda 2011). Very extensive safety evaluation was done for sulphonylurea compounds, in the context of preclinical development and later concerning clinical safety, in particular cardiovascular safety and drug interactions (Krentz et al. 1994; Krentz and Bailey 2005; Sehra et al. 2011). This was followed by long and acrimonious considerations about recombinant human insulin and difference related to the use of animal insulins (Richter and Neisser 2005), a controversy which no longer appears relevant due to the widespread development of biosimilar human insulin preparations (worldwide). The discussion about the growth effects of insulin and insulin analogues on cell proliferation was resumed in the context of in vitro studies in cell lines, including cancer-derived cell lines (Leroith 2007; Sandow 2008, 2009; Sommerfeld et al. 2010; Stammberger and Essermeant 2012). The relevance of this important approach was resolved by the conclusion that the clinical evidence from epidemiology studies was the important source of information (Simon and Balkau 2010; Muessig et al. 2011). The current task is now to develop drugs even in the presence of class preclinical findings, by finding an explanation for their toxic mechanism which may show that the adverse effects observed are not related to the proposed use in clinical conditions (Ettlin et al. 2010a, b).

Safety pharmacology of the endocrine pancreas as a target organ for drug effects is a different and specific approach by methods of toxicology (Wilson and Longnecker 1999; Thomas and Thomas 1999; Longnecker and Wilson 2002) and histopathology (Greaves 2007c). Problems arising from effects on the exocrine pancreas are also addressed by these methods; they are identified less frequently during preclinical drug evaluation but may become apparent with prolonged clinical use.

There are established reference compounds for the toxicology of the exocrine pancreas, namely, azaserine (Christophe 1994) and cerulein (Kern 1980), and of the endocrine pancreas, namely, alloxan and streptozotocin (Lenzen and Panten 1988; Szkudelski 2001), which may serve as comparators because their mechanisms of action have been elucidated.

Each of the pharmacological classes affecting the endocrine pancreas (Davis 2006; Nolte 2009) has its own history and particular problems identified during the development phase. There is now an impressive array of methods in early toxicology, based on receptor profiling, in vitro cell-based assays, and molecular biology methods directed at the development of early biomarkers suitable for preclinical toxicology

development (Dambach and Gautier 2006; Pavanello 2006, Collings and Vaidya 2008) and translation to clinical toxicology (Muller and Dieterle 2009). Some of these biomarkers may find their definitive use in regulatory toxicology (Godsaid and Frueh 2006), after validation for specific target organs such as the kidney (Vaidya et al. 2008, 2010), the endocrine pancreas, and hormone-responsive endocrine tissues, for example, the gonadal system. More recently, biomarkers have acquired an important role in the assessment of the effects on the skeletal system and connective tissue, for example, in the evaluation of bisphosphonates, calcitonin, and parathyroid hormone. Examples of established biomarkers for damage to the exocrine pancreas are the enzymes amylase and trypsin; new biomarkers are being established (Walgren et al. 2007).

The studies on mechanisms of action may be suitable for identifying new therapeutic options or specific pharmacological mechanisms, but they are not relevant for the preclinical safety evaluation for the design of phase 1 studies. There are a number of important considerations concerning the use of receptor profiling of new chemical entities as well as for compounds with established hormone activity. The test systems for receptor affinity and signaling are based on cells or cell lines which reflect only one particular organ, whereas frequently receptors are found in a number of target organs responding at different doses and with different activation kinetics. An example is compounds which act both on endocrine target organs and on the central nervous system. It is then difficult to assess which receptor category gives rise to the relevant biological effects. This needs to be clarified by targeted endocrine evaluation as well as safety evaluation of centrally mediated effects including neurotoxicity. A particular case is incretin mimetics which have clinically relevant effect on appetite control and satiety. Safety pharmacology will usually be required to assess the relevance of centrally mediated effects in contradistinction to effects mediated by the gastrointestinal tract.

In the development of new methods to assess pancreatic safety pharmacology in biological systems (which can be related to classical studies in pharmacology and regulatory toxicology), there is an increasing recourse to animal models based on transgenic animals. These models may be closely related to toxicological pathology (Longnecker 2004; Hruban et al. 2006), and they may address specific tasks of exploratory pharmacology (Srinivasan and Ramarao 2007; Kumar et al. 2012) including reproductive toxicology (Jawerbaum and White 2010).

One specific area in application of safety pharmacology to the study of chemicals and compounds in the environment ("endocrine disruptors") is the inclusion of targeted investigation in the OECD 407 protocols (Hectors et al. 2011), often including the toxicological assessment in vitro of natural compounds used as nutritional enhancement (Reiter et al. 2011) and searching for leads in drug research (Ahrén 2009). The particular relevance of this approach was that animals were treated for 10–28 days including a maximum tolerated dose; parameters of endocrine function such as measurement of hormones were included during method development and assessed for diagnostic relevance. These programs started with the exploration of defined chemical entities, mainly established drugs such as synthetic gonadal and adrenal steroids. As an example, in the context of effects on the endocrine pancreas (beta cell function), bisphenol A was addressed as an endocrine disruptor (Alonso-Magdalena et al. 2008, 2010). The involvement of estrogen receptors (ER-alpha and ER-beta) in the regulation of pancreatic beta cell function has been explored (Nadal et al. 2011) and may be a future topic of safety pharmacology.

Studies on endocrine safety pharmacology are part of the "supplemental safety pharmacology studies" described in the ICH guideline S7A. These supplemental studies are designed and performed to evaluate potential adverse effects on organ system functions (endocrine organs and target tissues of hormones) which are not addressed by the general methods of the core battery or by repeated-dose toxicity studies. The definition clearly requires that the specific pharmacology of the test substance be known starting point for supplemental safety pharmacology. This knowledge will inform the investigator about decisions related to selecting test articles of endocrine safety pharmacology. The strategic objective is to bring information from two different sources to bear on the problem and to initiate the translational dialogue of pharmacology and toxicology. The classical source of information, namely, toxicological pathology with defined methods of morphology and (more recently) molecular biology of endocrine tissues (at autopsy), and the new source of information, that is, methods for functional assessment of endocrine organs and endocrine target tissues (during the study), provide information for the interpretation (mechanistic toxicology). This is obviously a process

leading from indications in biochemistry and molecular biology to the exploration and confirmation in targeted animal studies. This can be done in parallel with toxicological pathology, in the repeated-dose toxicology studies where possible and in satellite groups when necessary. The findings at the functional level and at the morphological level are shared and compared for consistency. The best opportunity prior to clinical studies is inclusion of hormone assays and hormone-related biomarkers as well as specific endocrine organ pathology in the regulatory toxicology studies. In the majority of cases, this will mean early treatment periods of 4 weeks (as in toxicology); there remain however numerous questions which are better addressed by targeted investigation included in the 6-month or 1-year toxicology. The final and most critical step in future development will be inclusion of such parameters in the lifetime toxicology performed in rodents, whenever the additional effort and expense appears justified. The critical task of assessing the relevance of findings for future therapeutic intervention in humans has been prepared in several instances by working within a group of compounds that already has a clinical history including long-term use and epidemiological studies, for example, about the relation of preclinical findings in animal toxicology to cancer risk in humans (Scarpelli et al. 1984; Scarpelli 1989; Longnecker 1999; Germann et al. 1999; Gopinath 1999).

The general pharmacology profiling of new drugs nowadays is predominantly performed by in vitro testing, followed-up by cell-based assays and studies in endocrine-related cell lines when available for pancreatic beta cells (Poitout et al. 1995, 1996; Skelin et al. 2010). This may be quite an effective procedure for preselection of candidate compounds with hormonal activity (Houck and Kavlock 2008). It is also an interesting approach to the receptor-mediated effects which can be studied on the isolated pancreas (Barseghian and Levine 1980) and on beta cell lines in relation to the effects of glucocorticoids (Pierluissi et al. 1986; Delaunay et al. 1997) and other hormones (Lenzen and Bailey 1984; Hectors et al. 2011). These in vitro explorations will need in each case to be followed-up by animal studies, the best option being the repeated-dose studies in the toxicology program. The studies of the pancreas proceed in parallel, with more flexibility in study design and (hopefully) providing the explanations for the questions which are raised by the finding in pathology and toxicology.

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14.6.3 Endocrine Pancreas Study in Rats

In the toxicology studies (2 weeks, 1 month, 3 months, etc.) morphological changes in the endocrine pancreas and less frequently in the exocrine pancreas are detected, often without an obvious relation to the pharmacological profile of the drug investigated. In general, there are predictions and early indications for a therapeutic potential, which provide direction for targeted investigation. There may be indications from receptor interaction of pancreatic beta cells, for example, with the glucocorticoid receptor (McMahon et al. 1988; Delaunay et al. 1997), of insulin-responsive tissues with the IGF-1 receptor (Sandow 2009) or with thyroid hormones and steroids (Lenzen and Bailey 1984). Any indications for interaction with the glucocorticoid receptor (GR), GLP-1, and glucagon receptor (GCGR) from early drug profiling may be followed by a search for adaptive changes during repeated-dose treatment (Brubaker and Drucker 2002; Hansotia and Drucker 2005; Drucker et al. 2012). The animal study reflects the sum of changes induced by a candidate drug, and the site of action at different levels can be investigated when characteristic analytical determinations are available. For more complex research questions of receptor signaling and receptor interaction (crosstalk), in vitro studies on cell lines will need to be added where the response in the regulatory toxicology system needs to be explored in more detail and when particular safety issues require mechanistic explanations.

In the selection of animal model, many studies are performed in adult normal rats, by including special analytical determinations and biomarkers in the regulatory toxicology studies (similar to the enhanced protocols in environmental toxicology). This approach is helpful, when necessary satellite groups are added, or separate satellite studies need to be designed. For antidiabetic compounds, pancreatic safety studies in normal rats are often inappropriate. The decision is then for diabetic animal models (Herling 2006; Mueller 2008; Tourrel et al. 2002; Srinivasan and Ramarao 2007; Kumar et al. 2012; Hempe et al. 2012; Tesch and Allen 2007; Masiello 2006; Alemzadeh et al. 2002) which may be directed more at diabetes type I by extensive destruction of beta cells or at diabetes type II by inducing insulin resistance, including the reduction of beta cell mass. Such studies often transcend the tasks of safety pharmacology, and they may be directed at identification of specific extrapancreatic effects, for example, on the vascular system (Murthy et al. 2010).

Safety issues arising from reproductive toxicology findings are addressed in specific models in, for example, pregnant animals (Jawerbaum and White 2010). In recent submissions for marketing authorization of incretin mimetics, diabetic animals were included in the primary pharmacology studies of exenatide and liraglutide (Byetta 2006; Victoza 2009) and also in the primary pharmacology of DPP4 inhibitors (Galvus 2007; Onglyza 2009; Trajenta 2011). The advantage of diabetic animals is that pharmacodynamic effects are more readily characterized and the preclinical chemistry is more characteristic of the mechanism of action.

Another species for diabetic animal models in toxicology is the mouse, including a large number of transgenic animal models (Longnecker 2004; Hruban et al. 2006). The situation is more complex for diabetes models in dogs. Previously, pancreatectomized dogs were used for insulin studies, whereas now, the rodent models are considered sufficient for safety assessment. In regulatory toxicology, the dog is the second species for long-term studies; it is also shown chosen for verification of specific issues (e.g., saxagliptin) and for the pharmacokinetic analysis.

14.6.3.1 Analytical Methods for Pancreas Functions

There are a number of analytical determinations which can be built into pancreatic safety pharmacology in animals. Evaluation of beta cell function is similar to clinical protocols (Weinstock and Zygmont 2010), fasting blood glucose, and the change after drug administration characterized the activity pattern (antidiabetic, hyperglycemic). The plasma concentrations of insulin and glucagon can be measured; measurement of C-peptide is an index of pancreatic secretory capacity which may change with loss of beta cells. The noninvasive C-peptide measurement using the urinary C-peptide/creatinine ratio is clinically validated and may be applied to any mouse in a similar manner (Bowman et al. 2012; Besser et al. 2011). The change in glycated hemoglobin (A1c) may be determined after 1 month or longer intervals of treatment as an index of efficient glycemic control or loss of control. Description for specific analytics suitable for incretin mimetics and gliptins is found in a number of publications (Gedulin et al. 2005; Pérez-Arana et al. 2010; Gil-Lozano et al. 2010; Nachnani

et al. 2010; Wu et al. 2012) and in the assessment reports of the European Medicines Agency (EMA). These documents are particularly valuable because the primary animal pharmacology of antidiabetic compounds is of not described in sufficient detail, whereas the clinical reports are very extensive for incretin mimetics (Byetta 2006; Victoza 2009) and for gliptins (Galvus 2007; Onglyza 2009; Trajenta 2011).

Dynamic function test may be included in satellite studies, in a similar manner as for clinical protocols (Weinstock and Zygmont 2010), namely, the glucose challenge (OGT) and insulin challenge, also including euglycemic clamp studies in rats.

PURPOSE AND RATIONALE

The study of endocrine pancreas function may be performed in normal rats or—in case of specific pharmacological knowledge about the mechanism of action—in rat models of type II diabetes and other related models (Tourrel et al. 2002; Masiello 2006). Assuming that there is no prior knowledge, young male or female rats (100 g initial body weight) or adult rats (2–300 g BW) are selected for the study.

PROCEDURE

Male and female rats of the same age are assigned to control and treatment groups (six animals per group), preferably at two dose sizes related to the toxicology studies.

Choice of animal models for the incretin mimetics (exenatide, liraglutide) are often diabetic rats because insulin release in these models is dependent on glucose levels.

The animals are treated in the same way as in the toxicology study, either by gavage or daily injections, up to 4 weeks. At intervals of 7 days, a test procedure is performed for the endocrine pancreas which may be blood sampling at the same time of the day (preferably in the morning) for the determination of hormone and metabolic parameters. The animals may have free access to feed or may be kept without feed during the night, prior to sampling.

Parameters to be investigated are blood glucose, insulin and proinsulin, glucagon, GLP-1 (total and active GLP-1), somatostatin, and other hormonal parameters (e.g., ghrelin). When an effect on glucose regulation is known or anticipated from pharmacology studies and early toxicology, glucose and insulin are determined prior to drug administration and 1–2 h after treatment. A time-action profile for glucose regulation at the higher dose level is recommended, when there are consistent effects on glucose regulation (duration of the effect). In any long-term study of 3 or 6 months duration, the assay of glycated hemoglobin (HbA1c) needs to be included. There are species-specific methods for the rat, dog, and mouse. It is recommended to perform an initial validation of the analytical methods for glucose-regulating hormones and related parameters, when performing studies in mice and dogs. There is a wide spectrum of suppliers and reagents, from which assays may be selected either in the form of single hormone assays or multiplex assays. There is also an array of commercially available biomarkers for metabolic and toxicology studies (websites of suppliers).

Biomarkers may also be determined during this study, depending on the drug-receptor profile, results of in vitro investigations, and cell-based studies which have been performed prior to development decision. Effects on the endocrine pancreas are much more frequent, and the related examinations take precedence. There is also the option of monitoring parameters of exocrine pancreas function, for example, enzyme levels and biomarkers related to exocrine pancreas function (Muller and Dieterle 2009, Walgren et al. 2007).

All specific investigations of blood sampling at particular days of the study, and urine samples when appropriate, place an additional burden on the animals and may interfere to a significant extent with the protocol of a regulatory toxicology study (Redfern and Wakefied 2006). The results of the satellite study need to be reviewed in the context of the regulatory toxicology which needs to follow an established protocol.

At the end of the treatment period, autopsies are performed, and tissue samples are processed for determination of hormone contents, for molecular biology studies, and for targeted histology and histomorphometry.

EVALUATION

For all quantitative parameters such as organ weights and hormone concentrations, group means are calculated, and the significance of differences is assessed by the appropriate statistical methods. The aim is to show that the drug was tested at doses which achieve biologically effective concentrations (later to be followed-up by pharmacokinetics). In relation to the regulatory

toxicology studies, this will provide proof of relevance and enable prediction of anticipated adverse events (AE) in phase 2 clinical pharmacology (early risk management plan).

Histology may be reported in descriptive terms, evaluated by a semiquantitative method, or by computer-assisted histomorphometric methods (to be used for quantitative statistical analysis). For advanced methods of in situ hybridization and gene expression, a description of the observed changes is generally appropriate together with quantitative data generated by computer-assisted analysis of gene expression profiles.

MODIFICATIONS OF THE METHOD

In the development of a drug candidate, there may be an established profile, for example, for an antidiabetic agent or a general project plan based on indications of receptor profiling. Studies on endocrine pancreas profiling with modern methods of endocrine evaluation have been performed, for example, with insulin analogues (insulin lispro, insulin aspart, insulin detemir, insulin glargine), GLP-1 agonists (exenatide, liraglutide), and DPP-4 inhibitors (vildagliptin, sitagliptin, saxagliptin, linagliptin). The classical compounds, for example, sulphonylureas, do not have the advanced methodology of hormonal assays but contribute considerably to the knowledge of biochemical mechanisms and toxicological evidence. They have in particular contributed to the study of mechanisms of action on ATP-sensiive K^+ channels (Inagaki et al. 1995, 1996; Miki et al. 1998; Quast et al. 2004; Thomas and Smart 2005; Gloyn et al. 2004).

CRITICAL ASSESSMENT OF THE METHOD

The endocrine pancreas safety study of rats is a satellite investigation providing additional evidence for understanding of results of the regulatory toxicology study is an important element, because it integrates the biological response of several systems including the currently important incretin hormone system.

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Websites for Suppliers and Reagents:

http://www.htrf.com/products/gpcr/binding/ligands/glucagon_glp1/
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http://www.meso-scale.com/CatalogSystemWeb/WebRoot/products/assays/metabolic.aspx
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14.6.4 Exocrine Pancreas Function

Effects on the exocrine pancreas are occasionally detected in studies with rats and mice; there are also a number of observations in carcinogenicity studies with currently used drugs and toxicants in evaluation of chemicals and environmental contaminants. Drug-metabolizing enzymes in endocrine, exocrine, and pancreatic tissue are inducible by certain chemicals. Many observations have been made in histopathology (Greaves 2007c); changes in pancreatic enzyme reactivity have been found in patients with chronic pancreatitis and pancreatic cancer. The current discussion about the risk of acute pancreatitis during treatment with exenatide and incretin mimetics shows that there is a need for evaluation and safety pharmacology, in particular for new development compounds of similar class. Acute pancreatitis has been found after administration of, for example, corticosteroids, diuretics, antidiabetics, and analgesic or anti-inflammatory agents (paracetamol, indomethacin, salicylates). It is therefore of interest to include monitoring of exocrine pancreas function in all regulatory toxicology studies related to the derived or related drug classes.

PURPOSE

Diabetic rat strains are predisposed to the development of acute and chronic pancreatitis (a characteristic toxicant in rats is in the cholecystokinin analogue cerulein, a compound which may cause maximum pancreatic secretion). Cerulein is also used as a diagnostic agent. Acute pancreatitis has been observed with several anxiolytic and antipsychotic drug candidates. Whereas the development of acute pancreatitis may be detected by changes in pancreatic enzyme excretion, chronic pancreatitis is more difficult to detect; focal tissue chronic inflammation with or without fibrosis has been described as a common finding in the exocrine pancreas of aged rats, to some extent also found in mice, hamsters, and beagle dogs (Greaves 2007c). The increasing prevalence in aged rats already indicates the impending problems in carcinogenicity studies, where chronic pancreatitis is a very frequent finding in histopathology. The finding of changes in endocrine pancreas function (controlled by glucose tolerance, serum insulin, and histology of pancreatic beta cells) was found to be associated with atrophy of pancreatic acinar cells in dogs and monkeys. Interestingly, long-term treatment with phenobarbital induced proliferation of pancreatic acinar cells together with that of endocrine pancreatic cells (Jones and Clark year?). The findings were associated with a distinct change in drug-metabolizing capacity of the pancreas and distribution of CP 450 enzymes.

PROCEDURE

Animal study of rats and dogs, biomarkers, and dynamic function test

14.6.4.1 Details of the Method

Pancreatic growth is regulated by cholecystokinin; there is a negative feedback mechanism on CCK by stimulation of intestinal trypsin. As a consequence, feeding with raw soy flour containing a trypsin inhibitor will remove endogenous trypsin and may stimulate the release of CCK with resultant pancreatic proliferation. A number of soy derivatives have been investigated in OECD studies for environmental toxicants. Studies with pentagastrin administration have shown an increase in pancreatic size and enzyme secretion, principally characterized by hypertrophy of acinar cells. A number of such studies were performed in the context of evaluating carcinogen and hamster treatment with nitrosamines (505). The continuous administration of CCK-8 induced increase in pancreatic weight, protein and DNA content, and thymidine labeling index. As a consequence, CCK-8 may be used as a model toxicant in studies on proliferation and apoptosis of the pancreas. Injections of isoprenaline have been shown to increase pancreatic weight. This confirms the relevance of including safety pharmacology of the exocrine pancreas when there are structural indications for effects on pancreatic enzymes. Characteristic changes in histopathology have been reviewed (Greaves 2007c), among them duct proliferation in rabbits treated with corticosteroids. There were also zones of exocrine pancreatic cells with increased eosinophilic staining of the cell cytoplasm. The future objective for safety pharmacology is to validate biomarkers of pancreatic enzyme secretion which can be monitored in long-term toxicology studies. In the presence of focal lesions and neoplasia of the exocrine pancreas found in carcinogenicity studies, functional assessment is important for risk assessment. The pancreatic acinar cell hypertrophy, hyperplasia, and adenoma in rats were modified by a number of therapeutic agents such as testosterone, estradiol, and cholecystokinin as well as cholecystokinin analogues. CCK is an important growth factor for normal pancreas; new drugs acting on gastrointestinal hormones may stimulate neoplastic growth of acinar cells by a CCK-related mechanism. One of the established toxicants is azaserine. Alterations of the exocrine pancreas may also be found in chronic toxicity studies with mice.

CRITICAL ASSESSMENT OF THE METHOD

Among the established compounds inducing pancreatic proliferation, there is ciprofibrate, a peroxisome proliferator tested in Fischer 344 rats. There are some indications that an increase in prolactin secretion may contribute to pancreatic tumor development. Prolactin secretion is one possible biomarker which may be detected by analytical studies in rodent toxicology. A mechanistic explanation for the development of pancreatic acinar carcinoma in Fischer 344 rats has been an effect on decreasing bile acid synthesis and changing bile composition during treatment with peroxisome proliferators (e.g., clofibrate). The change in bile acid synthesis may increase CCK secretion and stimulate acinar cell proliferation by CCK-A receptors (IUPHAR database). Human safety studies of hypolipidemic agents have not disclosed any similar development for peroxisome proliferators. In nonhuman primates, beta cells are

the main cell population, glucagon cells comprise about 10%, and somatostatin-secreting D cells are a much smaller population. The technical analysis by immunohistochemistry of the prevalent cell types in rat pancreas is time consuming and difficult; it would be helpful to have hormone measurements which could replace risk assessment by histomorphometry. Particular factors to be monitored are glucagon and somatostatin, which might be measured in the circulation (basal levels and dynamic function tests), or by molecular biology methods for the glucagon and somatostatin-related receptor proteins. Such methods ex vivo might contribute to replace the time-consuming histomorphometry.

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14.6.5 Pancreatic Cell Lines and Ion Channels

Pancreatic ion channel signaling has been studied in HEK293 cells and many other models. The in vitro effects of sulfonylureas and glinides, as an example of using safety assays, have focused on the function of ion channels in the beta cell membrane, an example for continuing the work on sulphonylureas (Müller and Geisen 1996; Kramer et al. 1996) which require mechanistic implications for the site of action. Much of this work on classical bioassays, pharmacological assays, and biochemical methods has been summarized (Herling 2006; Mueller 2008). The examples of glimepiride, nateglinide, and repaglinide are of particular relevance for the studies on ion channels in pancreatic cells. These compounds have been tested on rat pancreatic ductal cells, concerning their interaction with the K(ATP) channels and the sulphonylurea receptor one (SUR1). The KATP channel is an octameric complex of the inward-rectifier potassium ion channel Kir6.2 and sulfonylurea receptor SUR1 which associate with a stoichiometry of Kir6.24/SUR14. The HEK 293 cell line has been discussed as a vehicle for isolated receptor channels, including the sulphonylurea channel (Thomas and Smart 2005). Application of the HEK-293 cell system to studies of sulphonylureas and glinides has been reported (Hu et al. 2000). In these studies, the direct interaction of nateglinide with K(ATP) channels in rat pancreatic beta cells was investigated as a suitable test system for orally active antidiabetic agents. Methods and concepts for the pancreatic sulphonylurea channels are discussed in the IUPHAR database of ion channels (Inagaki et al. 1995, 1996; Miki et al. 1997, 1998). The selectivity of ion channels for insulin secretagogues (sulphonylureas and glinides) have been discussed by Quast et al. (2004) in relation to the effect on pancreatic beta cells and the presence of KATP channels in coronary myocytes. They found a different selectivity for the pancreatic beta cell membrane over the cardiovascular KATP channels when comparing long sulphonylureas (glibenclamide) and short sulphonylureas (nateglinide). For the safety pharmacology evaluation, this means that oral antidiabetic agents need a specific profiling with regard to their effects on ion channels. The mechanisms of insulin signaling in pancreatic beta cells have been elucidated by studies with tissue-specific knockout animal models of the insulin receptor (Kulkarni et al. 1999a) and related studies on the insulin signaling in cultured beta cell lines (Kulkarni et al. 1999b). Due to the intrinsic differences of these models when compared with the situation in toxicological studies in rats and mice, studies in cultured cell lines and animals with

targeted knockout have remained a scientific approach; there is currently no direct relation to safety pharmacology.

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14.6.6 Animal Models of Antidiabetic Activity

There are some specific issues related to safety evaluation of GLP agonists and gliptins by pancreatic toxicology in repeated-dose studies. Evidence is in part summarized by the EMA assessment reports and the related scientific discussion; the specific questions concerning safety evaluation are reviewed. The preliminary test is whether any dose-related response can be obtained in the animal species used in toxicology or whether a different animal species is to be select. One example is the evaluation of exenatide in repeated-dose studies where the response of the mouse is similar to that of the intended therapeutic use, whereas the paradoxical response of the nondiabetic rat is probably due to the release of catecholamines. This precludes the use of the rat in conventional toxicology studies with exenatide, because the response is different and gives rise to different histological findings.

Selection of normal rats for safety pharmacology may not always be appropriate. This has developed for single-dose studies and repeated-dose protocols. In studies with exenatide, nondiabetic mice and rabbits were suitable. The injection of exenatide reduced plasma glucose in both species. Nondiabetic rats

however showed a paradoxical hypoglycemic response, and studies were therefore performed in *diabetic* mice and rats. There is a wide selection of models for diabetic rats and with a considerable range of sensitivity to drug effects. In the exenatide studies, a reduction in fasting glucose and an acute increase in insulin secretion were shown in the diabetic models in the presence of elevated glucose levels. One-month treatment was associated with a decrease in A1c. In these studies, the effects observed were increased insulin secretion, suppressed basal and postprandial glucagon secretion, and improved insulin sensitivity. Specific effects on the adrenocortical steroid secretion were investigated (Gil-Lozano et al. 2010). A once-a-month depot injection for exenatide has been evaluated in rats (Gedulin et al. 2005). Effects on the gastrointestinal tract are not usually investigated in safety pharmacology studies. They are however relevant for the pharmacological profile of GLP analogues (Nachnani et al. 2010). In the case of incretin mimetics, in particular of the GLP-1 agonists, inclusion of studies on gastrointestinal gastric emptying has shown consistent drug effects and is recommended for safety pharmacology evaluation of new compounds in this class.

In this context of safety pharmacology of GLP analogues, the evaluation of pancreatic exocrine secretion is of relevance (Kern 1980; Greaves 2007). This was done in some animal studies with exenatide and liraglutide (Malhotra et al. 1992; Vrang et al. 2012). Exenatide was examined for its effect on cholecystokinin-stimulated secretion of amylase and lipase in the rat. CCK-8 injection is a test of exocrine pancreas function. Another test was pentagastrin-stimulated gastric acid secretion in cannulated rats.

There were some specific issues in the safety pharmacology of the gliptins. Gliptins are small molecules which require extensive profiling. The primary screening procedure for saxagliptin was its ability of binding of appropriate radioligands to 42 receptors and ion channels or to inhibit the action of 14 different proteases. No significant effects were observed in this initial screen. This test of receptors and enzyme profiling is important because effects on GLP-1 receptors have been found with liraglutide, effects which were different from those anticipated. In rodents, GLP-1 receptors were found on C cells of the thyroid, and receptor activation was shown, in a species-specific manner different from human and monkey receptor activation.

The safety evaluation for *saxagliptin* as a selective DPP-4 inhibitor (DPP4) was done by in vivo efficacy testing in Sprague-Dawley and Zucker rats and in the Zucker FA/FA rats, a model for type II diabetes with several pharmacodynamic endpoints: plasma GLP-1, insulin and glucose levels postglucose challenge, and fasting glucose levels. It is important to note that the testing needs to be done in the presence of increased glucose concentrations, because both the gliptins and the GLP agonists are active in the presence of increased glucose concentrations, but not when glucose levels are in the normal range (Augeri et al. 2005; Tahara et al. 2009; Gallwitz 2008).

The single-dose toxicology studies were performed including the monitoring for 2 weeks after a single dose (standard toxicology procedure). In principle, hormone parameters could be included in such a protocol, in parallel to the regulatory toxicology. Repeated dose toxicity was evaluated in mice, rats, dogs, and cynomolgus monkeys at exposure markedly higher than the therapeutic exposure in a number of nonclinical toxicology studies.

Vildagliptin as a small molecule and DPP4 inhibitor was initially studied for its biochemical activity and subsequently by in vivo studies in rats and monkeys. Studies in diabetic rats and insulin-resistant monkeys (cynomolgus) demonstrate a dose-related glucose lowering effect. Chronic effects were studied in *prediabetic* insulin-treated monkeys (the specific model is discussed by Ahrén (2006)). The particular details were discussed with regard to an increase in beta cell mass in neonatal rats and improved beta cell function in streptozotocin-induced diabetic mice. The effects on the gastrointestinal tract were examined in relation to the established effects of an incretin mimetics. Vildagliptin and the other gliptins have no significant effect on gastric emptying in experimental models, for example, in monkeys. One safety issue different from that of other gliptins was modulation of immune function; this effect is outside the scope of endocrine-mediated effects but has considerable relevance for risk assessment. An interesting aspect is the safety assessment of drug combinations in the primary drug application. Vildagliptin was administered to Zucker fatty rats together with nateglinide, and additive effects on glucose parameters were found. The evaluation was complicated by findings of gastrointestinal symptoms in dogs which were however unrelated to the characteristic effect of incretin mimetics on a delay in gastric

emptying and decreased gastrointestinal motility. The dogs in this vildagliptin study had diarrhea and soft feces. Carcinogenicity studies were performed in mice and rats with no findings that gave rise to concern. In particular, there was no effect reported on the exocrine pancreas after long-term treatment. A matter of concern with tumors is noted in the mouse carcinogenicity study which might be the result of an effect on the pituitary-gonadal axis. There was an upregulation of genes related to milk production, suggesting that hormone-initiated changes are occurring in the mammary gland of mice. These findings are not of relevance for humans.

Another well-documented oral antidiabetic agent is *linagliptin*. The DPP4 inhibition after a single dose is long-lasting making this a once-a-day medication. As with the other members of this class, linagliptin can stimulate glucose-dependent insulin secretion, achieve suppression of glucagon secretion, and to a limited extent delay gastric emptying (not confirmed in humans). Linagliptin was tested both in normal and diabetic rats, with characteristic glucose-induced elevations of insulin and prolonged elevations of endogenous GLP-1. It was found that the effect on blood glucose in diabetic rats was dependent on the severity of insulin resistance. Animal models were Zucker diabetic fatty rats. Another antidiabetic model was treatment of cynomolgus monkeys, a monkey model with similarities to type 2 diabetes. Cynomolgus monkeys have been used as an animal model for other antidiabetic compounds with reproducible results; the diabetic condition depends on the increased food intake of the animals in captivity (insulin resitance and diabetes associated with obesity). Diabetic mice were treated for 14 and 28 days with a consistent effect on glycated hemoglobin (A1c). In the secondary pharmacodynamic studies on gastrointestinal function in rats, there was no decrease of gastric emptying. The effect of pharmacodynamic drug interactions was studied combining linagliptin with metformin. This combination acts by different mechanisms, and there was an added effect on glycemic control when comparing monotherapy and combination therapy. In pharmacokinetics studies, there was no indication that linagliptin does cross the blood-brain barrier. The studies were performed because centrally mediated effects of incretin medics on food intake have been suggested from studies with, for example, exenatide. In the repeated-dose toxicity studies in rats, there were effects on reproductive organs, thyroid, and lymphoid organs at high concentrations, conceivably by interaction with hormone-related enzyme systems. After initial testing, no repeated-dose toxicity studies were performed in dogs because of secondary cardiovascular changes which were considered species-specific and preclude the use in toxicology. Repeated dose toxicology was therefore done in cynomolgus monkeys. Intravenous administration of linagliptin was associated with atrioventricular block and symptoms of pseudo-allergy. These studies were not further pursued because the route of administration will not be used in clinical therapy. In the 2-year carcinogenic rodent studies, there were no carcinogenic effects in either mice or rats.

In summary, the primary pharmacology of the gliptins (vildagliptin, saxagliptin, and linagliptin) is extensively studied and documented. The results confirm that both pancreatic safety studies and inclusion of endocrine parameters are advisable, in particular referring to findings with linagliptin. There were no indications that the general principle of DPP4 inhibition has any characteristic effect on hormone systems other than the endocrine pancreas. The findings described as pseudo-allergy merit a more detailed discussion. Pseudo-allergic reaction became apparent without prior sensitization and was associated with significantly increased plasma histamine levels. This is reminiscent of nonspecific histamine-releasing effects observed with LHRH antagonists which contain multiple basic amino acids. The structural reason for the pseudo-allergy observed with linagliptin has not been followed-up. Clinical study results indicate that pseudo-allergic found with linagliptin is not of relevance for humans. The interest of monitoring plasma histamine levels as a biomarker for pseudo-allergy is evident from these studies.

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14.7 Pharmacology of Bone

14.7.1 General Considerations

With increasing knowledge of the regulation of bone as a hormone-dependent organ system, hormones involved have been characterized in biochemical and functional detail, with regard to calcitonin, parathormone, and calciferols (vitamin D group). A number of validated biomarkers are available for the structural changes occurring in bone, referring in particular to osteoporosis and metastatic bone disease also to changes in end-stage renal failure (ESRF). A detailed discussion of biomarkers which may be included in animal studies for safety pharmacology of bone is presented there.

The task of this section on endocrine safety pharmacology is to evaluate the use of existing methods for early investigation of effects on bone, to be detected prior to planning studies in clinical pharmacology for a drug candidate. This may be a new chemical entity (NCE) or an established compound with effects on bone predicted from early experiments but not well characterized. The critical question is how studies can be included in the preclinical profile, in particular whether they can be added to the regulatory toxicology studies. Any dose-related effects on bone are likely to have an effect on compound development and need to be detected at an early stage whenever there are safety concerns and open questions.

Bone is an endocrine-dependent organ tissue that may undergo profound changes with prolonged therapy. One aspect is the structural change and loss of mineral content after ovariectomy in animals and the related situation in aging humans that affects both men and women (fracture risk). Another aspect is the prevention of such changes in the bone, for example, by antiresorptive agents (e.g., bisphosphonates and steroidal compounds). Endocrine safety pharmacology can contribute during the early phase of drug evaluation, to recognize the need for targeted studies in phase 2 clinical development.

A number of drugs are known to affect bone metabolism to some extent and different mechanisms, among them are heparin, warfarin, cyclosporine, glucocorticoids, medroxyprogesterone acetate, thyroid hormones, selective estrogen receptor modulators (SERM), atypical antipsychotics (clozapin), and certain antibiotics.

As outlined further by the FDA and EMEA, supplemental studies must be conducted outside the safety pharmacology core battery, when there is a cause for concern. This includes effects of the test substance on renal/urinary parameters, on parameters of the autonomic nervous system, the gastrointestinal systems, and other organ systems such as skeletal muscle, immune, and endocrine functions. In this context, Henriksen et al. (2010) have reviewed the options for targeted investigation of effects on the bone by using specific biomarkers which are validated for research on animal models and in a similar manner have been applied in clinical studies. In preclinical studies and regulatory toxicology, the task is to identify indications for a potential effect on bone in clinical studies. The application of such methods is justified by the gain in information, using established animal models extending their predictive power. In the clinical application, biomarkers are helpful as an initial indication for clinical efficacy which subsequently has to be supported and confirmed by clinical outcome studies. During the early development of bisphosphonates, biomarkers of bone resorption were the leading indicators for a consistent clinical effect and at that time were designated "surrogate endpoints."

Safety pharmacology of the skeletal system is related to the secretion of calcitonin, parathormone, calciferols, the electrolyte balance of calcium and phosphate, and finally to the manifestations of the effect of drugs and hormones on bone structure. The characteristic findings in toxicological pathology (Woodard et al. 2002) and histopathology have been reviewed (Greaves 2007b). There are a number of therapeutic drugs with indications in diseases with manifestations on bone diseases (ICD-10), for example, osteitis deformans, postmenopausal osteoporosis, and end-stage kidney disease (Friedman 2006; Bikle 2009). In safety pharmacology of the bone, assays related to hormonal regulation of bone and cartilage as an endocrine-dependent organ system are performed in a search for preclinical evidence for mechanisms induced by drug candidates or investigational drugs. The classical regulatory toxicology studies often detect changes in bone formation in reproductive toxicology, with no direct relation to a signal for future clinical use. Studies in normal mice and rats will often miss skeletal changes which are difficult to assess and may require histomorphometry of the bone. Direct hormone assays for

calcitonin and parathormone are difficult to perform and interpret. There are some enzyme assays in conventional clinical chemistry which may indicate that the specific pharmacology needs to be extended and/or reinvestigated and the inclusion of biomarkers of bone turnover is certainly to be recommended.

This situation has improved considerably with the introduction of bone-related biomarkers which may be easily included in regular toxicology and in targeted satellite investigation in animals. There are some interesting examples for well-documented drugs, for example, calcitonin, parathormone, teriparatide, several bisphosphonates (Licata 2005), and calcimimetic cinacalcet, and there are of course the classical observations of steroid-induced osteoporosis related to the effects of chronic therapy with glucocorticoids and their derivatives in asthma, chronic obstructive pulmonary disease (COPD), and related indications. In the early profiling of a drug candidate, interaction with a particular set of receptors, for example, steroid receptors or the calcium-sensing receptor (Theman and Collins 2009), may have been found and give rise to a more detailed safety assessment.

Duration of treatment in rats is based on bone remodeling cycles (six cycles recommended). The ovariectomized rat (OVX) is frequently used for such studies (Lelovas et al. 2008).

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14.7.2 Bone Safety Study in Rats

In the context of regulatory studies, there are two problems which need to be considered concerning the standard inclusion of biomarkers for increased bone turnover. There are a number of therapeutic agents which are known to affect bone turnover; therefore, drug candidates might be addressed if they belong to one of the classes already known to affect bone turnover (Rosen 2012).

PURPOSE

The specific bone safety model in adult female rats is the ovariectomized rat (OVX).

The intact female rat is not suitable to detect subtle changes. Moreover, male rats are not adequate models for bone turnover studies, even if antiandrogens would cause an interesting change in bone turnover.

PROCEDURE

The preferred selection for a bone safety study is therefore based on adult ovariectomized female rats, preferably treatment to be started at least 4 weeks after ovariectomy. Inclusion of two dose levels may be sufficient; preferred duration of treatment is 6 or 12 weeks. This model is particularly useful for compounds with antiresorptive activity (calcitonin, parathormone, calciferols, bisphosphonates, etc.).

EVALUATION

The result of finding a significant change indicated by the selected biomarkers needs to be assessed with regard to dose-related changes and a follow-up by histomorphometry. It depends on the extent of the biomarker change whether histomorphometry of specific bone areas is to be included (for details, see Bagi et al. 2011) or whether the result is translated to inclusion of similar biomarkers in the proposed clinical trial.

14.7.2.1 Biomarkers of Bone Turnover

An extensive review for clinically validated biomarkers for osteoporosis management is provided by Garnero (2008). In this review, the urinary osteocalcin fragments are addressed, the tartrate-resistant acid phosphatase, regulators of osteoclastic and osteoblastic activity currently under investigation, and markers for the posttranslational modification of bone matrix proteins (type I collagen).

There is a wide selection of analytical methods available; biomarkers following increased bone turnover have been characterized extensively in clinical studies, and the general principle of investigating biomarkers as an exploratory approach with a high probability of relevance is well established (Calvo et al. 1996; Henriksen et al. 2010; Frank and Hargreaves 2003; Dambach and Gautier 2006; Williams et al. 2006; Garnero 2008; Wagner 2009). Selection of an analytical method at the time of planning a safety study is based on an Internet research to collect some experience with the sample acquisition and determination in a small preliminary study (Leeming et al. 2006; Bay-Jensen et al. 2010; Herrmann and Seibel 2008).

CRITICAL ASSESSMENT OF THE METHOD

A detailed discussion of parameters related to the ovariectomized model is provided by Lelovas et al. (2008). The histomorphometry methods are reviewed with reference for specific sites to be investigated. For limited safety pharmacology, the application of biochemical markers is of practical interest. For the biomarkers, there are numerous assay methods available. Markers of bone formation are alkaline phosphatase and osteocalcin, and markers of bone resorption for the rats are pyridinolin, tartrate-resistant acid phosphatase, and urinary type I collagen cross-linked in telopeptides (CTX-I). The markers of bone turnover represent bone metabolism changes in the whole skeleton; they are not suitable for regional assessment. The markers of bone turnover are very valuable for the assessment of drug candidates with indications for hormone effects on bone (due to structural considerations or by receptor screening). Densitometry is a precise noninvasive method, but requires special equipment and cannot be recommended for toxicology studies. Histomorphometry remains limited for detailed information of regional bone changes. Although a description for methods applying

mechanical stress is available, these methods are outside the scope of safety pharmacology. The review by Lelovas et al. (2008) provides an excellent assessment of the options in extended mechanistic studies. A similar review for the pharmacology, pathology, and preclinical studies with ibandronate is provided by Russell (2006). The species included are rats, dogs, and monkeys. The review shows that evaluation of bone changes in the rat requires treatment periods of preferably 6 months; ovariectomy is performed in adult rats aged 10–12 months.

Given the complexity of bone turnover, any studies in cell culture, for example, with neonatal rep osteoblasts, have the disadvantage of being with no direct relation to the practical therapy (Yan et al. 2009). The bone safety study and rats is to be preferred as a practical satellite study enhancing the interpretation of any findings in long-term regulatory in rats.

14.7.2.2 Parathormone and Teriparatide

There are some practical examples where inclusion of safety pharmacology studies for the effects on hormonal-dependent bone may be appropriate (Forsteo 2004). Detailed indications for clinically established compounds are provided by the EMA assessment reports. The human parathyroid hormone sequence (1–34), teriparatide, was evaluated in animal models in rats, mice, rabbits, and monkeys. Monkeys and rabbits are considered remodeling species but in bone physiology do not mimic that seen in humans. Endpoints in skeletal changes were analysis of bone mass (DXA absorptometry), bone architecture by histomorphometry, and mechanical testing. For the practical application in safety pharmacology, studies in ovariectomized rats are relevant. It was summarized that teriparatide stimulated both modeling and remodeling of bone in adult animals. In drug interactions, selective estrogen receptor modulators (SERM) administered with teriparatide increased bone mineral density in trabecular bone. For the assessment of drug interaction studies in mechanistic studies, biomarkers and bone imaging are suitable; however, interaction studies are rarely part of endocrine safety pharmacology. Interaction studies with vitamin D3 were performed in rat, rabbits, and monkey and showed similar bone responses. The safety pharmacology may then turn to specific questions such as the effect of excessive calcium mobilization on kidney function. Teriparatide is an interesting example for limited relevance of carcinogenicity detected in a lifetime studies. There was formation of osteosarcoma in lifetime studies in rats, but not when the study duration was limited to 12 months in the rat.

14.7.2.3 Bisphosphonates

Studies on bone quality have been performed with several antiresorptive bisphosphonates. Models used in the evaluation of ibandronate were reviewed (Bauss and Dempster 2007). Studies were directed at bone mass, strength, and architecture. Biomarker determinations were also applied in these studies. Intermittent and daily regimens of bisphosphonates have frequently been compared, and there are clinical preparations administered once a week, once a month, or by infusion (in the treatment of metastatic bone disease). The details of studies with alendronate in osteoporosis with regard to the supporting preclinical data were summarized (Bauss and Russell 2004). The animal models of preclinical studies included for animal models the rat, dog, mini pig, and monkey. In the rats, dogs, and monkeys, the osteoporosis-like condition was initiated by ovariectomy (estrogen depletion). In mini pigs, bone loss was induced by glucocorticoid treatment. Corticoid treatment is a clinically relevant risk condition, where osteoporosis has been frequently observed as a critical adverse long-term effect. The studies compared the continuous and intermittent treatment, in particular with regard to developing preparations for intermittent use (once a month). Additional models of secondary hyperparathyroidism and bone loss induced by immobilization (both models in rats) were applied. In clinical studies, ibandronate was found to reduce the risk of vertebral fractures, and the studies also support the use in metastatic bone disease.

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14.7.3 Animal Models

14.7.3.1 Animal Tests of Bone Structure and Drug-Induced Changes

An early study was performed by Balena et al. (1993) with alendronate using a rat model of hyperthyroidism. Alendronate was tested for prevention of thyroid hormone-induced bone loss. Ten-week-old Sprague-Dawley rats were treated with thyroxine 250 μg per kilogram per day, to induce bone changes. After 3 weeks of treatment, histomorphometry parameters of cancellous bone remodeling were assessed in the tibia and in vertebra. Thyroxine treatment caused significant bone loss associated with increased bone turnover. At the time of the study (1993), the authors did not apply any of the biomarkers for bone turnover currently used to describe bone metabolism changes. Thyroid hormone in this model induces high bone turnover and bone loss in the tibia which can be prevented by alendronate through an inhibition of osteoclast activity. After 3 weeks' treatment, there was no significant change in the vertebral bone (spinal column). The consequence is that studies need to be performed for a longer time when distinct changes in histomorphology of vertebral bone are to be induced in the model.

There are several well-established animal models for the effect of calcitonin on skeletal metabolism (Wallach et al. 1999). The methods for evaluation of the response of bone to the hormone generally include histomorphometry in rats. Similar models were applied to the evaluation of parathormone (full sequence and active sequence). In these models, calcitonin and parathormone as well as calciferols were assessed (Peppone et al. 2010). Bone density was assessed by a variety of methods, and studies of mechanical properties of connective tissue with regard to breaking strength and tensile strength were sometimes included. Many models are based on time-consuming evaluation and complex endpoints; the studies were subsequently extended and refined by enhancement with validated biomarkers. The initial animal models were focused on detailed examination of the bone and cartilage, with a view to treating postmenopausal osteoporosis and more recently also targeting osteoarthritis. A recent update of the methods used in rodent models of aging bone was provided by Syed and Melim (2011). There are now comprehensive research models in mice and rats which evaluated hormone concentrations,

biochemical effects, inflammatory cytokines, oxidative stress, nutrition, and the effect of exercise on the skeleton. The segment suitable for safety pharmacology is based on the response of adult ovariectomized rats preferably with 3-month treatment. The research methods for new factors involved in bone pathophysiology ("bone is an endocrine organ") are in general not suitable for safety evaluation of drug candidates, due to their limited predictive power. They do provide an outlook for future development of animal models to osteoblasts and osteoclasts activity separately and include adipocyte cells. The step of translation to the clinical situation for the effects of salmon calcitonin treatment was reviewed by Karsdal et al. (2009a). They reviewed the use of biomarkers of bone turnover in postmenopausal women. The analytical determinations included measurement of plasma calcitonin levels and changes in the bone resorption marker serum CTX-I and the urinary cartilage degradation marker CTX-II measured up to 5 h after treatment. Bone resorption and cartilage degradation markers displayed a comparable response, during the early posttreatment observation period. The parameters were applied to evaluation of recombinant calcitonin injections and new oral calcitonin formulations. Karsdal et al. (2009b) also discussed the specific value of biochemical markers for the development of a better understanding of pathophysiology. There is currently an FDA Critical Path Initiative of improving the validation of specific markers for drug discovery.

14.7.3.2 Calcitonin

The ovariectomized rat model of postmenopausal bone loss was described by Kalu (1991) and subsequently frequently applied for the assessment of antiresorptive agents including calcitonin and the bisphosphonates. At this time, the antiresorptive activity of calcitonin was extensively evaluated, and research indicated that there are calcitonin-related peptides which might have similar therapeutic activity. It turned out that the specific antiresorptive activity is closely associated with the calcitonin structure (Zaidi et al. 1987). Research on the molecular pharmacology of the calcitonin receptor family is continuing (Purdue et al. 2002); the methods are currently not applicable to safety evaluation. The complexity of the calcitonin receptor family needs to be further explored, and therapeutic activity is currently restricted to calcitonin formulations.

14.7.3.3 Recombinant Human PTH

In the context of osteoporosis research, animal models have been reviewed and discussed by several groups, including Kimmel (Kimmel et al. 1993; Kimmel 2002). This group has evaluated the effects of recombinant human parathormone full sequence (1–84) and active sequence (1–34), synthetic parathormone teriparatide. In many of these studies, the ovariectomized rat model (OVX) was applied.

Cheong et al. (2011) investigated the use of tetracycline bone-seeking label in rats after ovariectomy and found that the excretion of label followed the presence in the skeleton over a period of 3 months after injection of the probe. This method may be suitable in satellite groups, in parallel to the toxicology studies if there is a question of a drug effect on accelerated bone turnover.

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14.7.4 Bone-Related Hormone Analytical Methods

The concentrations of calcitonin, parathormone related peptides and partial sequences and calciferols may be measured in satellite studies in rats, dogs and monkeys. There is considerable selection of analytical methods for the determination of calcitonin, procalcitonin, parathormone (intact sequence and partial sequences), and the calciferols (vitamin D group). Methods for calcitonin assay may be directed at monitoring pharmacokinetic concentrations after treatment and endogenous secretion in animal studies. Recently, the importance of thyrocalcitonin as an indicator of thyroid cancer (Rosai 2003; Kratzsch et al. 2011) has become a very controversial issue, when thyroid C-cell proliferation was found with an incretin mimetic and studies are required to assess the relevance of these findings for human risk assessment (Bjerre Knudsen et al. 2010). Reference ranges for the clinical assay are established (D'Herbomez et al. 2007). Interestingly, pro-calcitonin is used as a biomarker for inflammation in sepsis and has acquired clinical relevance (Maruna et al. 2000; Kratzsch et al. 2011).

There are several assay methods for intact parathormone (1–84) and partial sequences including the active sequence (1–34), teriparatide (Carter and Howanitz 2003; Fuleihan and Jüppner 2012). The group of assays for calciferol (vitamin D3) is characterized by a number of different methods which have been recently reviewed due to problems of sensitivity and specificity (Lensmeyer et al. 2006; Glendenning et al. 2006).

Application to safety pharmacology studies of hormone-dependent bone is certainly an approach which may extend the information provided by biomarkers of bone turnover. Considering the complexity of the analytical methods and their problems of specificity and handling of samples, these hormone determinations will only be included in very specific situations and for pharmacokinetic monitoring.

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Skin Pharmacology

15

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15.1 In Vivo Percutaneous Absorption Assays

PURPOSE AND RATIONALE

Percutaneous absorption, also known as dermal or skin absorption, is a term that refers to the transport of a chemical from the outermost layer of the skin (stratum corneum) to the systemic circulation. Percutaneous absorption assays are essential for the safety assessment of chemicals making contact with the skin surface, such as pesticides, topically applied pharmaceuticals, industrial chemicals, and cosmetics, as well as in the development of drugs for dermal or transdermal application.

PROCEDURE

Percutaneous absorption can be determined by applying a known amount of chemical to a specified surface area of laboratory animals or human volunteers. Radiolabeled chemicals, usually carbon-14 (^{14}C) or tritium (^{3}H), are widely used for analytic convenience. When the study is performed in laboratory animals, the amount of chemical and/or metabolites recovered from urine, feces, cage washing, blood, skin bound residues*, and the remaining carcass are added, and the percutaneous absorption is expressed as the total percentage recovery from the applied dose. Expired air should be collected when volatile metabolite is excreted in quantity $>5\%$ of applied dose.

If the animals are not sacrificed, or the study is conducted with human volunteers, an indirect measure of absorption is performed by monitoring urine, feces, and blood. The amount of chemical that is absorbed, but not measured in the monitored fluids, is corrected for by determining the amount of chemical excreted

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following parenteral administration. The percutaneous absorption is calculated by the amount excreted after topical application divided by the amount excreted after parenteral administration of the chemical and is expressed in terms of percentage of applied dose. In the absence of intravenous studies, the average percutaneous absorption rate can be estimated by mass balance. It is calculated by the amount excreted divided by the amount applied.

At the end of the experiment, a full mass balance of the applied dose should be performed. An adequate mean recovery is in the range of 90–110% (OECD).

Note: *According to OECD (2008), skin bound residues (residues recovered from the application site after it has been washed) should be added to the absorbed dose depending on the bioavailability of skin bound radioactivity. If bioavailability cannot be determined, skin bound residues should be considered absorbed.

EVALUATION

The advantage of in vivo assays is that it relies on a physiologically and metabolically intact system (OECD 2004a). In vivo studies in humans are the gold standard and provide definitive data for the assessment of the absorption of chemicals through human skin.

Percutaneous absorption rates in rat, rabbit, and mouse are generally higher than in humans while the skin permeability of pig and monkey more closely resembles humans. There are interspecies differences in routes of excretion of some chemicals as well. These knowledge should be considered when selecting an animal model and designing the experiment. If the objective of the assay is to predict human percutaneous absorption, then the rate and extent of skin absorption in the laboratory animal should be quantitatively the same as in man or consistently related to the absorption in humans by a constant ratio.

CRITICAL ASSESSMENT OF THE METHOD

As monkey and pigs are comparatively more difficult and expensive to maintain, rat is the most commonly used species for animal in vivo studies, having the advantage that information from other toxicity studies is mostly obtained from this species and is therefore directly comparable and the disadvantage of overestimation of human percutaneous absorption. Increasing resistance from animal protection organizations to the use of live animals in testing of chemicals associated with the difficulties in conducting these pharmacokinetic assays—collecting excrement for relatively long period of time (24 h +), requirements for specialized cages, for specialized protective apparatus to avoid ingestion of the chemical or contact with the cage, and space requirements for housing animals individually—constitutes the major limitations of animal in vivo assays. The major limitations of human in vivo assays are the possible systemic and local toxicity of applied chemicals and the fact that their conducts are subject to strictly ethical considerations and conditions.

Radiolabeled chemicals are widely used for analytic convenience; however, they may not provide accurate estimates of bioavailability. For example, a comparison of the bioavailability of nitroglycerin and the level of radioactive tracer indicates the overestimation of available drug by as much as 20%. This corresponds to the metabolism of the drug to an inactive form.

MODIFICATIONS OF THE METHOD

15.1.1 Tape Stripping

Rougier et al. observed a linear relationship between the amounts of chemical present in the stratum corneum that has been washed 30 min after application and the total amounts excreted in the urine within a 4-day period. The good correlation between the two measurements suggested the value of the tape-stripping approach as a simple and minimally invasive method for estimating percutaneous absorption in laboratory animals and humans. In this assay, the stratum corneum of the skin exposed to the chemical is removed sequentially by successive application of adhesive tape. The amount of chemical present in the stratum corneum recovered by the tape is then determined. This method has been used to estimate bioequivalence of topically applied chemicals. However, it does not have a regulatory imprimatur.

15.1.2 Residual Analysis Methods

The determination of the material "lost" from the skin surface may also be considered as a measure of

percutaneous absorption. Two approaches have been described. (A) Single-point measurements of drug disappearance: The difference between the applied dose and the recovered residue from the application site is assumed to be the amount of drug absorbed. The disadvantages of this method are that recovery of the chemical is difficult and total recovery from the skin is not guaranteed. Volatile materials may leave the surface without penetrating. In addition, skin may retain a reservoir of the chemical that has not entered the circulation. (B) Continuous monitoring of drug uptake: The chemical (radiolabeled or containing a spectrophotometric marker) in the outer skin layers is monitored over time for the disappearance of radioactivity or of spectral signal, and absorption is assessed from the decay of the respective signal.

15.1.3 Cutaneous Microdialysis

Cutaneous microdialysis measures the amount of a chemical in the extracellular space beneath the skin of human volunteers or laboratory animals using perfused dialysis. The microdialysis probe may be implanted into the dermis or into the subcutaneous tissue. The blood flow underneath the skin application site of the chemical is mimicked by continuously passing a receptor fluid (perfusate) through a microdialysis tubing (a thin hollow tube formed by a semipermeable membrane) and collecting it in a collector (dialysate). The technique is based on the passive diffusion of the chemical across the microdialysis tubing, and the amount absorbed into the receptor fluid is then measured, and the results are usually expressed in terms of relative recovery. Microdialysis may be useful to assess local bioequivalence/bioavailability after topical application of a chemical. The challenge of the assay is the measurement of lipophilic chemicals and those with high protein binding, which are poorly diffused into the receptor fluid (perfusate). A limitation rests in the inability to quantitatively collect all of the dialysate and obtain mass balance data.

15.1.4 Whole-Body Autoradiography

The whole-body distribution of a topically applied radiolabeled chemical in the laboratory animal skin is determined in frozen cross sections of the entire carcass. Whole-body cross sections include representative samples of all major tissues and are evaluated qualitatively and/or quantitatively for radioactive content using autoradiography or autoradioluminographical techniques.

15.1.5 Pharmacodynamic Method

Pharmacodynamic responses in skin function can be used to assess absorption of topical dermatological products. The method is rather qualitative than quantitative. It represents an accepted approach to establish bioequivalence between different formulations of certain topical drugs, e.g., the vasoconstriction or "blanching" to evaluate topically applied corticosteroids.

15.1.6 Skin Biopsy

Despite the advantage of offering a "snapshot" of chemical disposition in the different skin layers, the invasive nature of the biopsy makes the method less useful.

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15.2 In Vitro Percutaneous Absorption Assays

PURPOSE AND RATIONALE

Recently, many efforts have been made to replace percutaneous absorption assays in the animal by in vitro assays. The excised skin from humans and/or laboratory animals are used to measure absorption of chemicals based on the assumption that stratum corneum is the principal diffusion barrier for percutaneous absorption, and as it is composed of nonviable cells, the permeability properties of skin are maintained after its excision from the body.

PROCEDURE

Most common methods of in vitro percutaneous absorption assays are performed with diffusion cells, which consist of a donor compartment (upper compartment) and a receptor compartment (lower compartment), between which a disc-shaped fragment of the chosen skin sample is stretched, epidermal side up. The underside of the skin is bathed with a receptor fluid, which for hydrophilic chemicals can be saline or an isotonic buffered saline. For lipophilic chemicals, a solvent mixture such as ethanol or bovine serum albumin should be added. Two types of diffusion cells may be used: the static or Franz diffusion cell (samples of the fluid from the receptor compartment are manually withdrawn periodically for analysis) or the flow-through diffusion cell (automatic sampling of the fluid that flows continuously through the receptor compartment).

The chemical to be studied, which may be radiolabeled, is applied to the epidermis for a specified time under specified conditions and then is removed by an appropriate cleansing procedure. Skin barrier integrity should be previously checked by an appropriate method. The fluid from the receptor compartment is sampled at time points throughout the experiment and analyzed for the test chemical and/or metabolites by liquid scintillation counting (LSC) or high-performance liquid chromatography (HPLC). The amount found in the receptor fluid at the end of the experiment is considered to be systemically available. For studies performed in accordance with the OECD methodology (2008), the residue in the skin is counted as absorbed.

At the end of the experiment, a full mass balance should be performed. An adequate mean recovery is in the range of 90–110% (OECD) or 85–115% (SCCP). Lower or higher recovery rates should be investigated and/or explained.

The choice of the skin model depends on the purpose of the study, the chemical under investigation, and also on the availability of the skin sample. Human viable skin (female abdominal or breast skin obtained from cosmetic surgery) is preferred, but alternatively, human nonviable skin (cadaver skin) may be used. Either split-thickness skin (stratum corneum+epidermis+upper dermis), prepared with a dermatome or epidermal sheets (stratum corneum+epidermis), prepared enzymatically by heat or chemically, are acceptable. The use of full-thickness skin (stratum corneum+epidermis+dermis) should be justified, as the lack of a functioning microcirculation below the in vitro epidermis leads the dermis to participate in the absorptive process, functioning as a permeability barrier to lipophilic chemicals. Frozen and/or cadaver skin preparations may lack enzymatic activity; therefore, it is not recommended for chemicals that undergo significant biotransformation in the skin.

Skin from laboratory animals may also be used. Pigskin is accepted and validated for cosmetic assays by the SCCP. Although rodent skin overestimates

human percutaneous absorption, it is used for toxicological studies because the majority of the studies in vivo were conducted in this species.

EVALUATION

In vitro assays offer some advantages over in vivo assays. Highly toxic chemicals can be studied in human skin, and large number of diffusion cells can be run simultaneously. In addition, these assays may be less expensive and easier to conduct.

Van de Sandt et al. (2000) and Cnubben et al. (2002) demonstrated that in vitro assays using viable human full-thickness skin samples correlated reasonably well with human in vivo percutaneous absorption of propoxur and ortho-phenylphenol, respectively, on the basis of the potential absorbed dose and the amount systemically available. For propoxur (solubility in water 1,900 mg/L), human full-thickness skin samples slightly overestimated the amount systemically available after 24 h. For *ortho*-phenylphenol (solubility in water 700 mg/L), full-thickness skin samples significantly underestimated the amount systemically available at the earlier time points, but after 48 h, it predicted very well the amount systemically available. This could be explained by the fact that lipophilic chemicals (*ortho*-phenylphenol) are partially retained in the dermis and are only slowly released into the receptor fluid. The epidermal sheet samples significantly overestimated human in vivo data in both studies. Perhaps, a better correlation would have been found if split-thickness skin were used instead of full-thickness skin or epidermal sheets.

CRITICAL ASSESSMENT OF THE METHOD

The disadvantages of in vitro assays include the many variables that must be standardized for results to be comparable between studies and conclusions to be made reliably. Variations of experimental parameters such as thickness and types of skin, amount applied, exposure duration, vehicles, type of diffusion cell, perfusate flow, air temperature, relative humidity, air flow, type of receptor fluid, and extent of chemical solubility in the receptor fluid have all shown to result in significant changes in chemicals absorption rates.

Van de Sandt et al. (2004) described an interlaboratory study where the in vitro absorption rates of benzoic acid, caffeine, and testosterone were compared. A major effort was made to standardize the study performance, but not all variables were controlled (skin thickness, diffusion cell type, and receptor compartment volumes). All laboratories assigned the absorption of benzoic acid the highest ranking of the three chemicals, but only seven out of nine laboratories utilizing human skin ranked the maximum absorption of caffeine to be higher than testosterone. The variation observed was attributed to human variability in dermal absorption and to the skin thickness. Skin thickness had a great influence on testosterone (most lipophilic chemical) absorption, and the maximum absorption rates were clearly higher in the laboratories using thin, dermatomed skin samples.

MODIFICATION OF THE METHOD

To overcome the legal and ethical limitations concerning the use of human and animal skin samples in in vitro assays, several artificial human skin models have been developed, and some of these are commercially available: Epiderm®, SkinEthic®, and Episkin®, which are reconstructed human epidermis (RHE) models. More recently, full-thickness models were also introduced to the market: EpiDermFT® and Phenion® full-thickness skin model.

Schäfer-Korting et al. reported a validation study that aimed to evaluate whether the commercially available reconstructed human epidermis (RHE) models, Epiderm®, SkinEthic®, and Episkin®, were suitable for in vitro percutaneous absorption assays. Nine chemicals, covering a wide spectrum of physicochemical properties, were tested in ten laboratories, under strictly controlled conditions having human epidermis sheets and 1-mm thickness pigskin as references. In general, permeation of the RHE models exceeded that of human epidermis and pigskin (SkinEthic was found to be the most permeable), but the ranking of the permeation through the three tested RHE models and the pigskin reflected permeation through human epidermis sheets. According to the authors, additional experiments employing at least 30 test chemicals should be performed to estimate the prediction of systemic human exposure by permeation experiments with RHE models. The major limitation of all three models is the still relatively weak barrier function, and when used in percutaneous absorption assays as alternatives to human and pigskin, product-specific overpredictability should be taken into account.

According to the OECD Guidance Document N° 28 for the conduct of skin absorption studies (2004a), reconstructed human skin models can be used if

obtained data from reference chemicals are consistent with those in the published literature. On the other hand, considering the insufficient barrier function of reconstructed skin models, SCCP (2006) recommends the use only of samples of natural origin for in vitro testing.

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15.3 Guinea Pig Sensitization Tests

PURPOSE AND RATIONALE

The prediction of the sensitizing potential of individual chemicals or finished products (capacity of inducing proliferation of specific memory T lymphocytes that can lead to cutaneous inflammatory reaction in subsequent exposures of susceptible individuals) can be investigated by animal test methods using guinea pigs. These assays are valuable in the safety assessment of chemicals coming in contact with the skin such as cosmetics, household products, pharmaceuticals for topical use, those present in the occupational environment, or any other with which humans my come into contact. Several tests will be described here.

PROCEDURE

15.3.1 General Principles

Male and/or female healthy young guinea pigs of the Hartley or Pirbright White strains with a starting body weight of 300–450 g have been shown to be optimal. If females are used, they should be nulliparous and nonpregnant. Animals are maintained in the facilities with temperature of 20 ± 3°C, relative humidity of 30–70%, and 12-h automatic light cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of vitamin C. Test sites are cleared of hair by clipping, shaving, or possibly by chemical depilation, depending on the test protocol used. Care should be taken to avoid abrading the skin.

The test animals are initially exposed to the test substance by intradermal injection and/or topical application with the intention of stimulating the immune system (induction exposure—simulates the first contact). Following a rest period, during which an immune response may develop, a subsequent exposure (challenge exposure—simulates subsequent exposures) to the test substance in a virgin skin area of the previously treated guinea pigs checks if the animals show allergic reactions to the tested substance.

A control group is necessary to ensure that the positive challenge reactions are of allergic origin and not due to skin irritancy. The guinea pigs of this group are treated in the same manner as the test animals, except that during the induction exposure, the use of the test substance is omitted. If it is necessary to clarify the results obtained, a second challenge (a rechallenge) should be considered approximately 1 week after the first one, and it is essential to incorporate a new set of control animals.

Skin reactions are evaluated by visual scoring and palpation of erythema, edema, and other skin clinical changes using various grading scales, e.g., the Magnusson and Kligman grading scale (0 = no visible change, 1 = discrete or patchy erythema, 2 = moderate and confluent erythema, 3 = intense erythema and swelling). An exceptional case is the optimization test, in which "reaction volume" serves as the parameter for evaluation of skin reactions. Reactions are considered positive when they are more intense in the test group than in the controls. The incidence of positives in the test group compared to the control group is used to classify the test substance as a sensitizer or not.

The sensitivity and reliability of the chosen technique should be assessed twice a year using substances known to have mild-to-moderate skin sensitization properties such as hexyl cinnamic aldehyde, mercaptobenzothiazole, and benzocaine. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a nonadjuvant test should be expected.

The tests differ significantly in route of exposure, use of adjuvants, induction interval, and number of exposures. The principal features of the most commonly used assays to predict sensitization are summarized in Table 15.1.

15.3.1.1 The Draize Test (DT)

This test employs intradermal injections for the induction and challenge exposure. A minimum of 20 animals is required for the treatment group and for the control group (OECD 1981). Days 0 to 20—Induction exposure through intradermal injections: One flank of the animals is cleared of hair. One intradermal injection (0.05 mL) at 0.1% of the test substance in an appropriate vehicle is given in the anterior flank. Then, nine subsequent intradermal injections (0.1 mL) at 0.1% of the test substance are given every other day into a new site on the same flank. The control group is injected only with vehicle. Day 34—Challenge exposure through intradermal injection: The contralateral flank of the animals is cleared of hair. One intradermal injection (0.05 mL) at 0.1% of test the substance is given in the anterior flank of both groups. Days 35 and 36—Assessment of sensitization: The test sites are visually evaluated and graded at 24 and 48 h after challenging. Although largely abandoned—largely because of the intradermal—rather than topical dosing, the method in experienced hands identifies many known human allergens.

15.3.1.2 Open Epicutaneous Test (OET)

This test employs open topical application for the induction and challenge exposure. A minimum of 6–8 animals is required for each treatment group (usually six groups) and also for the control group (OECD 1981). Days 0–20 or 28—Induction exposure through open topical applications: One flank of the animals is

Table 15.1 Principal features of guinea pig sensitization assays

Feature of test	Draize	Open Epicutaneous Test (OET)	Beuher assay	Freund's Complete Adjuvant Test (FCAT)	Optimization test	Split adjuvant	Guinea Pig Maximization Test (GPMT)
Number in test group	20	6–8	10–20	8–20	20	10–20	20–25
Number in control group	20	6–8	10–20	8–20	20	10–20	20–25
Introduction							
Exposure route	Id	Open epicutaneous	Patch	Id	Id	Patch	Id and patch
Number of exposures	10	20–21	3	3	9	4	1 id; 1 topical
Duration of each patch	No patch	Continuous (no patch)	6 h	–	–	48 h each	48 h patch
Concentration	0.2	Nonirritating	Slightly irritating	5–50%	0.1%	0.1–0.2 ml	Maximum tolerated
Test group(s)	TS	TS	TS	TS in FCA	TS in FCA		TS, TS + FCA, FCA
Control group	None	Vehicle only	Vehicle only	FCA only	–		FCA, FCA + V, V
Site for dosing	Left flank	Right flank	Left flank	Shoulder	Back (flank first injection)	Mid-back	Shoulder
Frequency of exposure	Every second day	Daily	Every 5–7 days	Every 4 days	Every other day	Day 0, 2, 4, 7	0 (id); day 7 patch
Duration (days)	1–18	0–20	0–14	0–9	0–2	0–9	0–9
Misc.			9 exposure version			Day 0 dry ice treatment FCA (id) day 4	Irritant does of SLS pretreatment
Rest period (days)	19–34	21–34	15–27	9–21; 22–34	22–34	10–21	9–30
Challenge							
Exposure route	Id	Open	Patch	Id; patch	Id	Patch	Patch
Number of exposures	1	2	1	2	2	1	1
Duration of exposure	–	–	24 h			24 h	24 h
Exposure day(s)	35	21 and 35	28	22; 35	14–28	22	21

FCA Freund's complete adjuvant, *SLS* sodium lauryl sulfate, *TS* test substance, *V* vehicle; *id* intendermal. Reproduced from Maibach and Patrick (2001) with permission

cleared of hair. A volume of 0.10 mL of the test substance in an appropriate vehicle is applied to an 8 cm^2 area of the flank of each treatment group for 20 consecutive days or 5 times a week for 4 weeks. Each treatment group is treated with different concentrations of the test substance (100%, 30%, 10%, 3%, 1%, and 0.3%). The test substance is applied to the same site daily unless a moderate to strong skin reaction occurs, requiring the change of application site. The control group is treated only with vehicle. Days 21 and 35 or 29 and 43—Challenge exposure through open topical application: The contralateral flank of the animals is cleared of hair. Twenty-four hours after the last induction treatment, 0.025 mL of the test substance at the minimal irritant concentration, the maximum nonirritant concentration, and some lower

concentrations is applied to a 2 cm^2 area of all animals. The same procedure is repeated 2 weeks after. Vehicle may also be involved at challenge, if indicated. Days 36, 37, and 38 or 44, 45, and 46—Assessment of sensitization: The test sites are visually evaluated and graded at 24, 48, and 72 h after challenging. This assay provides not only irritation and sensitizing data but also dose-response evaluation.

15.3.1.3 Buehler Test

This test employs an occluded topical patch technique for the induction and challenge exposure. A minimum of 20 animals is required for the treatment group and a minimum of 10 animals for the control group (OECD 1992). Days 0, 7, and 14—Induction exposure through occluded topical application: One flank of the animals is cleared of hair. An occlusive patch (2 × 2 cm) or chamber loaded with the test substance in a suitable vehicle is placed on the test area and is held in contact to the skin for 6 h. This procedure is repeated on day 7 and 14 on the same test area. The concentration of the test substance for each induction exposure should be the highest to cause mild irritation. The control group is treated only with vehicle. Day 28—Challenge exposure through occluded topical application: The contralateral flank of the animals is cleared of hair. An occlusive patch (2 × 2 cm) or chamber loaded with the test substance in a suitable vehicle is placed on the contralateral flank of both groups and is held in contact to the skin for 6 h. An occlusive patch or chamber with only the vehicle may also be applied when relevant. The concentration of the test substance should be the highest nonirritating dose. During the induction and challenge exposure, the patch is held in place by keeping the animals in a special restrainer, which prevents their movement and enables attachment of the occlusive patch with a rubber dam, slightly pulled and fastened to the restrainer. Days 29 and 30—Assessment of sensitization: 24 and 48 h after removal of the patch, the reactions are visually evaluated and graded. Widely utilized for a generation, this method as with the other assays requires meticulous attention to the original protocol.

15.3.1.4 Freund's Complete Adjuvant Test (FCAT)

This test employs intradermal injections (with adjuvant) for the induction exposure and open topical applications for the challenge exposure. A minimum of 8–10 animals is required for the treatment group and also for the control group (OECD 1981). Day 0, 4 and 8—Induction exposure through intradermal injections: A 6 × 2-cm area across the shoulders of the animals is cleared of hair. An intradermal injection (0.1 mL) of 1:1 FCA and the dissolved test substance at a concentration of 5% are given on days 0, 4, and 8. The control group is injected only with FCA. Days 21 and 35—Challenge exposure through open topical application: One flank of the animals is cleared of hair. A volume of 0.025 mL at up to six concentrations (including the minimal irritant concentration and the maximum nonirritant concentration and lower concentrations) is applied to a 2-cm^2 area of the flank. The same procedure is repeated 2 weeks later. Days 36, 37, and 38—Assessment of the sensitization: The test site is visually evaluated and graded 24, 48, and 72 h after challenging.

15.3.1.5 Optimization Test

This test employs intradermal injections (with and without adjuvant) for the induction exposure and both intradermal and occlusive topical application for the challenge exposure. A minimum of 10 animals is required for the treatment group and also for the control group (OECD 1981). Day 0 to 21—Induction exposure through intradermal injections: One flank, back, and shoulder regions of the animals is cleared of hair. One intradermal injection (0.1 mL) at 0.1% of test substance in 0.9% saline is given three times a week into the back area for 1 week. On the day of the first injection, an intradermal injection is also given to one flank. During the second and third weeks, intradermal injections (0.1 mL) at 0.1% of test substance in 1:1, FCA/saline, are given three times a week to the area across the shoulders (total of ten intradermal injections). Controls are injected only with saline during week 1 and FCA/saline during weeks 2 and 3. Day 35—Challenge exposure through intradermal application: The contralateral flank of the animals is cleared of hair. Two weeks after the last induction application, all the animals are given an intradermal injection (0.1 mL) at 0.1% of test substance in 0.9% saline in the contralateral flank. Day 45—Challenge exposure through occlusive topical application: The back region of the animals is cleared of hair. After a rest period of 10 days, an occlusive patch loaded with the maximum nonirritant concentration of the test substance is placed on the back area and is held in contact to the skin for

24 h. Assessment of the sensitization after intradermal and topical challenges is done 24 h after the intradermal injections during the first induction week as well as 24 h after the intradermal challenge injection, skin reactions are measured by multiplying the two greatest perpendicular diameters and the skinfold thickness (with a skinfold caliper) to obtain a "reaction volume" for each animal. If the reaction volume of the challenge injection is greater than the reaction volume of the first induction week, the animal is considered sensitized (positive). For final evaluation, the number of "positive" animals in the test group is statistically compared with the number of animals in the control group that showed nonspecific reactions of comparable intensity. After 24, 48, and 72 h of the removal of the occlusive patch, the skin reactions are evaluated according to a standard rating scale. The significance of differences in the number of positive animals between the treated group and the controls are again assessed.

15.3.1.6 The Split Adjuvant Test

This test employs pretreatment of induction site, occlusive topical application combined with intradermal injection (with adjuvant) for the induction exposure, and occlusive topical application for the challenge exposure. A minimum of 10–20 animals is required for the treatment group and also for the control group (OECD 1981). Days 0 to 9—Induction exposure through occlusive topical application and intradermal injection: The skin of the shoulder region is shaved to the glistening layer and then treated with dry ice for 5–10 s. A dressing with a 2-cm^2 opening window is fixed over the test area. On day 0, 0.2 mL semisolid or 0.1 mL liquid test substance is spread over the test area and covered with filter paper, fixed with adhesive tape, and kept under occlusion for 48 h. On day 2, the filter paper is lifted from the test site, the test material (0.2 mL semisolid or 0.1 mL liquid) is reapplied, and the filter paper covering is replaced. On day 4, two intradermal injections (0.1 mL) of FCA are given symmetrically into the induction area, followed by application of the test material (0.2 mL semisolid or 0.1 mL liquid). On day 7, the procedure of day 2 is repeated. On day 9, the dressing is removed. Day 21—Challenge exposure through occlusive topical application: An area of 2 cm^2 of the dorsal back region of animals is cleared of hair. A volume of 0.5 mL semisolid or 0.1 mL liquid test substance, in a concentration half of the induction, is spread over the test site and covered with filter paper, fixed with adhesive tape, and held in contact to the skin for 24 h. Days 22, 23, and 24—Assessment of the sensitization: The test site is visually evaluated and graded 24, 48, and 72 h after challenging.

15.3.1.7 Guinea Pig Maximization Test (GPMT)

The guinea pig maximization test employs intradermal injections (with adjuvant) combined with occlusive topical application for the induction exposure and occlusive topical application for the challenge exposure. A minimum of ten animals is required for the treatment group and at least five animals for the control group (OECD 1992). Day 0—Induction exposure through intradermal injections: The shoulder region of the animals is cleared of hair. One pair of intradermal injection (0.1 mL) of a mixture of 1:1 FCA/water of the test substance at the highest concentration to cause mild-moderate irritation in an appropriate vehicle and of the test substance at the highest concentration to cause mild-moderate irritation formulated in 1:1 FCA/water is given in the shoulder region. One of each pair lies on each side of the midline. Control animals are injected only with FCA/water, undiluted vehicle, and vehicle/FCA/water. Day 7—Induction exposure through occluded topical application: The shoulder region is again cleared of hair. A filter paper (2×4 cm) loaded with the test substance in a suitable vehicle at the highest concentration to cause mild-moderate irritation is placed on the injection site and is then covered with approximately 4×8-cm occlusive surgical tape and secured in place with an elastic bandage wrapped around the animal. It is held in contact to the test area for 48 h. If the test substance is nonirritating, the test site is pretreated with 10% sodium lauryl sulfate in petrolatum 24 h before the topical induction application (day 6) to provoke an irritant reaction. Control animals are patched only with the vehicle. Day 21—Challenge exposure through occluded topical application: The flank region of the animals is cleared of hair. A patch loaded with the test substance at the highest nonirritating concentration is applied to one flank, and a patch with the vehicle may also be applied to the other flank when relevant. It is held in contact to the test area for 24 h. Days 23 and 24—Assessment of sensitization: 24 and 48 h after removal of the patch, the reactions are visually evaluated and graded. This assay mandates extensive operator background knowledge of its complexity and training.

EVALUATION

The first guinea pig sensitization method was developed by Draize in 1944. However, the sensitivity of the method to detect known human sensitizers such as benzocaine, mercaptobenzothiazole, neomycin, and nickel sulfate was very low. The sensitivity of the further developed methods was increased by enhancing the immune system through the addition of Freund's complete adjuvant during the induction period. Methods with topical application were also developed to simulate the exposure situation in humans.

In the original OECD guideline of 1981, seven tests were considered to be acceptable (the Draize test, open epicutaneous test, Buehler test, Freund's complete adjuvant test, optimization test, split adjuvant test, and the guinea pig maximization test). In the updated version of 1992, the guinea pig maximization test (GPMT) and the Buehler test were given preference over the other methods, whose use was left to special occasions. The GPMT is a highly sensitive adjuvant method for hazard allergenicity screening of chemicals. It may overestimate the sensitization hazard of many chemicals, but on the other hand, a negative GPMT gives a large safety margin. The Buehler test was designed to detect moderate to strong sensitizers prior to testing in man. It is less sensitive than the GPMT and may underestimate the sensitization potential of chemicals.

The advantage of the guinea pig tests is the inclusion of induction and elicitation phases (challenge exposure) in the assay. Also, the long tradition for using the guinea pig as an animal model has given extensive experience with this laboratory animal, and the performance of the various test methods is documented. Marzulli and Maguire Jr. evaluated several guinea pig test methods by analyzing the frequency with which their results agreed with those obtained with the human Draize test, using ten chemicals. Three rounds of each test were carried out on each of the ten chemicals (i.e., a total of 30 evaluations/tests). An agreement between guinea pig and human data was observed in 14 cases with the Draize test, in 10 cases with the Buehler test, in 29 cases with the guinea pig maximization test, and in 22 cases with the split adjuvant test. The adjuvant techniques (GPMT and split adjuvant test) showed significantly greater agreement with human predictive findings than the nonadjuvant techniques (Draize and Buehler). Comparing the adjuvant techniques one with another, the GPMT test was superior. False-negative results showed the highest frequency with the Draize and Buehler methods and the lowest frequency with the GPMT.

CRITICAL ASSESSMENT OF THE METHOD

Many factors may influence the outcome of guinea pig assays, leading to considerable interlaboratory variation. Interpretation of test results may vary widely, as the visual evaluation of skin reactions may be subjective, and in case of testing colored materials, the reaction evaluation may be impaired. Intradermal injections have to be given carefully, as deep injections (subcutaneous or intramuscular) can lead to a lower sensitivity of the guinea pig tests. Other disadvantage of guinea pig assays is that, with the exception of the open epicutaneous test (OET), dose-response relationships are not included in the standard protocols. Dose-response relationship assessment is important because the induction and the elicitation of allergic reactions is concentration dependent, and there is a threshold dose below which no significant effects are seen. Moreover, guinea pig assays are usually time consuming. The time required for a full test varies from 3 to 6 weeks.

MODIFICATIONS OF THE METHOD

The Draize test has undergone modifications to enhance the sensitivity of this animal assay. In the modified (Sharpe 1985), the concentration of the test substance was increased both in the intradermal induction exposure (2.5 times the intradermal challenge concentration) and in the challenge exposure (maximum nonirritant concentration). An open topical application was included on the challenge exposure with the test substance at maximum nonirritant concentration. The guinea pig maximization test was modified to study materials unsuitable for testing by intradermal injection. In the modified guinea pig maximization test (1985), the FCA was injected intradermally, but an occluded topical application instead of intradermal injection of the test substance was performed in the induction exposure. Andersen et al. (1995) refined the GPMT by applying a multiple dose design at the induction exposure in order to provide information of the relative sensitizing potency of six chemicals. The scientist motivated to seek detail will find this in Andersen et al. (1995) and Marzulli and Maibach's Dermatotoxicology (Klecak 2008).

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15.4 Sensitization Tests in Mice

15.4.1 Local Lymph Node Assay (LLNA)

PURPOSE AND RATIONALE

The local lymph node assay (LLNA) is based on the knowledge that the induction phase of skin sensitization is associated with, and dependent upon, proliferation of lymphocytes in the lymph nodes draining the site of test substance application.

PROCEDURE

Healthy female mice of CBA/Ca or CBA/J strain (nulliparous and nonpregnant), 8–12 weeks of age are selected. Other strains and males may be used when sufficient data can demonstrate that strain and/or gender-specific differences in the LLNA response do not exist. Animals are maintained in the facilities with temperature of 22 ± 3°C, relative humidity of 30–70%, and 12-h automatic light cycle. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

At least three concentrations of the test substance are evaluated in separate groups with a minimum of four animals each. A control group is treated only with the vehicle (VC). Positive controls (PC) are used to assess the reliability of the assay by performing it with sensitizing test substances for which the response is well known, such as 25% hexyl cinnamic aldehyde in acetone, olive oil (4:1), or 5% mercaptobenzothiazole in *N,N*-dimethylformamide.

Solid test substances should be dissolved or suspended in an appropriate vehicle and diluted if appropriate. Liquid test substances may be applied neat or diluted. Recommended vehicles are 4:1 acetone/olive oil, methyl ethyl ketone, *N*,*N*-dimethylformamide, propylene glycol, and dimethyl sulfoxide.

Three consecutive concentrations from the series 100%, 50%, 25%, 10%, 5%, 2.5%, 1.0%, 0.5%, etc. are tested. The highest concentration from the three to be tested should be the maximum that does not induce systemic toxicity and/or excessive local skin irritation. In the absence of this information, an initial pretest is necessary.

Twenty-five microliters of the appropriate dilution of the test substance is applied to the dorsal surface of each ear for 3 consecutive days. The control group is treated only with the vehicle (VC). Five days after the first exposure, 250 μL of sterile phosphate-buffered saline (PBS) containing 20 μCi (7.4 × 105 Bq) of tritiated (3H)-methyl thymidine is injected into all test and control animals via the tail vein. Five hours after injection, animals are humanely killed. Draining auricular lymph nodes from each mouse are excised and placed in PBS individually (individual animal approach) or placed in PBS pooled with lymph nodes from other animals of the same group (pooled treatment group approach).

A single-cell suspension of lymph node cells (LNC) is prepared by gently passing the nodes through 200-μm-mesh stainless steel gauze. The LNC are washed twice with an excess of PBS, and the DNA is precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18 h. Pellets are resuspended in 1 mL TCA and transferred to scintillation vials containing 10 mL of scintillation fluid for 3H counting.

Lymphocyte proliferation is measured by estimation of DNA synthesis, which involves the incorporation of the labeled nucleoside [tritiated thymidine ([3H]dT)] into genomic DNA. Incorporation of 3H-methyl thymidine is measured by a β-scintillation counter as disintegrations per minute (DPM). Depending on the approach used, the incorporation is expressed as DPM/mouse (individual animal approach) or DPM/treatment group (pooled treatment group approach).

The ratio of tritiated (3H)-methyl thymidine incorporation into each treated group and into the vehicle treated control group (VC), termed the stimulation index (SI), is calculated. When using the individual animal approach, the SI is derived by dividing the mean DPM/mouse within each test substance group by the mean DPM/mouse for the VC group. When using the pooled treatment group approach, the SI is obtained by dividing the DPM/treatment group within each test substance group by DPM/VC group. The material is considered a sensitizer when SI $\geq$ 3 (OECD 2010a).

The relative potency (defined as a function of the amount of chemical required for skin sensitization) of contact allergens can be measured by deriving from dose responses in the LLNA an EC3 value, which is the concentration of the chemical required to provoke a threefold increase in the proliferation of LNC compared with controls (i.e., an SI of 3). Additional details are found in the ICCVAM website.

EVALUATION

LLNA offers advantages in the lower number of animals used, lower cost, an objective and quantitative end-point measurement, and less time required for conducting the assay in comparison to various guinea pig assays. Moreover, the LLNA provides advantages with regard to animal welfare. The nonrequirement of a challenge exposure and the nonuse of adjuvant reduce animal pain and distress. The inclusion of several concentrations tested during the induction exposure provides opportunity to combine hazard identification with quantitative estimation of relative sensitizing potency.

Warbrick et al. (1999) examined the stability and consistency of LLNA responses induced by the contact allergen paraphenylenediamine (PPD). Analyses were conducted once a month over a 4-month period in each of two independent laboratories. In all assays and in both laboratories, PPD elicited a positive response, with minor differences, confirming the stability of LLNA responses both with time and between laboratories.

The LLNA EC3 values (the concentration of chemical necessary to obtain a threefold stimulation of proliferative activity in draining lymph nodes) have been reported to correlate well with the sensitization potencies of human contact allergens. Gerberick et al. (2001) assigned five potency rankings [(strong, moderate, weak, extremely weak, and nonsensitizing) for 21 chemicals based on quantitative data from human repeat patch test studies reported in the literature [human maximization test (HMT) and the human

repeat insult patch test (HRIPT)] together with their clinical experience and compared these with the potency rankings derived from LLNA EC3 values. They showed that the potency of 12 out of the 15 chemical allergens was similarly classified. For the nonsensitizers, each of the six chemicals evaluated and classified as nonsensitizers for humans were classified identically in the LLNA.

The local lymph node assay (LLNA) is currently recognized as a stand-alone test method for the purposes of hazard identification (i.e., a method that can be used not only for the identification of skin sensitization hazard but also for confirmation of the absence of such hazard), and it has now been adopted formally by several regulatory agencies in the USA.

CRITICAL ASSESSMENT OF THE METHOD

The main disadvantages of the LLNA are the need for radioactive material and the restriction of the evaluation to the induction phase. As shown in previous publications, false-positive results to certain nonsensitizing skin irritants [e.g., sodium lauryl sulfate (SLS)] and false-negative results to metal allergens (in particular nickel) may be obtained.

MODIFICATIONS OF THE METHOD

15.4.1.1 The Reduced LLNA (rLLNA)

The protocol for the rLLNA is identical to that of the traditional LLNA, with the exception that instead of testing three concentrations of each test substance, only the highest concentration that does not induce systemic toxicity and/or excessive skin irritation is tested. This way, it is possible to reduce the number of animals used to perform the test in up to 40%. The rLLNA is recommended to distinguish between skin sensitizers and nonsensitizers in cases that do not require dose-response information (ICCVAM 2009).

15.4.1.2 Nonradioactive LLNA Methods

The LLNA: DA is a similar method to the LLNA that measures the adenosine triphosphate (ATP) content as an indicator of lymphocyte proliferation, instead of the incorporation of the labeled nucleoside [tritiated thymidine ([3H]dT)] into genomic DNA. The ATP content in the lymph nodes (known to correlate with living cell number) is measured by the bioluminescence method, which utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin. The emitted light intensity, which is related to the ATP concentration, is given in relative luminescence units (RLU). The SI is derived by dividing the mean RLU/mouse within each test substance group by the mean RLU/mouse for the vehicle-treated control group (VC).The material is considered a sensitizer when SI $\geq$ 1.8 (OECD 2010).

The LLNA: BrdU-ELISA (enzyme-linked immunosorbent Assay) is also an alternative method similar to the LLNA that measures the incorporation of 5-bromo-2-deoxyuridine (BrdU) into genomic DNA as an indicator of lymphocyte proliferation. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. Its incorporation is measured by ELISA (utilizes anti-BrdU antibody labeled with peroxidase, which reacts with BrdU to produce a colored product, whose absorbance at 370 nm is determined with a microplate reader with a reference wavelength of 492), and the result is given as BrdU labeling index. The SI is derived by dividing the mean BrdU labeling index/mouse within each test substance group by the mean BrdU labeling index/mouse for the vehicle-treated control group (VC). The material is considered a sensitizer when SI $\geq$ 1.6 (OECD 2010). Taken together, the LLNA is a precise assay for hazard identification. Its use in risk assessment requires many strategies and interpretation—to minimize withdrawing many chemicals from potential use by man because of the many compounds that are so identified as allergens, but with appropriate concentrations of use may be well tolerated in man.

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15.4.2 Mouse Ear Swelling Test (MEST)

PURPOSE AND RATIONALE

The basic principle of the mouse ear swelling test (MEST) is the assessment of skin sensitization by quantitatively and objectively measuring the increase in mouse ear thickness with a micrometer following the challenge exposure with potential sensitizers.

PROCEDURE

Healthy, 6–8-week-old female CF-1 or Balb/c mice are fed with a diet supplemented with vitamin A acetate at 250 IU/g of feed for 2 weeks prior the beginning of the assay. A minimum of ten animals is required for the test and control group and at least eight for the pretest group. A pretest is performed in order to establish the minimal irritating concentration to the abdomen and the highest nonirritating concentration to the ear of the animals. Two mice are used to test each concentration of the test substance, and this way, at least four concentrations are evaluated. Acetone, methyl ethyl ketone, and 70%, 80%, and 95% ethanol in water have been shown to be acceptable vehicles based on solubility and chemical compatibility with the test substance. Days 0, 1, 3, and 5—Induction exposure: The abdomen of the animals is clipped free of hair. Next, two intradermal injections of FCA (20 μL) are given at separate sites along the borders of the test area that is then tape-stripped until the skin appears glossy. A volume of 100 μL of the test substance at the minimal irritating concentration is topically applied to the test area of the test group. Control group receives 100 μL of the vehicle. On days 1, 3, and 5, the tape stripping and application of test material (test group) and vehicle (control group) are repeated. Day 10—Challenge exposure: 20 μL of the test material at the highest nonirritating concentration is applied to the ventral and dorsal surfaces of the left ear of all test group animals and to five control animals. A volume of 20 μL of 100% vehicle is applied to the ventral and dorsal surfaces of the right ear. Days 11 and 12—Assessment of skin sensitization: Ear thickness is measured 24 and 48 h after challenging using an Oditest spring-loaded caliper. Positive results are defined as animals whose test ear is at least 20% thicker than the control ear. The percentage of respondents in the test group is then calculated. The degree of ear swelling of the test group should also be calculated by dividing the thickness of the ear to which the test material was applied by the thickness of the vehicle-treated control ear. One or more positive responses (20% or greater swelling compared to the control ear) in a group of 10 animals indicate that the test substance is a sensitizer. A negative response indicates that the substance is not a moderate or strong sensitizer. Day 17—Rechallenge: It can be performed using the five remaining naïve control group, if it is desired to clarify ambiguous findings. A positive control is recommended and should show 0.05% dinitrochlorobenzene (DNCB) in 70% ethanol to be a strong sensitizer. The procedure presented here was based on the description of Gad (1994), modified from Gad et al. (1985, 1986).

EVALUATION

The mouse ear swelling test (MEST) was developed to overcome the disadvantages of the existing guinea pig based tests. It provides a lower cost, shorter length of test, and an objectively graded end point (making unnecessary the subjectively grading skin erythema). The possibility of rechallenging is an advantage from the other murine test, the LLNA.

Gad et al. (1986) published a large study, in which a MEST was employed to evaluate a battery of 72 test substances [for which the sensitizing potential in the guinea pig maximization test (GMPT) and/or in humans was already known].These included 49 known positives and 23 known negatives. Only one known positive sensitizer was not properly identified, giving a false-negative rate of 1/49 or 2%. All 23 nonsensitizers, including contact irritants such as benzoic acid and sodium lauryl sulfate (SLS), were correctly identified.

CRITICAL ASSESSMENT OF THE METHOD

The sensitivity of the MEST has been questioned. Dunn et al. (1990) conducted an investigation at two independent laboratories for the purpose of evaluating

the ability of the mouse ear swelling test (MEST) developed and validated by Gad et al. (1986) to detect contact sensitization induced by 26 chemicals. They found a high incidence of false negatives for either weak (HS, PABA, Sudan III, methyl methacrylate, croton oil) or moderate (benzoyl peroxide, nickel sulfate, ethylenediamine, monothioglycerol) human contact sensitizers. Contrary to results reported by Gad et al., their findings suggested that MEST is a useful model for identifying strong contact sensitizers but is not reliable for detecting weak and moderate ones.

Although the MEST is quantitative, it has a degree of variability based on individual interpretation of caliper measurements. The type of micrometer used may also affect interpretation of the test results. When van Loveren et al. (1984) compared the spring-loaded caliper, the screw with friction thimble micrometer, and the sliding caliper, the spring-loaded instrument, which applies the least pressure to the ears, was considered to give the most accurate measurements of the thickness of the ears at different times after challenge.

MODIFICATIONS OF THE METHOD

Sailstad et al. (1993) evaluated four protocols with variations of the mouse ear swelling test (MEST) using the strong sensitizer 2,4-dinitrofluorobenzene (DNFB) and three weaker sensitizers, glutaraldehyde, formalin, and an *azo* dye (Solvent Red 1 [SRl]). For one of the groups, the protocol required all animals to be fed a supplemented diet with 0.477 g/kg of vitamin A acetate (approximately 15 times the amount found in standard chow) for 4 weeks prior to sensitization. All four protocols of the MEST detected the strong sensitizer (DNFB); however, only the vitamin A–supplemented protocol detected the weaker sensitizers. They concluded that feeding the animals with a vitamin A–supplemented diet proved to be the best procedure to enhance the test sensitivity for weak sensitizers. Other previous studies (Miller et al. 1984; Maisey and Miller 1986; Thorne et al. 1991) demonstrated similar effects of vitamin A–supplemented diet. It is believed that a vitamin A–supplemented diet has an adjuvant effect that enhances cell-mediated immune responsiveness. With this knowledge, the MEST protocol was optimized. On the original MEST design of Gad et al. (1986), mice were fed with conventional diet. However, on the current version of the protocol (Gad 1994), mice are fed with a diet supplemented with 250 IU/g of vitamin A acetate, starting 2 weeks before the induction exposure.

Studies have tried to turn the assessment of sensitization response on the MEST more objective by measuring cellular proliferation on the mouse ear instead of measuring skin thickness. Cornacoff et al. (1988) compared the sensitization response on the mouse ear by measurement of the influx of inflammatory cells which have been prelabeled (125[I]-iododeoxyuridine) with the classical measurement of increase in tissue thickness. The study demonstrated that both the radioisotopic method and the classical method detected significant reactivity to the strong sensitizer oxazolone and DNFB. Both methods were capable of detecting reactivity to only one of the three weak sensitizers tested. The authors concluded that the radioisotopic approach was comparable to the classical approach and also more sensitive and less subjective. Lee et al. (2003) attempted to assess sensitization response on the mouse ear using a nonradioisotopic end point (BrdU incorporation into DNA as an alternative to labeling proliferating cells with radioisotopes). However, the method failed to find a significant difference in ear response between the mice treated with allergens and those treated with irritant. This, like the LLNA, is a useful screening assay and, like the LLNA, requires many other steps for risk assessment.

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15.5 Human Sensitization Assays

PURPOSE AND RATIONALE

Provided that the risk of inducing contact sensitization in volunteers is judged to be minimal (based on assessment of preclinical sensitization data), human sensitization assays may be conducted for premarketing safety assessment of new cosmetics or household products to confirm that humans will not respond adversely to chemicals contained therein. Sensitization assays should be conducted in accordance with accepted codes of ethics for the conduct of human testing [e.g., The Declaration of Helsinki, Good Clinical Practice (GCP) guidelines] and conform to any applicable national regulatory requirements and legislation. Volunteers should be informed of the purposes and the potential risks of the test and sign an informed consent.

PROCEDURE

There are several human sensitization tests available. They vary with regard to the number of induction patch tests, the placing of the patches, the grading scale, the scheme of induction exposure (continuous or with rest intervals), and the use of a maximization step. Basically, each test is divided into three consecutive phases: An initial or induction phase, during which repeated patches insult the skin; a period of rest, during which an immune response may develop; and a final challenge or elicitation phase to determine whether sensitization has occurred. Subjects are randomly selected, and each protocol has a standard list of inclusion or exclusion criteria. Volunteers with clinically active dermatitis, immunological disease, routine use of anti-inflammatory drugs, immunosuppressive drug therapy, or with a known preexisting sensitization to the chemical being tested are usually excluded. The concentration at which the chemical is evaluated is determined by integrating prior sensitization test results in animals, the desire to exaggerate use concentrations, and prior experience. There are three basic predictive human sensitization tests in current use: (1) the human repeat insult patch test (HRIPT), (2) the modified Draize human sensitization test, and (3) the maximization test. Principal features of human sensitization assays are summarized in Table 15.2.

15.5.1 Human Repeat Insult Patch Test (HRIPT)

The three most used HRIPTs include (1) the Draize human sensitization test (1955, 1959), (2) the Shelanski/Shelanski test (1953), and (3) the Voss/Griffith test (1969, 1976).

In the Draize human sensitization test, an occlusive patch containing the test substance is applied to the upper arms or back of the volunteers and is held in contact with the skin for 24 h. The patch is then removed, and the skin is allowed to rest for 24 h. Then, a second patch test is applied to a new skin site. This process is repeated every other day three times a week until 10 induction patches are applied. Each induction site is evaluated for erythema and edema after removal of the patch. Ten to 14 days after application of the last induction patch, a challenge patch is applied to a new site for 24 h and subsequently evaluated for erythema and edema. The response after challenge is compared to the responses reported after the early induction patches and the incidence of sensitization are reported.

In the Shelanski/Shelanski test, an occlusive patch is applied on the upper arm for 24 h in a similar fashion (24-h contact and 24-h rest) to the Draize RIPT described above. However, the patch is placed on the same test site each time, and a total of 15 induction patches are applied. The test site is evaluated before application of the new patch, and if inflammation has developed, the patch is placed on an adjacent uninflamed site. The induction site is evaluated for erythema and edema after removal of the patch. Two to 3 weeks after application of the last induction patch, a challenge patch is applied on a new site of the upper arm for 48 h and subsequently evaluated for erythema and edema. The response after

Table 15.2 Principal features of human sensitization assays

Feature	Complete	Shelanski/ Shalanski	RIPT Draize	Griffith–Voss–Stotts	Modified Draize	Human maximization
Number of subjects	200	200	200	200	100–200	25
Introduction						
Exposure site	Upper arm	Upper arm, same site	Upper arm or back; naïve site for each exposure	Upper arm same site	Upper arm or back, same site	Upper arm or lower back same site
Number of exposures	1	15	10	9	10	5
Duration of exposures	24–72 h	24 h	24 h	24 h	48–72 h	48 h
Frequency of exposures	–	3 per week	3 per week	3 per week	3 per week	24 h between patches
Evaluation schedule	At removal, 24, 48 h	At removal	At removal	48–72 h	30 min are removal	Before each application
Miscellaneous	4-week usage period	Fatiguing index		Pilot group	Continuous exposure	SLS/irritation as adjuvant
Rest period duration		14–21 days	10–14 days	14 days	14 days	14 days
Challenge						
Exposure site	Upper arm	Upper arm	Upper arm	Upper arm	Upper arm or back	Lower back, upper arm
Duration of exposure	24–74 h	48 h	48 h	24 h	72 h	SLS 1 h; 48 h
Evaluation of schedule	AT removal, 24, 48 h	At removal	At removal	48 and 96 h	At removal, 24 h	At removal, 24, 48 h
Miscellaneous			Naïve test site	Original and naïve sites	Naïve test site; may use two 48-h exposures	At removal, 24, 48 h sensitization index

RIPT Repeat Insult Patch Tests, *FCA* Freund's complete adjuvant, *SLS* lauryl sulfate, *TS* test substance, *V* vehicle, *id* interdermal
Reproduced from Maibach and Patrick (2001) with permission

challenge is compared to the responses reported after the early induction patches and the incidence of sensitization is reported.

In the Voss/Griffith test, quadruple occlusive patches are applied to the same site of the upper arm for 24 h in a similar fashion (24-h contact and 24-h rest) to the Draize RIPT described above. This process is repeated on 3 consecutive weeks for a total of nine induction applications of each material per subject. If primary irritation occurs, the succeeding patch is applied adjacent to the original site. Twenty-four hours after removal of the patches, the induction site is evaluated for erythema and edema. Seventeen days after application of the last induction patch, a challenge patch is applied on the original test sites and on the opposite virgin arm. Patches are removed after 24 h, and the test sites are evaluated for erythema and edema after 48 and 96 h.

Stotts (1980) presented detailed examples of proper interpretation of human repeat insult patch tests. Sensitization is characterized by delayed appearance of response, by challenge reactions stronger than reactions early in the induction phase, and by persistence and/or increased responses through delayed readings. Weak responses in a few subjects when the material has not produced irritation in the panel suggest a weak sensitizer and a sudden increase in intensity of reactions during the second or third week of the test (following the fourth or fifth application) can be indicative of a strong sensitizer with a short induction period. A very strong reaction by one subject following the first or second patch

application suggests a preexisting sensitization. Jordan and King (1977) proposed modifying the challenge procedure to two consecutive 48-h patch periods, and Marzulli and Maibach (2003) proposed that the investigator, rather than the volunteers, remove the patches.

15.5.2 Modified Draize Human Sensitization Test

The HRIPT was modified by Marzulli and Maibach (1974) to provide for continuous induction patch exposure (without rest period between patches). Occlusive patches with the test material (at use concentration as well as at higher concentrations) are applied three times weekly (Monday, Wednesday, and Friday) for 48 or 72 h (weekends) to the same site of the upper lateral portion of the arm until ten patches have been applied. The patches remain in place until approximately 30 min before the application of the new one, and skin reactions are graded (l = erythema, 2 = erythema and induration, 3 = vesiculation, 4 = bulla formation) during this interval. If moderate inflammation has developed, the next patch is moved to an adjacent skin site. This produces a continuous exposure of 504–552 h compared to a total exposure period of 216–240 h for HRIPT of comparable induction applications. Following a rest period of approximately 2 weeks, the challenge patch (at nonirritant concentration) is applied to a new site and allowed to remain for 72 h. Test sites are evaluated at patch removal and after 24 h.

15.5.3 Human Maximization Test

The human maximization test, designed and later modified by Kligman et al. (1966, 1975), employs irritancy as an adjuvant. Test materials are tested during induction at a concentration that produces a moderate erythema within 48 h. If materials are nonirritating, the test site is pretreated with a 24 h patch of 5% sodium lauryl sulfate (SLS). Additional SLS patches may be applied before each patch application until a brisk erythema is achieved. The induction concentrations should be at least five times higher than use levels. Patches are applied to the upper arm or lower back, and up to four different materials can be tested at once. Five sets of patches are applied on the same site for 48 h each, with a 24-h rest period between removal and reapplication. Following a 2-week rest period, a new site is pretreated with SLS patch for 1 h followed by a 48-h patch application with the test substance. Test sites are evaluated at patch removal and then reexamined 24 and 48 h after. The number of subjects developing a positive response is reported, and a sensitization index based on percentage of subjects responding is assigned to the test material. The assay is rarely employed today.

EVALUATION

The advantages of experimental induction of skin sensitization in human volunteers are that exposures are controlled, several doses can be tested, and extrapolation of the test results from one species to another is not needed. The FDA developed a guidance document (1999) for evaluating skin sensitization to chemicals in natural rubber products and selected the modified Draize test as the test of choice.

Because no standardized test guidelines are available for the human sensitization assays, methods may vary from laboratory to laboratory. When reporting test data, it should contain a brief description of the test protocol, the scoring criteria used, and the method used for rating skin responses.

CRITICAL ASSESSMENT OF THE METHOD

Human skin sensitization assays have the disadvantages that large cohorts of volunteers are needed to give a reliable result and a number of them may drop out during the course of the test (usually for reasons unrelated to the test). Studies conducted in human volunteers are subject to stringent ethical considerations, and the investigator is required to obtain approval from an Institutional Review Board (IRB) (USA) or Ethics Committee (Europe). Because the human maximization test may produce strong skin reactions, it may be considered unacceptable nowadays.

Skin responses are evaluated by subjective visual scoring, and consequently, the degree of reliability with which the available signs are read and interpreted is dependent on the experience and judgment of the investigator. The greatest demand is the investigator ability in distinguishing between primary irritation and sensitization. Positive assays require careful

interpretation—to extrapolate from hazard—to risk for a given use situation.

MODIFICATIONS OF THE METHOD

In the interest of reducing animal use, in vitro alternatives for skin sensitization assays are under development.

15.5.4 Peptide Reactivity Assay

One characteristic of a sensitizer chemical is its ability to react with proteins prior to the induction of skin sensitization. Measuring chemical reactivity to peptides has a potential utility for evaluating skin sensitization hazard (Gerberick et al. 2004). In order to determine whether and to what extent reactivity correlates with sensitization potential, Gerberick et al. (2007) evaluated 82 chemicals (sensitizers of different potencies and nonsensitizers) for their ability to react with reduced glutathione (GSH) or with two synthetic peptides containing either a single cysteine or lysine. The test chemical was mixed with the two synthetic peptides and with glutathione, in separate reactions. Following a time allowed for reaction, the samples were analyzed by high-performance liquid chromatography (HPLC) to monitor the depletion of glutathione or of the two peptides. Generally, nonsensitizers and weak sensitizers demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent sensitizers displayed moderate to high peptide reactivity. Classifying minimal reactivity as nonsensitizers and low, moderate, and high reactivity as sensitizers, it was determined that the method gave a prediction accuracy of 89%.

15.5.5 Immune Cell Activation Assays

Based on the knowledge that skin sensitization is dependent upon recognition of the sensitizer chemical in the skin by Langerhans cells (LC), many efforts have been done to exploit LC responses to chemicals in vitro (chemical-induced changes in phenotype or function of these cells) for the identification of sensitization hazard. After skin contact with immunogenic chemicals, LC are activated and upregulated a set of cell surface markers (e.g., CD83 or CD86), secrete various cytokines, such as interleukin (IL)-1b, and downregulate proteins involved in antigen uptake such as aquaporins.

However, since Langerhans cells (LC) constitute only 1–3% of all epidermal cells, human peripheral blood mononuclear cell-derived DC (PBMC-DC), THP-1 cells (human monocytic leukemia cell line), and U-937 (human histiocytic lymphoma cell line) are being used as surrogates of LC in the development of in vitro model systems for predictive skin sensitization tests.

Aeby et al. (2004) reported the use of a human peripheral blood mononuclear cell-derived DC (PBMC-DC) test system to assess skin sensitization potential based on the measurement of cell surface CD86 expression by flow cytometry and interleukin (IL)-1b and aquaporin 3 gene expressions by quantitative real-time polymerase chain reaction (QRT-PCR). In presence of the sensitizer 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), the percentage of the CD86 bright cells increased up to approximately 200% of control at 30 h. On the other hand, the irritant sodium lauryl sulfate (SDS) did not induce relevant modification of the CD86 bright population after 24 or 30 h. A similar effect was observed on the modulation of IL-1b gene expression. Incubation with TNBS for more than 6 h induced relevant increases in IL-1b gene expression and reached 800% of control after 30 h. Exposure to SDS induced 80% of control after 30 h. Analysis of AQP3 gene expression demonstrated a pronounced decrease of AQP3 expression (down to 25% of control at 30 h in the cells exposed to TNBS). SDS did not affect AQP3 gene expression at any of the tested time points.

Uchino et al. (2009) described the development a three-dimensional human skin model composed of dendritic cells, keratinocytes, and fibroblasts (VG-KDF-Skin). This type of culture system develops a fully differentiated epidermis with a stratum corneum, which besides evaluating skin sensitization, allows for the topical application of test materials and provides a barrier system for skin penetration. Sensitizers and nonsensitizers were applied to the surface of the skin model for 1 h. When 2, 4-dinitrochrolobenzen (DNCB), α-hexyl cinnamic aldehyde (HCA), and 2, 4-dinitrofuruolobenzen (DNFB) were applied, the VG-KDF-Skin induced the expression of CD86 and significantly induced IL-1α and IL-4 release. On the other hand, DMSO and isopropanol (nonsensitizers) did not induce the expression of CD86 or cytokine release. Sodium dodecyl sulfate (SDS, an irritant but

a nonsensitizer) did not induce the expression of CD86 or IL-4 release but did significantly induce IL-1α release.

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15.6 Irritation Tests in Animals

15.6.1 Draize-Type Tests

PURPOSE AND RATIONALE

Draize et al. (1944) described a method to evaluate the potential for skin irritation (reversible local inflammatory changes without the involvement of an immunological mechanism) and corrosivity (irreversible tissue damage) that might result from intended or accidental exposures to chemicals and finished products. It has been modified to varying extents by regulatory authorities over time; however, all Draize-type tests evaluate skin primary irritation based on an occluded *single application* of the test material on albino rabbits.

PROCEDURE

According to Draize et al. (1944), the backs of six albino rabbits are clipped free of hair. Four areas of the back of each animal, placed approximately 10 cm apart, are designated for the position of the patches. Two of them are abraded by making four epidermal incisions (two perpendicular to two others in the area

of the patch). The compound is applied so that there are three applications to intact skin and three to abraded skin. This way, four compounds can be tested per series of six animals. The material (0.5 mL in the case of liquids or 0.5 g in the case of solids) to be tested is introduced under the patches, which consist of two layers of light gauze cut in squares (2.5 cm on the side). These are secured to the area by thin bands of adhesive tape. The entire trunk of the animal is then wrapped with rubberized cloth or other occlusive impervious material to retard evaporation of the compound and hold the patches in position. The wrappings are removed 24 h after application, and the test sites are evaluated according to the Draize scoring system for erythema (very slight erythema = 1, well-defined erythema = 2, moderate to severe erythema = 3, and severe erythema to slight eschar formation = 4) and edema (very slight edema = 1, slight edema = 2, moderate edema = 3, and severe edema = 4). This way, the total possible score for primary irritation is 8. Test sites are evaluated again 72 h after application using the same scale, and the final score is the average of the 24-h and the 72-h readings. Evaluations of abraded and intact sites are recorded separately. The combined averages of the 24-h and 72-h scores for the intact and for the abraded skin are referred as "the primary irritation index." Compounds producing primary irritation index of 2 or less are considered mildly irritating, whereas those with index from 2 to 5 are moderate irritants and those above 6 are considered severe irritants.

EVALUATION

The Draize test provides tremendous value in warning consumers, workers, and manufacturers of potential dangers associated with specific chemicals so that appropriate precautions can be taken. It can be done prior to human testing to offer guidance and reduce the risks of testing on man. Its predictive validity for the human response was assessed by Phillips et al. (1972). They compared the relative irritancy of 12 chemicals in man and rabbit utilizing a variation of the standard Draize rabbit irritancy test. The Draize rabbit test accurately predicted the severe human skin irritants and nonirritants but failed to separate the mild and moderate skin irritants. Several chemicals considered unsafe by the rabbit test proved nonirritating to human skin. Rabbit skin generally tends to be more easily irritated than human skin under similar conditions. An inherent difference in the permeability of rabbit and human skin probably accounts for a major portion of the increased irritation observed in the rabbit testing. However, there are a few examples of instances in which irritancy testing on animals has failed to indicate human irritancy (Nixon et al. 1975). Anatomical, physiological, and biochemical differences between the rabbit and humans may have prevented direct prediction of the expected irritancy in man from data on experimental animals.

CRITICAL ASSESSMENT OF THE METHOD

All Draize-type tests are used to evaluate corrosion as well as irritation; however, vesiculation, ulceration, and severe eschar formations are not included in the Draize scoring scales. When chemicals that are severe dessicants or severe escharotics are tested, dilute solutions in a bland nonirritating solvent should be done. Sufficient dilution needs to be made so that the responses elicited may be graded by the scale.

The subjectiveness of the scoring system and difficulties in the interpretation of the results contribute to intra- and interlaboratory variability, making comparison between different laboratories difficult. Animal welfare is another point of criticism. Animal welfare groups have long targeted the Draize test procedures as inhumane and unnecessary.

MODIFICATIONS OF THE METHOD

Modifications of the Draize test are currently required by regulatory authorities worldwide. The modifications relate to the number of animals tested, the duration of test material exposure, the application on intact versus abraded skin, and the number and frequency of skin evaluations. The Federal Hazardous Substance Act (FHSA), Department of Transportation (DOT), Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and OECD guidelines are contrasted to the original Draize methods in Table 15.3.

The Organization for Economic Co-operation and Development (OECD 2002) guidelines and the United States Environmental Protection Agency acute dermal irritation guidelines (EPA 1998) give special attention to animal welfare improvements and to the evaluation of all existing information on the test substance prior to animal testing. It is important to avoid the unnecessary use of animals and to minimize any testing that

Table 15.3 Comparison of skin irritation test based on the Draize method

Feature	Draize	FHSA	DOT	FIFRA	OECD[a]
Number of animals	3[b]	6	6	6	3
Abrasion	Abraded and intact	Abraded and intact	Intact	2 abraded and 2 intact	Intact
Dose liquids	0.5 ml undiluted	0.5 undiluted	0.5 ml	0.5 ml undiluted	0.5 ml
Dose solids	0.5 g	0.5 g in solvent	0.5 g	0.5 g in moistened	0.5 g in moistened
Wrapping materials	Gauze and rubberized cloth	Impervious material			Semiocclusive
Length of exposure	24 h	24 h	4 h	4 h	4 h
Evaluated at[c]	24, 72 h	24, 72 h	4, 48 h	0.5, 1, 24, 48, 72 h	05, 1, 24, 48, 72 h
Treatment at removal	Not specified	Not specified	Skin washed	Skin wiped, not washed	Skin washed
Excluded from testing				Materials pH <2 or >11.5	Materials pH <2 or >11.5

[a]Although other species are acceptable, the albino rabbit is the preferred species
[b]Draize tested four materials on six rabbits. Three abraded and three intact sites with each material
[c]Times listed are after removals for FIFRA and OECD. Time listed for Draize, FHSA, and DOT are after application of the test material
DOT Department of Transportation, *FHSA* Federal Hazardous Substance Act, *FIFRA* Federal Insecticide, Fungicide, and Rodenticide Act, *OECD* Organization for Economic Cooperation and Development
Reproduced from Maibach and Patrick (2001) with permission

is likely to produce severe responses in animals. It is recommended that prior to undertaking the described in vivo test for corrosion/irritation of the substance, a weight-of-the-evidence analysis be performed on the existing relevant data: human and animal data; results of testing of structurally related substances, physicochemical properties, and chemical reactivity (substances exhibiting pH extremes such as 2.0 and 11.5 may have strong local effects); and results from in vitro or ex vivo tests. Substances that have demonstrated corrosive or severe irritant properties need not be tested in animals. It can be presumed that such substances will produce similar severe effects in vivo. When determination of corrosivity or irritation cannot be made using a weight-of-the-evidence analysis, an in vivo sequential testing strategy is recommended. It begins with an initial test using one animal and up to three test patches are applied sequentially. The first patch is removed after 3 min. If no serious skin reaction is observed, a second patch is applied at a different site and removed after 1 h. If the observations indicate that exposure can humanely be allowed to extend to 4 h, a third patch is applied and removed after 4 h, and the response is graded. If the results of this test indicate the substance to be corrosive to the skin, further testing should not be performed. If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals for an exposure period of 4 h. Other modifications are that the skin of the rabbits are not abraded, the exposure period is reduced to 4 h, the responses are scored at 60 min, and then at 24, 48, and 72 h after patch removal, animals should be observed up to 14 days after removal of the patches to determine the reversibility of effects. The grading of skin responses is similar to the Draize scale. Chew et al. provided extensive clinical background that adds to the clinical implications of such testing. Dermatotoxicology background is found in Marzulli et al. (ref) [7th edition—Dermatotoxicology].

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15.6.1.1 Non-Draize Animal Studies

PURPOSE AND RATIONALE

These are animal assays that evaluate the ability of chemicals to produce cumulative irritation and is based on *repeated applications* of the test material. Many such tests have been described in the literature, but only a few have been studied extensively enough to mention. Even those used more often are not as well standardized as Draize-type tests, and many variables have been introduced by multiple investigators.

PROCEDURE

1. Repeat animal patch (RAP) test—Justice et al. (1961) compared the irritation potential of surfactants. Solutions are applied to the clipped back of immobilized albino mice with a saturated cotton-tipped applicator. The test site is covered with a rubber dam to prevent evaporation. This process is repeated seven times at intervals of 10 min and then the skin is subsequently microscopically evaluated for epidermal erosion.
2. A 16-day cumulative irritation test in rabbits—Marzulli and Maibach (1975) presented a new animal test for evaluating the skin-irritant capacity of cosmetics, drug preparations, and ingredients intended for repeated application. Substances are applied 14 times (uncovered) to the skin of six rabbits over a 3-week period on Mondays to Fridays, except for the last Friday. Each test site is scored every subsequent day, except Sundays (16 readings). The two parameters evaluated throughout the test as indices of skin irritation are redness (cumulative irritation score) and fluid accumulation (changes in skinfold thickness). The score used is 0 for no erythema, 1 for barely perceptible erythema, 2 for well-defined erythema, 3 for moderate to severe erythema, and 4 for beet-red erythema to slight eschar and/or disruption of epidermal intactness. At the conclusion of the test the cumulative irritation score for each animal (maximum $16 \times 4 = 64$) is obtained, and the mean cumulative score for six rabbits is recorded as an index of skin irritation. Changes in skinfold thickness are evaluated by deriving the mean difference from the daily differences between test and control-site readings on each rabbit and using the average of these mean differences for six rabbits as an index of skin irritation.
3. A 5-day dermal irritation test—performed in rabbits to compare various consumer products (MacMillan et al. 1975). The back of the animals are shaved, and 0.5 mL of the test material is spread over a 5×4.5-cm skin area. The test sites are protected from grooming by placing the animal in a leather harness or Elizabethan collar. After 4 h, sites are cleaned and graded using the Draize scoring system. The procedure is repeated every day for 5 days. The authors showed good agreement between the assay and the 21-day human patch tests of liquid detergents, after bath colognes, and hair preparations; the technique was less satisfactory for other types of materials.
4. The guinea pig immersion assay—used to evaluate the irritancy of aqueous detergent solutions and other surfactant-based products. Ten guinea pigs are placed in restraining devices that are immersed in a 40°C test solution for 4 h. The apparatus is designed to maintain the guinea pig's head above the solution. Immersion is repeated daily for three treatments. Twenty-four hours after the final immersion, the flank is shaved, and the skin is evaluated for erythema, edema, and fissures. A photographic grading scale for this assay was presented in MacMillan et al. (1975). Only materials of limited toxic potential are suitable for this assay because systemic absorption of a lethal dose

is possible. Concentration of test materials varies somewhat but is usually below 10% to limit systemic toxicity of the agents. A second group of animals is usually tested with a reference material as a control for the material of interest.

5. Open application procedure in guinea pigs—irritant skin reactions from repeated open applications of organic solvents are studied macroscopically and microscopically (Anderson et al. 1986). Aqueous solution of sodium lauryl sulfate (SLS) in low concentrations is used as the reference irritant substance to rank the solvents. Ten microliters of solvent or 5 μL of aqueous solution of SLS are applied openly three times daily for 3 days in 1-cm^2 areas of the shaved flank. Sites are than evaluated visually for erythema and edema. Biopsies samples are taken, stained with May-Grunward-Giemsa and assessed under oil immersion for epidermal thickness and dermal infiltration. A composite score reflecting the day of first visible reaction (day 1 = 3 points, day 2 = 2 points, day 3 = 1 point, no response = 0 point) and the macroscopic evaluation, the epidermal thickness, and the cellular response (response as for 2% SLS = 3 points, response as for 1% = 2 points, response less than 1% SLS = 1 point, no response = 0 point) are used to rank the chemicals. This method provides information on the pathogenesis of the response to each chemical, but the extensive processing may limit its application to special studies.
6. The mouse ear test—Uttley and Van Abbey (1973) applied undiluted shampoos to one ear of mice daily for 4 days. The degree of inflammation was quantified visually as vessel dilation, erythema, and edema. A reference material was tested on another group of mice, and the two were compared.
7. Finkelstein and colleagues (1963, 1965) test—they described a method to test for low-irritancy compounds using rabbits, rats, or guinea pigs. Application sites are pretreated with an irritant to make them more reactive. The animal is anesthetized, its abdomen is shaved, and circular areas of 1 in. in diameter are marked out. These areas are painted with a 20% solution of formaldehyde and then are allowed to dry for 5 min. This is repeated three times and then the test materials are impregnated on circular white cotton flannel pads of 1 in. in diameter and placed over the presensitized test sites. A control substance of known irritancy is tested in each study. Pads are secured in place and then the entire trunk is wrapped in polyethylene. A 0.5% solution of trypan blue is then injected subcutaneously away from the test sites to enhance visualization of the response by increasing test sensitivity. The dye serves as a marker for plasma leakage because it spontaneously binds albumin. After 16 h, patches are removed, and the accumulation of blue dye in the various areas can be estimated on a scale from 0% to 100%.
8. Brown (1971)—both open and closed exposures are used to rank surfactants for skin irritation potential. Tests ranged from 6-h patch exposures each day for 3 consecutive days in rabbits to daily open applications to the skin of rabbits, guinea pigs, or hairless mice for up to 4.5 weeks.

EVALUATION

The Draize-type tests for skin irritation consist of a single application of the test material on rabbits under occlusion. These tests may be most useful in identifying substances that may irritate skin after accidental contact. Since topical drug preparations are applied for days or even weeks, and cosmetics may be applied for a lifetime, it was felt the importance to develop animal tests based on repeated application to establish the safety of preparations intended for prolonged use. Marzulli and Maibach (1975) compared the response obtained on rabbits after a single occluded application (Draize method) and after 16 uncovered applications (cumulative irritation). The results suggested that the multiple-application technique can provide more definitive information about chronically applied substances that have a relatively mild irritation potential. They also compared the results obtained by this method with data from a 21-day occlusive test conducted in man. Results on 60 test materials showed a significant correlation between the cumulative irritation scores obtained on rabbits and those obtained on man.

CRITICAL ASSESSMENT OF THE METHOD

As the Draize-type tests, non-Draize animal studies are criticized regarding animal welfare concerns.

MODIFICATIONS OF THE METHOD

Some animal assays have been developed to quantify irritant response. Humphrey et al. (1993) measured

Evans blue dye recovered from rat skin after exposing the skin to inflammatory agents. Trush et al. (1994) described a procedure for the assessment of the dermal inflammatory response to numerous irritants by measuring the level of myeloperoxidase (MPO) enzyme in polymorphonuclear leukocytes of young CD-1 mice 16 h after application of the test agent on the shaved back. Chew et al. provided extensive documentation that aid in the clinical interpretation of such assays. Dermatotoxicology background is found in Marzulli et al. [7th edition—Dermatotoxicology].

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15.7 Human Irritation Tests

15.7.1 Single-Application Irritation Patch Tests

PURPOSE AND RATIONALE

Following a screening test in animals (for materials of unknown or unfamiliar composition) and with proper attention to ethics, irritation tests on human volunteers are preferred to animal tests if it is important to avoid the uncertainties of interspecies extrapolation. Single-application irritation patch tests measures acute skin irritation potential and is relevant to the identification of skin irritation hazards to humans.

PROCEDURE

The National Academy of Sciences (National Academy of Sciences and Committee for the Revision of NAS Publication 1138 1977) recommends a 4-h single-application patch test protocol for routine testing of skin irritation in humans. Ten volunteers are selected, and patches may be applied to the interscapular area of the back or to the dorsal surface of the upper arms. Eight to ten patches can be applied to each volunteer. Abrasion is not recommended. Instead of the wrapping around the body of the animals, a large piece of porous adhesive tape can be used to hold patches on the volunteers. A single exposure of 4 h is suggested, though it may be sufficient to use shorter exposures with strong irritants or very volatile materials. Volunteers are instructed to remove patches immediately if pain or discomfort occurs. After the exposure period, patches are removed and the skin is cleansed. Test sites are evaluated 30 min to 1 h, and 24 h after patch removal. Persistent reactions may be evaluated for 3–4 days. The Draize scale for redness and swelling can be used or an integrated scale based on the scale of Marzulli and Maibach: 0 = no response, 1/2 or + = questionable or faint, indistinct erythema, 1 = well-defined erythema, 2 = erythema with slight to moderate edema, 3 = vesicules or papules, 4 = bullous, spreading, or other severe reaction.

EVALUATION

The human 4-h patch test provides an opportunity to identify chemical acute skin irritation potential, providing data which are inherently superior to that given

by a surrogate model, such as the rabbit. It requires no species or system extrapolation of data for prediction of skin irritation potential as it is performed on the relevant species.

CRITICAL ASSESSMENT OF THE METHOD

Human testing is subject to strict ethical approval. This requires adherence to rigorous testing guidelines and overall protection of the well-being of the volunteers. They must read, understand, and sign an informed consent form prior to entry into the study. They should also be informed of all possible adverse reactions such as skin irritation and the possibility of postinflammatory hyperpigmentation. Ethical questioning comes from the fact that in human toxicological tests like this, some degree of intention knowingly "adverse" reaction may be produced without any direct health benefit to participants. However, these reactions are almost always mild or moderate in severity, are limited to small sites of chemical or product exposure, and quickly recover. In practice, the 4-h human patch test has been shown to be inherently low in risk, a fact well documented by an extensive published literature.

Single-application irritation patch tests measure acute skin irritation potential; however, for prediction of the cumulative or chronic skin irritation potential of ingredients or products intended for prolonged use, the repeat application irritation patch tests are more appropriate.

MODIFICATIONS OF THE METHOD

A standardized procedure for evaluating the irritation potential of new chemicals in man as a replacement for the Draize rabbit test has been proposed by Basketter et al. (1994). The method has been tested in several different laboratories, and results seem to be reproducible (Griffiths et al. 1997). The central tenet of this protocol is that skin responses to test materials are compared with the ones obtained after application of a meaningful positive control (20% SLS). It is then possible to determine statistically whether the test material has produced a level of skin irritation which is similar to, greater, or lower than the positive control (for the evaluation of the results of the test, what is measured is the number of subjects who had a positive irritant reaction after an exposure of up to 4 h. It is the incidence of positive responses, not the severity that is the basis for evaluation). Test materials showing statistically greater incidences of response (or no statistically significant difference versus the control) would be classified as "irritating to skin." Each test subject is exposed to the undiluted test material in occlusive chambers (Hill Top Chambers) and to a 20% solution of sodium laurel sulfate. The length of exposure begins with a 15-min exposure with evaluation at removal and at 24 and 48 h. If no response is observed, then another set of patches are applied for 30 min. This process of patching, evaluation, and patching for a longer exposure interval is repeated until the subject responds to the SLS exposure or until a 4-h exposure has been completed.

In a series of patch testing experiments to compare interspecies skin irritancy, Nixon et al. (1975) applied a 4-h Draize-type procedure (including abrasion) to evaluate a range of household products. Skin responses were evaluated for erythema and edema at 4, 34, and 48 h after application of the patches. Detail is provided by (Chew and Maibach 2006).

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15.7.2 Repeat Application Irritation Patch Tests

PURPOSE AND RATIONALE

Kligman and Wooding (1967) described a new method for bioassaying substances intended for chronic exposure, as a means of evaluating any irritant effects that

may be cumulative with repeated exposure. As with single-application patch tests, many investigators (e.g., Lanman et al. 1968; Phillips et al. 1972) developed their own version of the repeat application patch test.

PROCEDURE

For weak irritants, Kligman and Wooding (1967) determined the IT50, which is the estimated number of days of continuous exposure which would produce a threshold reaction in 50% of the population. For strong irritants, they determined the ID50, which is the estimated concentration needed to produce a discernible irritant reaction in 50% of the population in 24 h.

The IT50 procedure: A minimum of ten subjects are included in the study. The patches consist of one centimeter square of Wcbril®, a highly absorbent nonwoven fabric, loaded with the test material. Occlusion is obtainable by securing the patches under overlapping strips of impermeable plastic tape (Blenderm®). The appropriate volume is that which is just sufficient to load the patch completely without overrun, approximately 0.05 mL per sq cm. The patches are reapplied once daily at exactly the same site of the back, and the end point is the number of days required for an unmistakable erythema to develop. When this threshold is reached, no further patches are applied at that site. Ten days of continuous exposure is the minimum required. For very mild materials, extension to 20 days may be necessary to obtain more than 50% of reactors in the sample. Sometimes, 50% or more of the subjects will have failed to react even after 20 days. Many products are so innocuous that none or only one or two subjects may react; these include the common vehicles such as lanolin, hydrophilic ointment USP, baby oils, and others. One may simply conclude that the products are essentially nonirritating. The Litchfield and Wilcoxon probit analysis is used to estimate the IT 50 value. For particular purposes, one may use either more subjects or a greater number of days of exposure.

The ID50 procedure: The minimum number of subjects is ten, as before. A pilot study is done to select a range of at least five concentrations which may be either evenly spaced or arranged in geometric progression, depending upon the material. Patches are applied as before, every subject being exposed to the entire range of concentrations. The patches are left on for a standard period and are not reapplied. Usually, 1-day exposure with immediate readings is performed, but contact time for strong irritants may be as low as 2–8 h. In such cases, the readings should be made at 24 h. The ID50 value is estimated trough the Litchfield and Wilcoxon probit analysis.

EVALUATION

Extensive experience with this assay indicates that it is most useful in evaluating low-grade irritants and compounds which have frequent and intimate exposure to human skin. The end points of Kligman and Wooding's (1967) method are "all or none" choices (quantal responses). The observer has only to decide whether there is or is not a reaction; he/she need not estimate its intensity. Furthermore, the all-or-none reaction depends upon a single quality, erythema, which is the common denominator of all irritation reactions.

CRITICAL ASSESSMENT OF THE METHOD

Besides all the ethic questions related to human studies (reported in the single application tests), these type of tests have a higher cost and are more time demanding. Laboratory personnel and subjects must be available for three consecutive weeks, including weekends. The subjects can leave the study without completing it. Another issue is whether the trauma or irritation to the skin from applying and reapplying adhesive patches for 21 days interferes with the proper evaluation of irritancy for the compositions being studied.

MODIFICATIONS OF THE METHOD

Modifications of the cumulative irritation assay have been reported. Intensity of response has been evaluated using other evaluation schemes, the interval between applications of fresh patches has been varied, and other methods of data evaluation have been proposed. The newer chamber devices have replaced Webril® with occlusive tape in some laboratories. Some investigators currently use cumulative scores to compare test materials and do not calculate the IT50. Kligman and Wooding performed their studies on surfactants in 10 days. Lanman et al. (1968) needed 21 applications to discriminate between baby lotions. Finkelstein et al. (1963, 1965) described tests using either a 5–6 or a 17–18-h exposure each day for 4 days. Test sites were evaluated 1 h after patch removal.

15.7.2.1 The Chamber Escarification Test

Repeated application patch tests on intact skin fail to predict some adverse reactions due to repeated application of materials to damaged skin (i.e., acne, shaved underarms, or sensitive areas such as the face). The chamber scarification test was developed to evaluate materials that would normally be applied to damaged skin. Light-skinned whites who developed severe erythema with edema and vesicles following a 24-h exposure to 5% sodium lauryl sulfate in Duhring chambers applied to the inner forearm are preselected as subjects. Five to ten volunteers constitute a test panel. Six to eight 10-mm^2 areas on midvolar forearm are scarified with eight crisscross scratches made with a 30-gauge needle. Four scratches are parallel, with another four at right angles. The test material is applied once daily for 3 days with readings made at 72 h. The reactions are graded on a five-point scale from 0 to 4.

15.7.2.2 Immersion Tests

Small differences in the mildness of soap cannot be demonstrated readily by means of normal usage tests with normal persons. It is consequently necessary to exaggerate the conditions in some manner so that the differences which exist may be magnified and determined objectively. In this manner, Kooyman and Snyder developed the arm immersion technique to compare the relative irritancy of two soaps or detergent products (Kooyman and Snyder 1942). In this procedure, solutions of two soaps are prepared and placed in pans or troughs in which the temperature is maintained at a constant level of 41°C (105°F). In order to simulate practical dishwashing conditions more closely, concentrations of 0.35% are used. One hand and arm of the subject is placed in each solution for a given period (10–15 min) and is then rinsed and patted dry with a soft towel. This immersion process is carried out three times a day at convenient intervals, and the test is continued until observable irritation is produced on one or both arms. The relative mildness of the two soaps may then be determined by an arbitrary scale for grading the degree of irritation. More recently, variations on the arm immersion technique have developed so that the antecubital fossa and the hands are separately tested. Variations incorporate different dosing regimens or measuring different end points.

15.7.2.3 Soap Chamber Technique

The "chapping" potential of bar soaps is evaluated with the soap chamber technique, developed by Frosch and Kligman (1979). While patch testing is useful in predicting erythema, it does not address the dryness, flaking, and fissuring observed with bar soap use. Using this method, 100 μL of an 8% soap solution is applied to the forearm via Duhring chambers fitted with Webril pads. Nonocclusive tape is used to secure the chambers. The exposure encompasses 5 weekdays, the first one for 24 h beginning on Monday morning. Fresh solutions are applied to the same sites for 6 h daily for the next 4 days. The sites are read on the Monday morning following removal of the chambers on Friday afternoon. During this 36-h interval, "chapping" becomes more noticeable, while erythema tends to moderate. If severe erythema at the test site occurs, the investigator must discontinue the study. Skin responses are evaluated with visual scoring of erythema, scaling, and fissures.

15.7.2.4 Bioengineering Methods

Bioengineering methods utilized to quantify test results include transepidermal water loss (TEWL), capacitance, ultrasound, laser Doppler flowmetry (LDF), spectroscopy, and chromametry (colorimetry). Most of the assays described were developed before the introduction of these bioengineering methods. These methods allow a more precise quantification of test results.

15.7.2.5 In Vitro Assays

There are three validated in vitro test methods that may be used to determine the skin irritancy of chemicals. They are based on a reconstructed human epidermis (RhE) model, which is comprised of nontransformed human-derived epidermal keratinocytes that have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers representing main lipid classes analogous to those found in vivo and are commercially available as EpiSkin™, EpiDerm™ SIT (EPI-200), and SkinEthic™ RHE test methods (OECD Test Guideline 439). The test material is applied to the three-dimensional RhE model, and irritant chemicals are identified by their ability to decrease cell viability below a defined threshold level.

Three validated in vitro test methods have been adopted as OECD Test Guidelines to be used for skin corrosivity testing. OECD Test Guideline 435 (2006) provides an in vitro membrane barrier test method, commercially available as Corrositex®. The test system is composed of two components, a synthetic macromolecular biobarrier (a proteinaceous macromolecular aqueous gel and a permeable supporting membrane) and a chemical detection system (CDS). The test substance is layered onto the upper surface of the membrane barrier, and penetration of the membrane barrier (or breakthrough) might be measured by a pH indicator dye or combination of dyes will show a color change in response to the presence of the test substance. OECD Test Guideline 431 (2004e) is based on a reconstructed human epidermis (RhE) model, comprised with the same material of that used for skin irritation that are commercially available as EpiDerm™ and EPISKIN™. The principle of the method is based on the premise that corrosive chemicals are able to penetrate the stratum corneum by diffusion or erosion and are cytotoxic to the cells in the underlying layers. Corrosive chemicals are identified by their ability to decrease cell viability below defined threshold levels. The OECD Test Guideline 430 (2004d) is the in vitro skin transcutaneous electrical resistance (TER) test. The test substance is applied for up to 24 h to the epidermal surface of skin discs (prepared from humanely killed 28–30-day-old rats) in a two-compartment test system in which the skin discs function as the separation between the compartments. Corrosive substances are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which are measured as a reduction in the TER below a specified level. For rat skin TER, a cutoff value of 5 kΩ has been selected. Details are found in Rougier et al.

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Safety Pharmacology Assessment of Biopharmaceuticals

16

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16.1 Introduction

Since the approval of the first recombinant protein therapeutic (insulin) in 1982, the number of biopharmaceuticals that have been approved as new medicines has grown steadily (Gosse et al. 1996; Lowe and Jones 2007). In one decade (1992–2002), 56 biopharmaceuticals were approved by FDA (Schwieterman 2006) and more than 170 biopharmaceuticals have been approved for clinical use worldwide (Giezen et al. 2008; Leader et al. 2008). Biopharmaceuticals have been successful as new therapeutics because of their high specificity for a given molecular target, favorable pharmacokinetic properties such as long half-life, low volume of distribution that provide better target coverage, and low "off-target" liability. This latter property translates into fewer non-clinical and clinical failures due to toxicity, when compared to small molecule therapeutics. A recent review of regulatory actions for safety concerns for all approved biopharmaceuticals in USA/EU (total: 174) indicated that the most frequent warnings (41 products) were related to immunomodulatory effects such as injection site reactions and infections, but none of these biopharmaceuticals have been withdrawn from human use (Giezen et al. 2008). However, recently two biopharmaceuticals were withdrawn from market - efalizumab (2009) because of increased risk of progressive multifocal leukoencephalopathy and gemtuzumab ozogamicin (2010) due to lack of efficacy and safety concerns (FDA.gov). This is in stark contrast with a large number of conventional "small molecule" drugs withdrawn from medical use because of safety issues, especially cardiovascular toxicity (Guengerich 2011).

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The safety pharmacology assessment of drug candidates is based on the International Conference on Harmonization (ICH) guidelines S6(R1) (2011), S7A (2000) and S7B (2005). The primary goal of these safety guidelines is to provide a framework for the functional evaluation of candidate drugs, thus assuring their safety for first use in humans. This is achieved by assessing the undesirable pharmacological effects of drug candidates on various organ systems. The general approaches toward safety pharmacology assessment of small molecules and biopharmaceuticals are basically the same in that the effects of a drug candidate on cardiovascular, central nervous (CNS), and respiratory systems should be investigated prior to first use in humans. However, they could differ in practice. Safety pharmacology evaluation of small molecules is typically conducted in dedicated studies. In contrast, for biopharmaceuticals, incorporation of safety pharmacology endpoints into repeat-dose toxicity and/or pharmacodynamic studies is recommended. In this chapter, we review the special consideration for safety pharmacology assessment of biopharmaceuticals.

16.2 Regulatory Guidelines for Safety Pharmacology Assessment

In contrast to traditional chemically synthesized drugs, biopharmaceuticals are therapeutics derived from biological processes or organisms. Typical examples of biopharmaceuticals are proteins such as monoclonal antibodies (mAbs), recombinant proteins, cytokines, growth factors, fusion proteins, and peptides (ICH S6(R1) (2011)). In the ICH S6(R1) guideline, a wide variety of biopharmaceuticals are broadly referred to as biotechnology-derived pharmaceuticals, but other synonyms include biotherapeutics, protein therapeutics, and "large molecules." The designation "large molecule" is a reference to the high molecular weight (1 to >140 kDa) and complex secondary, tertiary, and quaternary structural characteristics of proteins (Crommelin et al. 2003), compared to classical "small molecule" drugs whose molecular weight is typically less than 500 Da (Ghose et al. 1999).

Biopharmaceuticals such as mAbs are engineered to specifically interact or modulate human cellular targets of diseases; as a result, this can make the non-clinical safety evaluation of protein therapeutics challenging, especially, the choice of the species to use for safety studies (Chapman et al. 2007; Bussiere et al. 2009). Such challenges can directly impact the in vivo safety pharmacology strategy used to assess biopharmaceuticals, especially if only a non-rodent species, such as the non-human primates (NHPs, i.e., cynomolgus monkey), is considered pharmacologically relevant (Green 1997; Chapman et al. 2007).

In this regard, the ICH S7A guideline (2000) recognizes that the traditional core battery of safety pharmacology studies may be inappropriate for biotechnology-derived products given their high specificity for human targets. For such agents, the guideline suggests inclusion of safety pharmacology endpoints in repeat-dose toxicity studies. It states that "For biotechnology-derived products that achieve highly specific receptor targeting, it is often sufficient to evaluate safety pharmacology endpoints as part of toxicology and/or pharmacodynamic studies, and therefore safety pharmacology studies can be reduced or eliminated for these products." While it is recognized that some safety pharmacology endpoints such as electrocardiographic parameters (ECG) can be collected from non-rodent toxicity studies, capturing high-quality safety pharmacology data requires skilful integration (i.e., right endpoint at the right time) and attention to the limitations of repeat-dose toxicity studies (i.e., small group size; interpretation of physiological/functional changes secondary to drug-induced toxicity) that may compromise the safety pharmacology assessment.

16.3 Integrated Safety Pharmacology Assessment

Because of limitation in the choice of animal test species for biopharmaceuticals, it is appropriate to monitor their potential undesirable pharmacological effects in repeat-dose toxicity studies. For biopharmaceuticals, there are several advantages of integrating safety pharmacology endpoints into a repeat-dose toxicity study including: ability to monitor acute (e.g., onset of delayed cardiac repolarization) and chronic effects (e.g., dysfunction due to myocardial structural alterations and QTc prolongation secondary to reduced hERG expression); opportunity to observe gender differences and whether there is recovery from toxicity after dosing stops; ability to relate toxicity; safety pharmacology and pharmacokinetic data from the

same animal (maximized data collection); and reduction in the total number of animals used, especially NHPs. This latter point is critical since NHPs tend to be used exclusively for toxicity studies for many types of biopharmaceuticals. Therefore, the opportunities to reduce NHPs use during non-clinical drug development should be considered (Chapman et al. 2007).

The integration of safety pharmacology endpoints into toxicity studies requires careful planning and thought to insure that best practices are used and that key toxicity and safety pharmacology endpoints are not jeopardized or compromised as a result of data collection. Another key point to consider is whether the pharmacokinetic profile of biopharmaceuticals is affected by antidrug antibodies which could alter their clearance and consequently the interpretation of study findings (Tabrizi et al. 2006). Depending on the safety concern with a novel biopharmaceutical, a dedicated and optimally designed safety pharmacology study may be needed in some cases (Vargas et al. 2008).

16.4 Cardiovascular Safety Pharmacology Assessment

Because biopharmaceuticals may require case-by-case safety pharmacology assessment during non-clinical development, alternative safety pharmacology approaches and experimental designs can be considered. Given that biopharmaceuticals generally have long half-lives, evaluations of cardiovascular effects that emerge after acute and chronic exposure are important to consider. Thus, a rational and cost-effective approach for biopharmaceuticals is the integration of cardiovascular endpoints into a repeat-dose toxicity study. For example, noninvasive ECG telemetry systems can enable a high-quality assessment of cardiac electrical activity in non-rodents and can be integrated into repeat-dose toxicity studies to optimize the evaluation of drug-induced changes in cardiac conduction and repolarization (Chui et al. 2009; Guth et al. 2009; Derakhchan et al. 2011). Such an approach can be used to collect high-fidelity multi-lead ECG data in long-term NHPs studies from 1 to 6 months duration (Vargas et al. 2010; Chui et al. 2011), particularly because NHPs are the common species used for biopharmaceuticals safety assessment.

Arterial blood pressure changes associated with the administration of biopharmaceuticals can be tracked using conventional implant telemetry in the cynomolgus monkey (Santostefano et al. 2012). Telemetric evaluation of arterial pressure using invasive or minimally invasive approaches enable the assessment of acute and chronic changes in blood pressure, are suitable for use in repeat-dose toxicity studies (Kaiser et al. 2010; McMahon et al. 2010), and would be a practical method to assess blood pressure changes associated with biopharmaceuticals. Lastly, blood pressure can be assessed using noninvasive approaches such as high-definition oscillometry (Schmelting et al. 2009), but such methods may not be ideal to assess drug-related effects because they require animal restraint, cause stress-associated elevation in blood pressure, and lack sensitivity to detect small changes in blood pressure (Kurtz et al. 2005; Wernick et al. 2012).

A key consideration with biopharmaceuticals is whether there is value-added safety in performing a voltage-clamp hERG assay to comply with the ICH S7B guideline. In contrast with "small drug" inhibitors of the hERG channel, large therapeutic molecules are not expected to interact with or inhibit hERG channel function directly or indirectly. A scientific review examined the hERG liability and QTc prolongation risk of biopharmaceuticals and concluded that mAbs and other protein that have very high target selectivity have low risk for blocking the channel, thus it is not appropriate to conduct the hERG assay as part of the non-clinical QT prolongation risk assessment (Vargas et al. 2008). Rather, the assay should be considered if there is a need to determine the mechanism of an in vivo QTc prolongation signal. The low likelihood of delayed cardiac repolarization with biopharmaceuticals is based primarily on the physical size and pharmacological characteristics of these agents which make them unable to cross the plasma membrane to interact with and inhibit the central pore or nonspecifically block the external "toxin-binding site" of the hERG channel. The recent demonstration that two specific anti-hERG polyclonal antibodies bind to epitopes on the hERG channel but do not interact with the external pore region further supports the low QTc prolongation risk of large molecule therapeutics (Qu et al. 2011). A recent perspective on safety assessment of biopharmaceuticals by Nakazawa et al. (2008) also recommends that the decision to conduct an in vitro hERG assay should be based on the results of an in vivo ECG evaluation.

16.5 Central Nervous System Safety Pharmacology Assessment

The potential undesirable pharmacological effects of biopharmaceuticals on the CNS can be evaluated in repeat-dose toxicity studies in accord with regulatory guideline (ICH S7A (2000). In traditional core battery safety pharmacology studies, rodents are typically used in the functional neurobehavioral assays conducted to support small-molecule drug development (see Chap. 3 "Central Nervous System (CNS) Safety Pharmacology Studies" and Chap. 6 "Respiratory Function Assays in Safety Pharmacology"), but for many types of biopharmaceuticals, rodents may not be a pharmacologically relevant species. Thus, if NHPs are the most relevant test species for safety assessment of biopharmaceuticals, then it is appropriate to assess CNS function during the repeat-dose toxicity studies in this species.

Behavioral changes indicative of adverse CNS effects, such as altered locomotor activity (e.g., hyper- or hypoactivity), changes in motor tone or mental alertness, and convulsion are generally detectable in non-rodent toxicity studies. Therefore, serious CNS adverse events associated with the administration of biopharmaceuticals can be identified from NHPs toxicity studies and used to support CNS risk assessment after acute and chronic exposure. This is advantageous because it eliminates the need to perform a dedicated neurobehavioral safety pharmacology study in this higher species. Specific testing methods have been developed and are available to improve neurobehavioral risk assessment in the cynomolgus monkey (Korte et al. 2007) and these evaluations could be conducted at various times during a regulatory toxicity study to assess the impact of a biopharmaceutical on CNS function.

However, based on their molecular size and physicochemical properties, biopharmaceuticals are not expected to penetrate into the brain and spinal cord, due to the restrictive nature of the blood-brain barrier (Pardridge 2005). As a result, biopharmaceuticals may have an inherently lower risk for causing undesirable pharmacological effects in the CNS (Pardridge 2005). In a recent internal survey (142 studies on 33 compounds) that examined convulsion incidence in neurobehavioral safety pharmacology and repeat-dose toxicity studies in rodents, we sought to assess the occurrence of CNS risk with a broad range of biopharmaceuticals and small molecules. None of the large molecules (0/11) caused convulsion in any repeat-dose toxicity study, whereas 14% of small molecules (3/22) caused convulsion in neurobehavioral safety pharmacology or toxicity studies (Amouzadeh and Vargas 2012). Overall, the physiochemical properties and results from internal data review supports the hypothesis that biopharmaceuticals have low potential to cause adverse neurobehavioral effects. The poor ability of biopharmaceuticals to access the CNS suggests that these agents may also have low inherent risk for inducing drug dependency and abuse. If a convulsion risk was detected during a NHPs toxicity study with biopharmaceuticals, which would be an unusual finding, a follow-up study using electroencephalography (EEG) may be needed to assess whether the adverse effect originated in the brain (e.g., CNS-mediated seizure) or had another cause (e.g., altered clinical chemistry or body temperature). The findings from such a specialized study could be a critical component of the CNS risk assessment and inform the decision to advance the compound into clinical development (see Chap. 2 "Safety Pharmacology: Introduction").

16.6 Respiratory Safety Pharmacology Assessment

The potential for biopharmaceuticals to affect the respiratory system can also be evaluated in repeat-dose non-rodent toxicity studies. Respiratory endpoints such as dyspnea, labored breathing, and breathing rate estimates can be monitored during cage-side clinical observations in NHPs. However, cage-side assessments are subjective, and may only have the sensitivity to detect clinically observable events like overt respiratory toxicity, and will be limited to the evaluation times designated in the study protocol. If a more detailed functional assessment of respiratory ventilation and mechanics is needed to support the risk assessment for biopharmaceuticals (e.g., triggered by an assessment of target liability), classic methods such as head-only or whole-body plethysmography can be adapted for use in the restrained or unrestrained NHPs (Foster et al. 2008; Iizuka et al. 2010; see Chap. 6 "Respiratory Function Assays in Safety Pharmacology"). In this latter scenario, a dedicated respiratory safety pharmacology study would be needed to evaluate the hypothetical

liability, given the need for special equipment, acclimation procedure, and staff expertise to conduct the NHPs respiratory function evaluation. In the future, it may be possible to introduce quantitative respiratory measurements into non-rodent toxicity studies, which would maximize animal use and allow for respiratory hazard identification of biopharmaceuticals. The use of jacket-based inductive plethysmography is an emerging noninvasive technology that can capture respiratory endpoints (breathing rate and tidal volume) continuously from unrestrained animals and has the potential to be fully integrated into long-term toxicity studies (see Chap. 6 "Respiratory Function Assays in Safety Pharmacology"), like jacket-based ECG evaluation.

16.7 Other Organ System Safety Pharmacology Assessment

The need for assessment of the undesirable pharmacological effects of biopharmaceuticals on systems other than cardiovascular, central nervous, and respiratory systems depends on a theoretical basis for potential adverse functional effects associated with the target, the organ distribution of the target receptor, and empirical observation during exploratory toxicity studies, including histopathological evidence. Therefore, supplemental safety pharmacology studies should be considered for biopharmaceuticals whenever there is an indication of a potential functional adverse effect. Similar to evaluation of the core battery of safety pharmacology studies, supplemental safety pharmacology endpoints may be integrated into multiple-dose toxicity studies, or they could be performed separately. For example, serum creatinine and blood urea nitrogen are biomarkers that are normally evaluated during toxicity studies and are used to monitor renal function. An important caveat to remember is that valid safety pharmacology methods in the NHPs model may be limited and available only in some key areas with established endpoints (e.g., renal or endocrine function and plasma catecholamines as an index of sympathetic nervous system tone). The future development of new biomarker approaches (e.g., KIM-1 for nephrotoxicity) and non-invasive methods (e.g., SmartPill® for GI transit) for application to NHPs studies represent new opportunities to integrate supplemental safety pharmacology endpoints into toxicity studies.

16.8 Summary

The goal of safety pharmacology evaluation of new drug candidates is to assess effects on cardiovascular, central nervous, and respiratory systems prior to first human exposure. This is guided by the ICH guidelines S6(R1) (2011), ICH S7A (2000) and S7B (2005). Although the general approaches toward safety pharmacology assessment of biopharmaceuticals and small molecules are similar, they differ in practice in that safety pharmacology of small molecules is usually evaluated in dedicated studies optimized to assess drug-induced functional effects, whereas the approach for biopharmaceuticals is case dependent and reliant upon the incorporation of safety pharmacology endpoints in repeat-dose toxicity studies. Overall, the safety pharmacology evaluation of biopharmaceuticals is driven by their unique pharmacological properties, specificity of action, and the choice of test species. In most cases, NHPs are the most appropriate test species for safety pharmacology and toxicity assessment. Since there is a desire to reduce NHP use during non-clinical development, especially in safety assessment studies, there will be an ongoing impetus to maximize the collection of important safety pharmacology endpoints in toxicity studies. This can be achieved by the careful integration of improved safety pharmacology methods (e.g., noninvasive cardiovascular and respiratory methodologies) into the design of toxicity studies used for regulatory filings.

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Magnetic Resonance Imaging in Pharmaceutical Safety Assessment

17

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17.1 Introduction

The high rate of attrition of drug projects through the pharmaceutical pipeline is a significant contributor to the increasing R&D costs seen in recent years. In 2004, the FDA released a report entitled "Innovation or Stagnation, Challenge and Opportunity on the Critical Path to New Medical Products" in which the alarm was raised that only 8% of the molecules that enter clinical development were successfully registered (http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.html). Recent data suggests that this figure had fallen to 4% by 2010 (Bunnage 2011). Many more fail in the preclinical stages of development. There is an urgent need for new tools to improve drug development and the critical path document specifically highlights imaging as one of the new technologies that has a potential to contribute. One quote from the report is particularly telling, "Often, developers are forced to use the tools of the last century to evaluate this century's advances."

Despite the explosion of potential biomarkers due to the "-omics" approaches, there is an acknowledged need to find and establish more sensitive, specific, and predictive biomarkers (Wehling 2006). ICI (now AstraZeneca) and Sandoz (now Novartis) introduced MRI into the pharmaceutical industry in 1983, and the use of imaging biomarkers to accelerate drug discovery and development has been well documented (Chandra et al. 2005; Pien et al. 2005; Beckmann et al. 2007). MRI has been successful in the pharmaceutical industry for the same reasons that it is popular in clinical practice; it is a noninvasive imaging technique with superb soft tissue contrast capable of delivering quantitative 3D information on organ anatomy and function (Beckmann et al. 2004;

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Maronpot et al. 2004). Because it is noninvasive, aside from the need to anesthetize animals to immobilize them during image acquisition, animals can be imaged on multiple occasions and studies can be designed so that each animal serves as its own control increasing the statistical power of experiments and allowing group sizes to be reduced. However, despite penetration into preclinical and clinical drug efficacy studies, there are relatively few reports of the use of MRI in drug safety studies. Toxicology accounts for approximately one third of attrition in development and is thus a major cost in the pharmaceutical industry. An informal survey of a number of preclinical imaging groups in the pharmaceutical industry showed that approximately 5% of effort (range 0–20%) was devoted to safety imaging studies. This seems a disproportionally small effort considering that MRI is a powerful tool that could potentially be used to reduce attrition in the late pipeline where it is most expensive. It is important to understand why MRI has not been more widely used in the drug safety arena before describing in detail a few of the MRI assays appropriate for preclinical safety studies.

There are three types of safety pharmacology studies conducted in the pharmaceutical industry: (1) single-dose core portfolio preclinical safety studies conducted to good laboratory practice (GLP), (2) supplemental studies of compound specific effects after chronic dosing that are conducted when results from the core battery of tests raise concern, and (3) "front-loading" safety studies conducted in the drug discovery function with the aim of designing safety liabilities out of the lead compound series.

The first type of study forms part of the legally required activities toward the registration of a pharmaceutical product. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) safety pharmacology guidelines recommend the use of unanesthetized animals, which is incompatible with the standard MRI experiment in which animals are anesthetized to prevent motion interfering with image quality. It is certainly feasible to habituate animals to the MRI environment; however, in practice, the results may not warrant the effort involved. In addition, it is unlikely that MRI assays will replace conventional endpoints or shorten the study duration. Thus, there is little incentive to routinely incorporate MRI assays in the core package.

The second type of study is investigational and is conducted when results from the core battery of tests raise concern (Ettlin et al. 2010). In almost all cases, the pharmaceutical industry prepares a comprehensive package of studies for the regulatory authorities that include effects after chronic dosing. The chronic-dosing regimen encourages the design of imaging experiments in which each animal acts as its own control, increasing statistical power with smaller group sizes and allowing longitudinal studies without the need to kill groups of animals at each time point; two factors that both separately and combined offer dramatic sparing of laboratory animals. In these investigational studies, there are no guidelines against anesthesia, although clearly one must consider the impact of anesthesia on each individual experiment. One of the most significant obstacles in incorporating MRI assays into investigational safety studies is that these studies are often on the critical path for drug development, and therefore there is an urgency that leaves little time to develop and evaluate sophisticated new assays. Thus, MRI is most appropriate to investigate adverse events that recur regularly in safety assessment departments so that the appropriate validation work with positive and negative controls can be in place before the technique is needed in earnest.

The third type of study is not designed with regulators in mind or conducted within dedicated safety assessment functions. It is now widely recognized that the pharmaceutical industry can no longer afford to start safety evaluation only after candidate selection, knowing that many candidates will quickly fail due to safety issues. The pressure is on to reduce attrition in the late pipeline by introducing safety screens in the early pipeline when it is still possible to design known safety liabilities out of the lead series. In future, we expect to see increased numbers of these early pipeline non-GLP safety pharmacology studies of pre-candidates conducted in the drug discovery functions for purely internal decision-making purposes. These "front-loading" studies are likely to be the most amenable to MRI as the drug project lifetime is sufficient to discover and develop the appropriate MRI assays.

Good laboratory practice is often considered a major hurdle in the use of MRI in safety assessment studies. GLP ensures that the data produced from nonclinical studies are of high quality, reliable, and valid. Since regulators use these data to authorize clinical trials and marketing of the end product,

it is important that they are correctly recorded and reproducible. An experienced, multidisciplined, and dedicated function is needed to ensure that such work is in compliance with legal requirements. The current generation of preclinical MRI scanners are not equipped with GLP software tools that would guarantee consistent spectrometer operation or data transfer in compliance with GLP. In principle, there is no reason why collaboration between MRI scanner manufacturers and the pharmaceutical industry could not produce GLP compliant MRI assays; however, the burden of GLP documentation makes compliance for complex and innovative assays impractical. In practice, regulatory agencies do accept investigatory studies not to GLP if the work is critical to a scientifically based risk assessment and has been conducted to an acceptable standard. In this case, there is still a definite advantage if protocols, data acquisition, transfer, archival, staff records, and so on are in accord with GLP principles.

Despite the obstacles mentioned above, the advantages of noninvasive imaging techniques to drug safety studies are obvious. It is possible to design longitudinal studies in which the same animal is studied at baseline and then at several time points while on study. Changes in individual animals can be quantitated and compared with baseline measurements either in simple percentage terms or, for example, using more sophisticated linear mixed-effect models (Brown and Prescott 1999), leading to a reduction in group size and obviating the need to sacrifice animals at each time point. Baseline data can be used either to select or deselect animals to be included in a study or as a basis for randomization between groups. And, of course, at the end of the study, the animal is still available for other, complimentary, analysis techniques. In general, the readout time for MRI endpoints is faster than that for histology leading to faster management go/no-go decisions. Some biomarkers are only amenable via MRI, for example, quantitation of intramyocellular lipid (IMCL) is straightforward with MR spectroscopy (MRS) but time-consuming with traditional microdissection techniques.

Further, the imaging biomarkers identified in preclinical safety assessment studies can also be used in clinical drug safety studies, as MRI is widely available and safe to use in volunteer studies. This can be an advantage for the preclinical safety assessment function as it provides feedback on translation of animal safety assessment studies to humans using the same endpoint. Clearly, one would not run MRI on all clinical safety studies but in those cases where there is no cheaper, simpler safety biomarker available and there is doubt about the degree of risk posed in man, for example, because of species differences or because the effect size in the placebo group is expected to be very high.

The conclusion is perhaps best put in a recent posting on the FDA website, "Imaging technologies provide powerful insights into the distribution, binding, and other biological effects of pharmaceuticals. As part of its Critical Path initiative, FDA has joined the National Cancer Institute (NCI), the pharmaceutical industry, and academia in a number of activities that will facilitate the development of new imaging agents and the use of medical imaging during product development. We believe that with a little effort on the part of all of us, imaging agents and technologies can contribute important biomarkers and surrogate endpoints during disease progression and contribute to the development of new therapies to treat disease" (http://www.fda.gov/cder/regulatory/medImaging/default.htm).

17.2 Liver Volume Measurement

PURPOSE AND RATIONALE

Liver hypertrophy is a frequent side effect in drug development caused by a wide variety of compounds. Because it is often the first indication of the hepatocarcinogenic potential of a drug candidate, liver weight is routinely monitored in safety assessment studies (Ou et al. 2001; Shoda et al. 2000). This is necessarily a terminal procedure so that longitudinal evaluation of hypertrophy must involve serial kills of groups of animals at the time points of interest. Assuming that the compound administered does not significantly change liver density, liver volume changes should be at least as sensitive as liver weight changes. Noninvasive serial MRI measurements of liver volume can reduce animal usage by following the same groups of animals over the time points of interest. In addition, the ability to measure difference from baseline instead of a single time-point liver volume usually increases the precision of treatment measurements, and the resulting increase in statistical power can be used to reduce group sizes.

PROCEDURE

Rats are anesthetized with isoflurane (1.5–2%, 0.6–1 l/min), and then MR images are acquired.

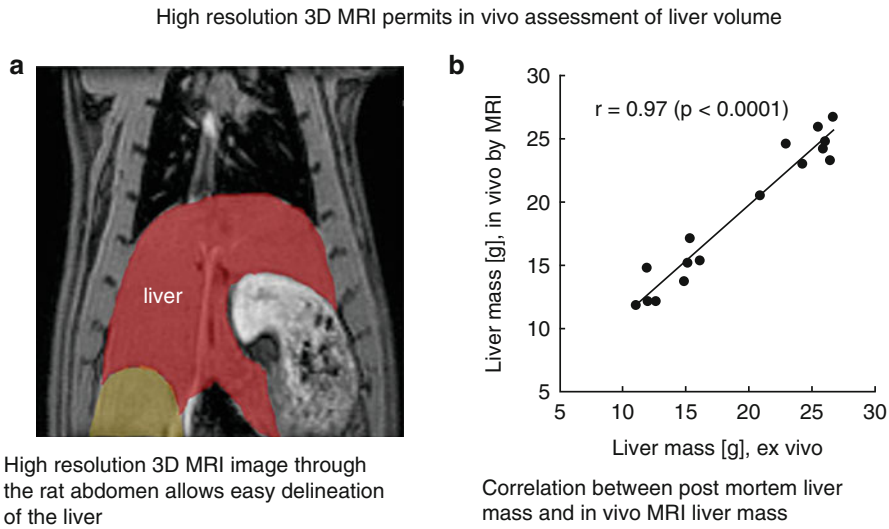

Fig. 17.1 MRI coronal section through a rat liver showing good contrast from surrounding tissues (**a**) and correlation with ex vivo wet weight (**b**) (Abdel Wahad Bidar, AstraZeneca, personal communication 2007)

A high-resolution 3D FISP scan is acquired (TE/TR 1.7/3.3 ms, FOV 50 × 50 × 50 mm, reconstruction size 256 × 192 × 192, NA 1, FA 20°). Individual 3D images take approximately 6 min to acquire. Spectrometer triggering is set such that data acquisition occurs during the expiratory phase of the respiratory cycle.

EVALUATION

Images can be evaluated with Analyze (Biomedical Imaging Resource, MN, USA). Liver volume is determined by manual segmentation of each slice using the ROI spline tool (Fig. 17.1). There is no loss in accuracy in liver volume estimation if only a subset of at least 6 evenly spaced slices are segmented instead of the full 60 slices through the liver. To improve liver segmentation at later time points, follow-up scans can be registered to the baseline scans (Hajnal et al. 1995).

MODIFICATIONS OF THE METHOD

Cockman et al. (1993) used a multislice spin-echo method and reported that respiratory triggering increased the accuracy of rat liver volume measurements. Hockings et al. (2002) and Hockings et al. (2003a) reported rat liver volumes obtained with a respiratory triggered segmented 3D fat suppressed inversion recovery snapshot readout sequence at both 7 T and 2 T and reported a correlation coefficient between in vivo MRI liver volume and post-mortem liver wet weight of 0.96 and 0.99, respectively. Tang et al. (2002) used a non-respiratory triggered multislice spin-echo method in rats and reported a correlation coefficient of 0.9 against liver wet weight with a systematic overestimation of MRI liver volume. The coefficient of variability of MRI precision was 2.3% and operator reliability for segmentation 2.9%. Garbow et al. (2004a) measured liver volume in mice with MRI at 4.7 T using an intraperitoneal injection of contrast reagent to increase contrast between liver and surrounding organs. The correlation coefficient between MRI volume and wet weight was 0.94.

CRITICAL ASSESSMENT OF THE METHOD

The correlation between MRI liver volume and liver weight has been established by a number of groups using a variety of MRI methods indicating the robustness of the technique. Its advantage over the direct measurement of liver weight is the dramatic sparing of animals as groups of animals no longer need to be sacrificed at each time point and because the ability to make within animal comparisons leads to greater precision and a reduction in group sizes. Hockings et al. (2002) reported a reduction in animal usage from 120 to 6 with the same level of precision. In order to measure liver volume with precision, it is necessary to produce good contrast between liver and surrounding tissues such as intercostal muscle, fat, spleen, stomach wall, and kidney. This can be done through judicious optimization of the MRI pulse sequence and timings. In addition, some researchers have used fat suppression pulses to null the signal from fat to enhance contrast to surrounding organs. Image

acquisition normally takes several minutes so motion from breathing and peristalsis in the GI tract can produce artifacts and blurring of the images. Fast imaging, averaging, breath holding, or respiratory triggering strategies can reduce motion artifacts from respiration. The respiratory-triggering strategy synchronizes data acquisition to the respiratory cycle and is the most widely applied strategy for preclinical liver volume determination. Peristaltic motility can be reduced by overnight starvation or the application of antispasmodics such as Buscopan; however, neither approach is usually necessary.

One possible confound for this experiment is that liver weight changes by up to 15% during the day as glycogen levels drop (Latour et al. 1999), and so care must be taken in longitudinal studies that animals are always imaged at the same time of day to reduce within animal variance. In addition, care must be exercised with the choice of anesthetic as anesthetics such as halothane are hepatotoxic and may influence the outcome of the study when there are several imaging sessions.

17.3 Cardiac Hypertrophy

PURPOSE AND RATIONALE

Measurement of cardiac function and morphology is a key part of the preclinical evaluation of experimental medicinal compounds. Blood pressure, heart rate, and electrocardiogram evaluation are part of the core portfolio of safety pharmacology studies carried out in conscious telemetry dogs. If results from the core battery of tests raise concern, then supplemental studies are conducted to measure endpoints such as left ventricular pressure, pulmonary arterial pressure, heart rate variability, baroreflex, cardiac output, ventricular contractility, and vascular resistance. However, many of these endpoints involve invasive surgery and so are only appropriate for acute single time-point studies. To date, there have been relatively few preclinical studies using MRI to measure cardiovascular function, especially in the dog which is a large animal species widely used in toxicology. MRI can be used to determine myocardial volume, wall thickness, and left ventricular (LV) and right ventricular (RV) end-diastolic and end-systolic lumen volumes (EDV and ESV, respectively). These parameters can be subsequently used to derive functional indices such as wall stress, degree of eccentric hypertrophy, stroke volume (SV), *cardiac output* (CO), and ejection fraction (EF). MRI studies are particularly suited to chronic-dosing regimen with multiple imaging time points in the same animals.

PROCEDURE

Adult male beagle dogs (Harlan UK) weighing between 9 and 14 kg are used. On days prior to scanning, food is withheld from approximately 4 p.m. Dogs are anesthetized with a bolus intravenous dose of propofol (approx. 10 mg/kg) followed by propofol (32–42 mg/kg/h) maintenance anesthesia and ventilated with medical air via an endotracheal tube. The dorsal metatarsal or femoral artery is cannulated for blood pressure measurements and to enable sampling of arterial blood for monitoring blood gasses to ensure adequate ventilation. ECG, capnography, pulse oximetry, body temperature, and arterial blood pressure are monitored throughout the scanning sessions on a Bruker Maglife C (Wissembourg, France). Body temperature is maintained with the aid of a thermostatically controlled heating blanket.

MRI scanning is performed in a 1 meter bore 2 T Bruker Medspec (Ettlingen, Germany) using a 28-cm transmit/receive birdcage resonator. ECG triggered segmented gradient-echo cine images are acquired during the expiration phase of the respiratory cycle as measured directly from the ventilator. An average of 16 frames per heart cine traverses approximately 80% of the cardiac cycle starting from end diastole. Other relevant imaging parameters are gradient-echo flip angle 20°, TE 3 ms, TR 8 ms, 1–3 averages, SW 100 kHz, image matrix 128 × 128, in-plane field of view 200 mm, four phase-encoding steps per frame, and linear traverse of k-space. Hence, the time resolution per cine frame is 32 ms. Each individual slice cine is acquired in about one to one and a half minutes depending on heart rate, so each set of multislice cines takes about 15–20 min.

To obtain true short axis views, scout imaging commenced with a mid-ventricular coronal slice allowing the vertical long axis (VLA) to be located by aligning another scout through the apex and mid-mitral valve, thus allowing for the leftward angle of the heart. From the VLA, the downward inclination of the heart is allowed for by taking a further scout lining up the apex and mid-mitral valve to generate the horizontal long axis plane (HLA). The scouts are acquired at end diastole (0 ms delay after the QRS wave) so that the

atrioventricular (AV) ring, which descends apically in systole, is in its most basal position. The first short-axis cine is then placed just forward of the AV ring on the HLA image, to cover the most basal portions of the right and left ventricles. Approximately 15 contiguous 5-mm-thick segmented gradient echo cines with no interslice gap are then sequentially acquired moving toward the apex and including the apical tip. In this way, the entire ventricle is imaged.

EVALUATION

Frames corresponding to end diastole and end systole are identified from each cine sequence and regions-of-interest (ROI) drawn around the left ventricular (LV) epi- and endocardial borders using ParaVision software (Bruker). The area of the ROIs is summed and multiplied by the interslice distance (5 mm) to calculate the end-diastolic and end-systolic volumes (EDV and ESV) of the whole ventricle and lumen. Other cardiac parameters are calculated as follows:

Stroke volume: $SV = EDV_{Lumen} - ESV_{Lumen}$
Cardiac output: $CO = SV \times \text{heart rate}$
Ejection fraction: $EF = (SV/EDV_{Lumen}) \times 100$

Left ventricle myocardial mass at end systole is calculated as:

$$\text{MassLV} = (ESV_{Ventricle} - ESV_{Lumen}) \times D$$

where D is the density of the myocardium (1.05 g/mL) (Hoffmann et al. 2001).

Left ventricle myocardial wall thickness in diastole is calculated from the epi- and endocardial areas at the slice where the epicardial area is maximum as follows:

$$\text{LVwall thickness} = \sqrt{\frac{\text{Area}_{LV}}{\pi}} - \sqrt{\frac{\text{Area}_{Lumen}}{\pi}}$$

The two ROIs used are assumed to be concentric and circular.

MODIFICATIONS OF THE METHOD

Markiewicz et al. (1987) examined eight pentobarbital anesthetized dogs and reported that cardiac output and stroke volume measured by ECG-triggered MRI correlated significantly with thermodilution measurements ($r = 0.73$ and 0.93, respectively). Shapiro et al. (1989) also used ECG-triggered MRI in dogs subjected to myocardial infarction and found excellent correlation between MRI-derived myocardial mass and wet weight ($r = 0.97$) and that MRI-derived myocardial mass measured in systole and diastole correlated closely ($r = 0.95$). Bambach et al. (1991) examined carbon monoxide–induced ventricular hypertrophy in rats using scan averaging instead of triggering to reduce artifacts from cardiac motion. They found that the mean outside diameter of the left ventricle plus interventricular septum (LV + S) showed a strong correlation with the duration of CO ($r = 0.73$, $p < 0.01$) and to the hematocrit ($r = 0.72$, $p < 0.05$). Rudin et al. (1991) used a dual respiratory-gated and ECG-triggered approach in two models of cardiac hypertrophy in rats. The correlation coefficient between LV mass determined by MRI and post-mortem LV weight was 0.99 and LV volume, SV, and EF in control animals showed statistically significant differences from cardiac hypertrophy animals. Siri et al. (1997) applied ECG-triggered MRI to murine hearts and found LV mass determined by MRI correlated well with LV weight ($r = 0.87$). This data demonstrated the dependence of LV mass estimates in the mouse on the geometric model of the heart used and show that MRI provides more accurate estimates of LV mass in mice than does two-dimensional-directed M-mode echocardiography. Slawson et al. (1998) used a dual respiratory- and cardiac-gated MR sequence in mice and obtained a correlation coefficient of 0.99 between MRI and post-mortem heart weight. Hockings et al. (2003b) used the method described above to measure dobutamine- and minoxidil-induced changes in cardiac function in dogs. They showed good correlation between cardiac output measured by MRI and cardiac output measured by thermodilution ($r = 0.94$) and that MRI could reliably detect acute changes in cardiac output induced by dobutamine infusion ($p = 0.01$) in small groups of animals ($n = 7$). Furthermore, they showed that MRI could detect LV enlargement induced by chronic administration of minoxidil and that the increase in EDV without an accompanying change in LV wall thickness indicated a preload-induced hypertrophy. Interestingly, the MRI technique was able to detect small amounts of pericardial effusion.

CRITICAL ASSESSMENT OF THE METHOD

MRI has become the gold standard imaging technique for the study of the human heart. The main advantages are that it is noninvasive and has pronounced contrast

between myocardium and blood and good temporal resolution allowing images to be acquired at any phase of the cardiac cycle. Thus, it is an accurate technique for measuring ventricular volumes independent of geometric assumptions, although clearly the precision with which myocardial geometry can be characterized depends on the number of image slices acquired through the heart and on the in-plane resolution. Image acquisition during end diastole and end systole allows the calculation of functional parameters such as stroke volume, ejection fraction, and cardiac output. One of the most important factors in the acquisition of artifact-free images is the quality of the MRI system's ECG and respiratory triggering. Cardiac exams in the clinic are usually conducted using breathhold rather than with respiratory gating because of the difficulty of obtaining a regular breathing cycle in conscious volunteers and patients. However, in anesthetized animals, breathing irregularities are not usually a significant problem and complications due to the increase in heart rate with hypercapnia during breathhold usually outweigh the time penalty involved in waiting for the respiratory gate. The studies described above indicate that combined respiratory gating and ECG triggering improve the precision of measurements.

Alternatives to MRI include echocardiography to measure LV wall thickness, lumen volume, and cardiac output (Coatney 2001; Collins et al. 2003; de Simone et al. 1990; Zhou et al. 2004), dye-dilution techniques such as bolus thermodilution to measure cardiac output (Siren and Feuerstein 1990), and implanted pressure transducers and flow probes to measure left ventricular pressure and blood flow parameters. Like MRI, echocardiography is noninvasive and has the further advantages that it provides low cost, real-time images with structural, functional, and hemodynamic information. Functional information is usually acquired in M-mode, and hence it is necessary to make geometrical assumptions that may not be applicable if heart morphology changes. In addition, the superior inter-study reproducibility of MRI in comparison with 2D echo leads to better reliability of observed changes and thus greatly reduced patient numbers in clinical trials (Grothues et al. 2002). Both dye-dilution and implanted pressure and flow probes are invasive techniques.

When planning functional studies, it is important to consider that most anesthetics cause cardiac and respiratory depression. For chronic studies, it may only be important to ensure that the depth of anesthesia is reproducible from imaging session to imaging session; however, for acute studies, it is necessary to consider interactions between the anesthetic and the test substance. The complexity of cardiac structure and function needs to be understood to devise a well-planned imaging protocol.

17.4 Hepatic Steatosis

PURPOSE AND RATIONALE

Hepatic steatosis is a side effect associated with a number of classes of compounds including some metal compounds, cytostatic drugs, antibiotics, and estrogens. In some cases, drug-induced hepatic steatosis patients can present with a rapid evolution of severe hepatic failure, lactic acidosis, and ultimately death (Diehl 1999). The absence of predictable correlation between abnormalities in liver enzymes and histologic lesions led Clark et al. (2002) to conclude that localized magnetic resonance spectroscopy (MRS) was the best noninvasive way to quantify liver fat in patients. This approach was favored because it avoids the risks associated with invasive liver biopsy. Lee et al. (1984) demonstrated that MRI can detect fatty infiltration of the liver clinically, and Longo et al. (1993) demonstrated that MRS is a reliable noninvasive method, comparable to computerized tomography (CT), for quantifying clinical liver steatosis in humans. Recently, Szczepaniak et al. (2005) used localized MRS to show a strikingly high prevalence of hepatic steatosis in the US population, and Cuchel et al. (2007) showed that treatment with BMS-201038 was associated with hepatic fat accumulation, a potentially serious adverse event. A trend toward increased hepatic fat was also seen by Visser et al. (2010) after treatment with mipomersen.

For 20 years, localized MRS has been used in medicine and biomedical research to obtain noninvasive biochemical information from living tissue (Koretsky and Williams 1992). The spectra obtained possess the very valuable property that the intensity of a given peak is proportional to the number of nuclei contributing to that peak provided that certain experimental precautions are taken. This allows a quantitative determination of a substance if there is an appropriate internal or external reference. In the case of localized in vivo 1 H spectroscopy, the water

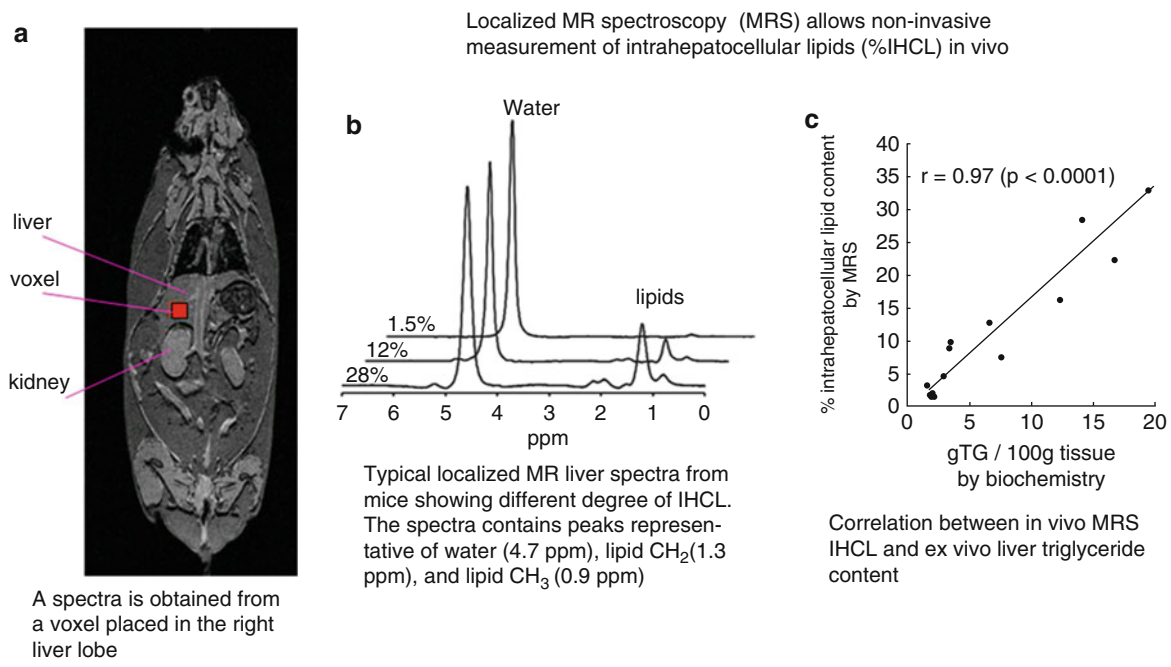

Fig. 17.2 (**a**) Coronal view through the liver of a Café diet mouse showing the position of the 2 × 2 × 2-mm-localized MRS voxel in the right lateral lobe of the liver, (**b**) in vivo–localized PRESS spectrum from three mice with different degrees of hepatic steatosis, and (**c**) correlation between in vivo MRS and ex vivo triglyceride measurements (Abdel Wahad Bidar, AstraZeneca, personal communication 2007)

signal is usually chosen as internal standard as the proportion of body water to ash and protein is relatively invariant. Single-voxel localized MRS allows spectra to be obtained with spatial resolutions down to 8 μL in some circumstances allowing localization of a volume of interest entirely within the liver in animals as small as mice (Fig. 17.2).

PROCEDURE

Isoflurane anesthetized mice or rats can be scanned in a dedicated small animal MRI system with a transmit/receive radiofrequency birdcage-design resonator. MRI and MRS acquisition are synchronized with the respiratory cycle to minimize artifacts (Schwarz and Leach 2000; Wilson et al. 1993). Scout multislice spin-echo images through the liver are used to determine voxel placement. Localized 1 H PRESS spectra (Bottomley 1987) with, for example, TE/TR 6/3,000 ms and 64 averages can be obtained. For the mouse, a 2 × 2 × 2-mm cube in the right lateral lobe adjacent to the portal vein and well removed from the surface of the liver and distinct hyperintense fatty deposits is appropriate to provide sufficient signal to noise.

EVALUATION

Quantification was accomplished by simulating the water signal (which was used as a chemical shift reference) at 4.7 ppm, and the fat signals at 2.1, 1.3, and 0.9 ppm, with an 80:20 Gaussian–Lorentzian lineshape using the Bruker XWINNMR package. Without knowing the average lipid chain length and degree of unsaturation, it is impossible to calculate a valid molar fat–water ratio, so the intrahepatocellular lipid (IHCL) content is expressed as the percentage of the sum of the fitted peak areas of the three fat peaks to the fitted water peak area.

$$\% \text{ IHCL} = 100 * \left(\frac{A_{(\text{lipid})}}{A_{H_2O} + A_{(\text{lipid})}} \right)$$

MODIFICATIONS OF THE METHOD

Hazle et al. (1991) used MRS to follow the time course of ethanol-induced liver steatosis in rats. Spectra were acquired without respiratory triggering, and lipid signal was normalized to signal from an external reference sample. Correlation between MRS normalized lipid signal and biochemically determined lipids

was moderate ($r = 0.52$). Ling and Brauer (1992) used respiratory-triggered MRS to examine the same model and were able to show that a 5.5-fold increase in lipid signal on treatment was matched by ex vivo analysis although a correlation coefficient was not given. Szczepaniak et al. (1999) used two animal models to show a close correlation between hepatic triglyceride measured by in vivo MRS and liver biopsy ($r = 0.93$). These researchers converted the MRS fat–water signal ratio to micromoles triglyceride/gram wet tissue by correcting for NMR relaxation and triglyceride proton density relative to water. Daubioul et al. (2002) used non-triggered localized MRS to show a reduction in hepatic steatosis in Zucker rats fed a dietary supplement with non-digestible carbohydrates. The spectra presented showed artifacts consistent with respiratory motion during acquisition. Hockings et al. (2003a) measured the MRS fat–water ratio in the livers of Zucker rats. They found a good correlation between MRS fat–water ratio and the fractional volume of intrahepatic fat determined by histology ($r = 0.89$) and were able to show that rosiglitazone treatment reduced liver fat content. Kuhlmann et al. (2003) reported similar findings in Zucker diabetic rats treated with rosiglitazone. Liver lipid levels in mice were examined by Garbow et al. (2004b). They reported that respiratory-triggered acquisition of spectra was important to remove the deleterious effects of respiratory motion and that the variation in MRS lipid content across the liver was typically less than 10%. The correlation coefficient between in vivo MRS and ex vivo wet chemistry lipid measurements was 0.95. Zhang et al. (2004) reported the use of a respiratory-triggered 3D three-point Dixon MRI method to determine liver fat–water ratio in rats treated with a microsomal transfer protein inhibitor known to produce hepatic steatosis. They reported a high level of reproducibility in in vivo measurements and were able to detect drug-induced steatosis, but the correlation coefficient against liver triglyceride and information on spatial inhomogeneity of lipid accumulation in the liver were not given.

CRITICAL ASSESSMENT OF THE METHOD

A number of both clinical and preclinical studies have shown a robust correlation between liver fat–water signal ratio measured by in vivo–localized MRS and ex vivo analysis. Most groups have used a short echo time PRESS sequence with respiratory triggering to reduce motion artifacts and water as an internal standard. Both liver biopsy and single-voxel localized MRS are hampered by sampling errors if fatty infiltrations are inhomogeneously distributed in the liver. In the clinical setting, alternative MRI or spectroscopic imaging techniques have been used to measure lipid content across the entire liver where there is a risk of fatty infiltrations. Preclinically, Ling and Brauer (1992) have shown that fat is distributed homogeneously throughout the liver in rats with ethanol-induced hepatic steatosis, and Garbow et al. (2004b) reported similar findings for wild-type and two transgenic strains of mice on low-fat or high-fat diets. Most researchers avoided the problem of potential inhomogeneous lipid distribution by selecting one region of the liver and always returning to the same region in serial time-point studies. For preclinical studies, it is clearly possible to kill groups of animals at each time point, but particularly when the within group variability is large in comparison to the measurement precision, the introduction of a noninvasive technology can result in a dramatic sparing of animals.

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Oncology Pharmacology

18

Jason H. Gill

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18.1 General Considerations

The development of anticancer pharmaceuticals and safety evaluation of such agents for human use is strongly dictated by the nature of the disease being treated. Cancer is a complex and heterogenic genetic disease involving an almost infinite number and combination of cellular and molecular perturbations. Unlike many other diseases, in its early stages cancer is often asymptomatic and remains hidden, with the problem only being identified later in the process once the cancer has become symptomatic and noticeable by the patient. Unfortunately, by this stage the cancer is commonly aggressive and in many cases has disseminated to other sites within the body. As a consequence cancer is often life-threatening with patients having a poor prognosis and significantly reduced life expectancy, factors which significantly alter the risk-benefit balance in this therapeutic area. Therefore the successful development of new treatments for cancer can have an immediate impact upon patient survival.

Until recently, the vast majority of agents used within the clinic were cytotoxics targeting nucleic acid replication or synthesis, many of which have been approved for clinical use since the 1960s. Due to the desire to obtain maximum efficacy, this class of drug are administered at or close to their maximum tolerated dose. Mechanistically these agents do not exclusively target cancer cells, and will also attack any rapidly proliferating cell type such as those in the digestive tract and bone marrow, commonly resulting in toxicity. As a consequence of this inherent toxicity, there is a high level of attrition during development of this class of agent (Walker and Newell 2009; Westhouse 2010). Despite a high prevalence of

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toxicity, a lack of alternative therapeutic avenues meant that the benefit of cancer treatment was deemed to be far superior to the unrequired toxic side-effects. Consequently, until recently, nonclinical safety evaluation studies were deemed of minor significance compared to therapeutic efficacy and rarely prevented progression of these drugs into first-in-human studies. In recent years, there has been a considerable shift in development of cancer therapeutics, particularly in terms of their safety evaluation.

Over the past decade, increased understanding of the molecular basis of cancer has advanced cancer therapy into an era of "targeted molecular therapeutics" (Collins and Workman 2006; Newell 2005). These new targeted drugs exhibit a broad range of therapeutic mechanisms, including inhibition of extracellular growth receptors, activation of cell death pathways, retardation of cell motility, kinase inhibition, and toxin delivery, to name a few. Despite many of these drugs significantly improving cancer treatment and patient survival, their targeted nature offers a set of potential liabilities and safety issues different to that of the cytotoxic genre.

The development of therapeutics to treat cancer is conceptually more difficult than for other non-life threatening diseases for two particular reasons; (1) The majority of cancer therapeutics have been developed to either kill the cancer cell or at least inhibit its proliferation, and thus have inherently high toxicity. (2) Initial clinical trials of new cancer therapeutics involve administration of the drug to the cancer patient population rather than healthy volunteers, due to the intended mechanism of action of these therapeutics. These patients would previously have received all current therapeutic strategies without success, are highly likely to be ill, and their disease will have been classified as terminal. Consequently, determination of safety of these drugs has several inherent complications, not least the drug mechanism and the intended clinical population.

Historically, the rationale for preclinical evaluation of any new pharmaceutical was determined by the potential risks the therapeutic posed to humans. For the reasons outlined above, such a framework is inappropriate for cancer drug development as it inadequately considers the short-life expectancy of the patient, the life threatening nature of the disease and the lack of efficacious therapeutics. In contrast to other therapeutic areas, more emphasis is required on potential benefits of new treatments for advanced cancer balanced by a requirement to manage any resulting toxicity. In order to reduce safety-related attrition of cancer therapeutics and simultaneously improve cancer treatment it is important that compounds with the greatest safety margin in nonclinical studies, involving significant efficacy and minimal toxicity, are identified and progressed. Taken together this supports the fact that the type and timing of nonclinical safety studies for anticancer therapeutics has significantly different goals and required outcomes relative to non-oncology therapeutics. These issues and requirements resulted in development of the ICH S9 guidelines, "Nonclinical Evaluation for Anticancer Pharmaceuticals" (ICH 2010a), announced in the Federal Registry. These are the only ICH guidance devoted to a specific therapeutic area.

The ICH S9 guidelines are not currently legally binding, but provide a minimum standard for harmonization of nonclinical study requirements in support of oncology therapeutics across the European Union, Japan, and the United States. The guidelines emphasize improved evaluation of pharmacological efficacy, and incorporation of toxicity endpoints which can reflect acceptable toxicity for this class of agent as a basis for clinical starting dose selection. The principle objectives of the S9 guidelines are to: (1) decrease the attrition rate of new cancer therapeutics, (2) increase the clinical dose escalation rate to attain an active dose, (3) reduce, refine and replace animal use for drug development studies, in line with international efforts, and (4) ultimately reduce the time taken for a cancer drug to enter the clinic.

The guidelines are strict with regards therapeutic area and relate specifically to the development of both small molecule drugs and biotechnology-derived therapeutics (including antibodies and peptides) for the treatment of cancer. Excluded from the guidelines are medicines for cancer prevention, vaccines, cellular therapeutics, and pharmaceuticals developed to treat the symptoms or side effects of cancer therapy. Specifically, the ICH S9 guidelines cover the development of therapeutics for "patients with advanced cancer whose diseases is refractory or resistant to current therapy, or where current therapy is not considered to be providing benefit" (ICH 2010a). A major underlying principle for the S9 guidelines is that, relative to non-cancer therapies, a higher health risk for a patient with advanced cancer is likely to be acceptable in light

of their limited life-expectancy. Accordingly, the S9 guidelines are not applicable to pharmaceuticals intended for patients with a long life expectancy. In this regard, the guidelines are non-prescriptive with the definition of advanced cancer or life-expectancy, implying that additional data requirements maybe applicable for patients with longer life-expectancy (Ponce 2011). Although not explicitly defined within the S9 guidelines, short life-expectancy is defined within the ICH S1A guidelines, in the context of nonclinical carcinogenicity studies, as < 2–3 years (ICH 1998). Therefore, defining patients with advanced cancer as being those with a median life-expectancy of less than 3 years appears a reasonable basis for application of the ICH S9 guidelines (Ponce 2011).

In principle, the nonclinical safety requirements outlined in the ICH safety guidelines, ICH S1A to ICH S8, are still applicable to the development of cancer pharmaceuticals and the submission of an IND application prior to clinical evaluation. However, as detailed in the ICH S9 guidelines, the counterproductive nature of a protracted drug development process for life-prolonging cancer pharmaceuticals led to modifications of the standard "safety package" such that long-term effects of the pharmaceutical is now defined unnecessary (or at least deferrable) for oncology therapeutics. Accordingly, the majority of assays and methodologies for evaluation of safety of cancer pharmaceuticals are covered in detail elsewhere in this book. This chapter specifically describes screening methodology of particular relevance to oncology therapeutics, not covered in detail in other chapters, and defines the ICH S9 guideline modifications and alterations from general drug screening practices suggested for oncology therapeutics.

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18.2 Nonclinical In Vitro Assessment of Toxicity

Conventional cancer therapeutics, principally antiproliferative or cytotoxic agents, are effective agents, with the downside in that they also demonstrate significant toxicities due to their effect upon cells and tissues with a high turnover (e.g., cells of gastrointestinal tract and the bone marrow) and those with metabolic or excretory functions (e.g., liver and kidney). Although the new generation of cancer therapeutics are designed to target specific molecular irregularities of cancer, many have also been shown to demonstrate a toxic effect upon other cell types. To reduce the high level of drug attrition with cancer therapeutics, significant effort has been placed on predicting likely toxicities and identifying therapeutics with high efficacy coupled to low toxicity as early as possible within the drug development cycle. Several methodologies have been developed for such goals.

18.2.1 Assessment of Cellular Toxicity: MTT Assay

PURPOSE AND RATIONALE

One of the initial stages of pharmacological screening within cancer therapeutic drug programmes is to determine the anticancer potential of a test therapeutic against cancer cells in vitro. At early stages in the drug discovery process the putative therapeutics are screened against a panel of human cancer cell lines to evaluate both patterns of tumor selectivity and potency (Shoemaker 2006). To improve screening efficiency and increase study throughput, the US National Cancer Institute (NCI) developed a 60 human tumor cell line screen (NCI60) to fulfill this objective (Shoemaker 2006). In these screens, therapeutics with common mechanisms of action demonstrate similar profiles of growth inhibition (Holbeck et al. 2010). In addition to

ascertaining the therapeutic potential and confirming proof of drug-target interaction, the same assays as used for efficacy can also be utilized to determine the effect of the therapeutic on non-cancer cell types, as either a secondary or off-target pharmacological effect.

In contrast to cancer cells, normal cells often have a lower proliferative index, are reliant on the heterogenous cellular nature of their host tissue for survival, and have a finite number of cellular divisions, making their in vitro culture very challenging. Over recent years, significant advances have been made with characterization of in vitro growth conditions for toxicological assessment of several human primary cell types, including renal, hepatic and haematological cells (Gomez-Lechon et al. 2010; Krishna et al. 2009; Li et al. 2003; McKim 2010; Valente et al. 2011). In addition, several laboratories have also developed toxicity assays using stem cells, characterized by their capacity to differentiate into many different cell lineages (Lee et al. 2011; Shuga et al. 2007, 2010). Despite these improvements, it is still common practice to utilize immortalized cell lines, such as HepG2 and HuH7 (liver), or HEK293 (kidney). An important benefit of the latter approach is that these cell lines are generally fully characterized in terms of morphology, behavior and biochemistry. On the downside, many of these cell lines do not recapitulate the full metabolic or differentiated status of the organ they represent.

Many screening tests have been developed for in vitro evaluation of cytotoxicity over the years, ranging from dye exclusion techniques of vital stains such as trypan blue or neutral red (Cavanaugh et al. 1990), to bioluminescent detection of released intracellular proteases following loss of membrane integrity (Cho et al. 2008) or quantitation of cellular ATP levels (Crouch et al. 1993; Shukla et al. 2010), to the most widespread technique which involves metabolism of tetrazolium salts by intracellular organelles of viable cells (Mosmann 1983; Shoemaker 2006). The most popular of these assays, the MTT assay, measures the reduction of the yellow MTT tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to dark purple formazan by succinate dehydrogenase present in mitochondria of metabolically active cells, with the amount of formazan created being directly proportional to the viable cell number. Importantly, no interference of the product with the substrate is observed with this assay, allowing it to be conducted in a 96-well plate format with spectrophotometric detection (Mosmann 1983; Shoemaker 2006).

PROCEDURE

The method for the MTT assay developed by Mossmann et al. is described below (Mosmann 1983; Shoemaker 2006). Cells are seeded into 96-well cell culture plates at a density dependent upon the cell-type and intended duration of therapeutic exposure, with the target of approximately 70% cell confluence at time of analysis. In the case of primary cells, the plate may be pre-coated with a matrix protein to aid attachment, if necessary. Following a period of monolayer establishment, normally 24 h, cells are then exposed to a dose range of the therapeutic for the experimental duration, which can range from 1 to 72 h dependent upon the objective of the study. After this exposure period, the MTT solution is added to each well, and the plate incubated at 37°C for 4 h. The insoluble formazan (purple) is then solubilized in dimethyl sulphoxide (DMSO) and the well absorbance read at 540 nm. The color generated is stable for a few hours at room temperature, but can be read directly following the crystal solubilization. Since the reduction of MTT varies from one cell type to another, standard curves showing the different metabolic activities must be determined for each cell type.

EVALUATION

The MTT assay is selective for metabolically active cells, showing a direct correlation between formazan absorbance at 540 nm and viable cell number (Mosmann 1983; Shoemaker 2006). From this data, dose-response relationships can be determined, as well as the IC_{50} (dose causing 50% reduction in cell number). In this context, an in vitro therapeutic index of a putative cancer therapeutic can be determined, through comparison of the IC_{50} of cancer cells versus that of normal cells. Ideally, a clear differential in cytotoxicity between the cancer and non-cancer cells is required, since agents anticipated to reach therapeutic plasma concentrations that are greater than or equal to the toxicity IC_{50} of the non-cancer cells have a high probability of dose limiting in vivo toxicity (McKim 2010).

MODIFICATIONS OF THE METHOD

One limitation of the MTT assay is the production of an insoluble formazan product, requiring solubilization

in DMSO prior to spectrophotometric measurement. Consequently, water soluble analogues of MTT have been developed, such as XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Scudiero et al. 1988). These water soluble variants do not require solubilization in organic solvents prior to colorimetric analysis, allowing immediate reading of cell number in the culture well without the requirement for further processing. Relative to MTT, the use of XTT also offers the advantages of being suitable for automation, and being more reproducible. On a negative note, a high background is obtained with XTT and this substrate is not reduced as easily as MTT by mitochondrial enzymes, often requiring electron couplers such as phenazinemethosulfate to promote rapid electron transport to XTT. Consequently, convenience must be balanced with lack of sensitivity when considering XTT versus MTT for this option.

The relative lack of stability of MTT and XTT and consequent requirement for preparation immediately prior to use, coupled with the fact that they have inherent toxicity, resulted in the development of several further improvements to this methodology, such as Alamar blue (Hamid et al. 2004). Whereas MTT was colorimetric, Alamar blue is converted by reduction in viable cells from a non-fluorescent substrate to a fluorescent pink non-toxic product (λ_{ex} 530 nm, λ_{em} 590 nm). Consequently, the Alamar Blue assay is both sensitive and highly reproducible (Hamid et al. 2004). The disadvantage to this method is the requirement for a fluorescence plate reader, rather than a spectrophotometer.

CRITICAL ASSESSMENT OF THE METHOD

The use of cytotoxicity assays, such as the MTT assay, is widespread across the drug discovery and development sector and is a valuable tool for initial evaluation of a therapeutic index. In order to interpret this data and relate it to the in vivo situation it is important that reference factors are also taken into consideration, including compound solubility and metabolic stability, and not just the value obtained from the MTT assay. Additionally, it is important to remember that the MTT assay, along with the majority of in vitro cytotoxicity assays, do not fully recapitulate the in vivo situation, in that they often do not address protein binding, metabolite activation and stability, and systemic pharmacology. These limitations must always be borne in mind or resolved when attempting to directly extrapolate in vitro observations to the in vivo setting (McKim 2010).

In order to improve assessment of drug efficacy and safety, it is strongly suggested that the MTT assay be integrated into a multiple parameter package of in vitro cytotoxicity assays (for instance, addition of a membrane integrity screen) (McKim 2010). This is especially important when appreciating that therapeutics can affect several subcellular targets, with varying exposure concentrations and times. The reliance specifically on the MTT assay (or any other single in vitro cytotoxicity assay) for predicting in vivo toxicity is beholding to the toxicity mechanism itself. For example, a therapeutic which inhibited mitochondrial reductases would provide erroneous results in the MTT assay (Hamid et al. 2004).

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18.2.2 Assessment of Haematological Toxicity: Colony Forming Unit Assay

PURPOSE AND RATIONALE

A major dose limiting toxicity of both conventional and molecular-targeted cancer therapy is myelosuppression. The rapid proliferation of haematopoietic stem cells and the motile and invasive nature of cells within the bloodstream results in this cell population being sensitive to a wide range of cancer therapeutics. Haematopoietic stem cells are pluripotent and can generate into almost all cell types of the immune system, generating cells of both lymphoid and myeloid lineages. In addition, these stem cells have the capability of self-renewal meaning that following division one cell replaces the stem cell whilst the other is committed to differentiate. An important goal for the successful development of cancer therapeutics is to predict whether it will be clinically toxic to the bone marrow, whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes, or erythrocytes), at what dose or exposure level the drug will be toxic, and when the onset and recovery are likely to occur. The assay which is currently used to predict myelosuppression, both clinically and nonclinically, is the colony-forming unit assay (CFU). Although several iterations of the CFU assay exist, each focusing on a specific haematopoietic cell type, assays focusing on granulocyte-macrophage precursors (CFU-GM) are routinely used to predict neutropenia, the commonest type of myelosuppression. In contrast to the majority of in vitro toxicity assays which report activity as an IC_{50}, the CFU-GM assay reports its activity based upon the IC_{90} value (Concentration inhibiting proliferation by 90%) as this has been demonstrated to be an accurate predictor of concentrations that produce dose-limiting haematological toxicity (grade 3 or 4 neutropenia) in the clinic (Parchment et al. 1998; Pessina et al. 2002, 2005). Following validation, the CFU-GM assay for predicting acute neutropenia using human derived cells was shown to be a valid substitute to the use of a second species, such as dog, for this purpose (Pessina et al. 2007). Importantly, the toxic concentrations calculated from the mouse and human in vitro CFU-GM studies have been shown to be predictive of human maximum tolerated doses in the clinic, through extrapolation of in vivo mouse toxicity data (Pessina et al. 2003; Pessina and Bonomi 2007).

PROCEDURE

The standardized protocol of the CFU-GM assay for evaluation of potential drug myelotoxicity developed by Pessina and Bonomi is described below (Pessina et al. 2007).

Bone Marrow cells (mu-BMC) are isolated from femur bone marrow of sacrificed mice (8–12 week old), with cells from three mice pooled together per experiment. For human studies, cryopreserved human cord blood mononuclear cells (hu-CBMNC) are used, since these are known to contain a high number of primitive haemopoietic progenitor cells. Each experiment normally consists of a linearity control (cells alone), therapeutic-vehicle control and six concentrations of therapeutic agent. Cells are cultured in a semi-solid methylcellulose-containing culture medium (MCM); primarily Iscove's modified Dulbecco's medium containing 1% Methylcellulose, 30% Fetal Bovine Serum, 1% Bovine Serum Albumin, 2 mM L-Glutamine and 10 ng/ml murine or human GM-CSF. Prior to cell addition, the respective volume of drug stock or drug-solvent (vehicle) is mixed with MCM. Cells are then added to each mixture, at 4×10^4 and 7.5×10^4 cells per dish for mu-BMC and hu-CBMNC, respectively. For the linearity controls, comparison plates should also be created containing 2.5×10^3 and 1×10^4 cells for mu-BMC and hu-CBMNC, respectively. The cell medium mixture (maximum volume

1 ml) is then added to 35 mm petri dishes, in triplicate. Cultures are placed in a humidified incubator maintained at 37°C, 5% CO_2, for 7 (murine) or 14 (human) days.

In many studies a preliminary dose finding study is first performed in order to identify both the Maximum Tolerated Concentration (MTC; complete inhibition of CFU-GM) and the Highest Non-Toxic Concentration (HNTC; highest dose that did not inhibit CFU-GM), with a secondary study performed at concentrations between these two values to identify the IC_{50} and IC_{90} values.

EVALUATION

Colonies are counted after 7 days (murine) or 14 days (human cells) by scanning the whole petri dish at low magnification for colonies. Aggregates containing 50 or more cells are defined as CFU-GM colonies, whereas smaller aggregates are termed clusters. Several different types of colonies will be present and should be counted as single colonies; Compact colonies (defined dense shape with few spread cells), Multifocal colonies (aggregates with potential halo of spread cells), diffuse and spread colonies (without apparent nucleus), and multicentric colonies (two or more central dense nuclei with common peripheral halo) (Pessina et al. 2003, Pessina and Bonomi 2007). It is advised that all dishes should be counted in a random fashion to reduce experimental bias. In order to determine the lower limits of acceptability for aggregates considered as a colony, it is advised that the highest drug level be examined first, since these colonies will be smallest and most difficult to define because of toxicity. Verification of colony growth linearity should be determined by comparison of the ratios between the number of colonies formed in low cell number control (2.5×10^3 or 1×10^4 cells per dish) and high cell number control (4×10^4 and 7.5×10^4 cells per dish for mouse and human, respectively) and testing the null hypothesis that the slope is equal to zero.

The IC_{90} value for the CFU-GM assay for both the mouse and human cells are calculated relative to vehicle control. The prediction of human Maximum Tolerated Dose (P-HuMTD) is calculated using the equation:

$$\mathrm{P-HuMTD} = \mathrm{Murine\ MTD} \times (\mathrm{Human\ CFU\text{-}GM}\ IC_{90}/\mathrm{Murine\ CFU-GM\ IC_{90}}).$$

Since pharmacokinetic differences across species may contribute to as much as fourfold difference in MTD, an accurate prediction of the myelosuppressive MTD is defined as that which lies within fourfold of the actual human MTD value (Parchment et al. 1998; Pessina and Bonomi 2007).

MODIFICATIONS OF THE METHOD

This CFU assay is primarily used to predict neutropenia, but can also be modified to predict clinical toxicity against other haematopoietic cell lineages, including lymphocytes, megakaryocytes and erthrocytes. The main differences between assays being the cytokine cocktail used to stimulate the progenitor cells. Drug-induced thrombocytopenia, a common toxicity of several anticancer therapeutics, is clinically defined as a dramatic decrease of platelet counts below 10^9/l, although this can be the result of bone marrow suppression or damage to circulating platelets. Since platelets are derived from proliferation and differentiation of megakaryocyte progenitors, the effect of therapeutics upon progenitor cells and consequent thrombocytopenia can be predicted using these cells in the CFU assay (CFU-Mk), with the standardized protocol previously reported (Pessina et al. 2009b). Comparison of the IC_{10}, IC_{50} and IC_{90} with maximal plasma drug concentrations (Cmax) in the mouse allows a robust prediction of the presence and degree of thrombocytopenia (Pessina et al. 2009b).

In light of the fact that the rat is the preferred species for nonclinical toxicology studies, the in vitro CFU-GM assay has now also been refined and optimized for this species, including the use of cryopreserved bone-marrow cells (Pessina and Bonomi 2007; Pessina et al. 2009a).

To determine potential haematological toxicity on a larger scale or for an increased throughput format, further assays have been developed using undifferentiated haematopoietic cells in liquid culture, in the presence of the respective cytokines and regulatory factors (Dal Negro et al. 2006a). Quantification of toxicity in these assays is performed either by manual counting of stained colonies, using flow cytometry, or through 96-well format luminescence assays which report intracellular ATP levels (Dal Negro et al. 2006a; Rich and Hall 2005).

A limitation of the standard CFU assay is that it only predicts direct toxicity and does not take into account a requirement for systemic or metabolic activation

(Dal Negro et al. 2006b). To achieve this goal, the methodology can be modified to include a metabolic activation step (liver microsomes, liver slices or isolated hepatocytes) to generate the relevant metabolites and determine their toxicity parameters, whether they result in synergistic or antagonistic effect upon haematotoxicity, and the effect of their detoxification (Dal Negro et al. 2006b). This new approach appears promising but has yet to be fully validated (Dal Negro et al. 2006b). In a further iteration of this assay, in vivo xenograft models have been developed containing humanized bone marrow to monitor haematotoxicity in a whole body system, with consequent reduced potential for interspecies differences (Cai et al. 2011). This humanized bone marrow mouse model was shown to be a feasible and physiologically appropriate tool with the potential to aid and improve screening for haematotoxicity (Cai et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

The CFU assay has great versatility for prediction of haematopoietic toxicity of cells from different lineages and shows a high level of correlation to the respective clinical toxicity. However, quantification of colony formation is still reliant upon manual observer interpretation of cell colonies and as such can be relatively subjective in nature. Alterations in the methodology toward liquid culture, as opposed to growth in semi-solid medium, have allowed the progression of the assay toward higher throughput screening of therapeutic agents and automated detection of colonies through technologies such as luminescent detection (Dal Negro et al. 2006a, b; Rich and Hall 2005). Although these modifications should in theory improve throughput and quality of data, methodological validation and comparison to the conventional CFU assay is still required to confirm the predictive value and benefits of these approaches.

It is well reported using in vitro clonogenic assays that interspecies differences exist with regards response of haematopietic cells to anticancer therapeutics (Kurtzberg et al. 2009; Masubuchi et al. 2004; Pessina et al. 2005). For example, mouse bone marrow is less responsive to several cancer therapeutics relative to human bone marrow, resulting in blood levels in nonclinical studies often been unattainable in human clinical trials (Cai et al. 2011; Kurtzberg et al. 2009). Therefore nonclinical assessments should be based on the results of several species (particularly human) prior to identifying a safe starting dose for clinical trial. As described above and in terms of neutropenia, the ratio of mouse to human CFU-GM IC_{90} has now been shown to adjust for the interspecies differences and has been accepted as a good predictor of human Maximum Tolerated Dose (MTD) (Pessina and Bonomi 2007).

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18.3 Nonclinical In Vivo Assessment of Toxicity

In reflection of the intended patient population, the severity and terminal nature of the disease, and the mode of action of cancer therapeutics, the selection of a clinical starting dose for cancer therapeutics has to be safe but must also be high enough to offer potential therapeutic efficacy. Consequently IND applications for cancer therapeutics must provide nonclinical data with clear translatability to clinical trials, as well as satisfying the other regulatory requirements. The main difficulty is in choice of the actual clinical starting dose, which must be balanced between choosing one which is low and thus safe, and one which is high enough to achieve as few dose escalations as possible to reach clinical maximum tolerated dose (MTD) or biologically effective dose.

Integrated evaluation of efficacy and toxicity for cancer pharmaceuticals has now led to many drug development organizations, particularly the National Cancer Institute (NCI), and several major pharmaceutical companies adopting a two phase cost-effective strategy to appraise potential safety aspects of cancer therapeutics; the first being a preliminary nonclinical assessment of toxicity in one or often two animal models, focused on identification of MTD, drug pharmacokinetics and identification of the potential therapeutic index. Successful fulfillment of program progression criteria then permits full-scale development, including comprehensive toxicity evaluation using the intended route, schedule and duration as the proposed clinical protocol and satisfaction of the regulatory requirements outlined in the ICH S9 guidelines.

In the majority of cases, the procedure and methodologies for evaluating safety of cancer pharmaceuticals is in agreement with other types of therapeutic and is addressed elsewhere in this book. Areas where there is deviance from the standard protocol are, (1) the modification of nonclinical rodent safety studies to facilitate attainment of maximum tolerated efficacious dose rather than the maximum effective dose, (2) the suggested modifications to the standard safety studies to accelerate development of this class of agent to the clinic, and (3) the mechanism by which the clinical starting dose is calculated, as outlined in the ICH S9 guidelines. These three areas will be discussed in the next section.

18.3.1 Determination of Nonclinical Maximum Tolerable Dose

PURPOSE AND RATIONALE

Many different types of nonclinical in vivo mouse model have been developed for efficacy assessment of cancer therapeutics, with the ever challenging goal of using a model which captures and is highly predictable for the clinical situation. These models range from autochthonous and spontaneous occurring tumor models, to human tumor xenografts in immunodeficient mice, to orthotopic tumor models, to genetically modified tumor and host models (Talmadge et al. 2007). The details of the advantages and disadvantages of these models with regards efficacy assessment is beyond the scope of this chapter, but is adequately reviewed elsewhere (Bibby 2004; Firestone 2010; Talmadge et al. 2007).

A comprehensive set of guidelines for the welfare and use of animals in cancer research have been compiled as a collaborative venture between academia, the pharmaceutical industry and government representatives, covering all aspects of developmental and therapeutic studies (Workman et al. 2010). All such studies are also still bound by the respective national legislation: USA Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals; UK Animals (Scientific Procedures) Act 1986; Public Health Service Policy on Human Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, National Institutes of Health, 2002), amongst others.

In addition to evaluating therapeutic efficacy, nonclinical animal models are also valuable for information relating to pathology, toxicity and ultimately

drug safety. Determination of efficacy and safety of small-molecule drugs or biopharmaceuticals (e.g., antibodies, protein therapeutics) targeted against cancer cells is initially conducted in rodents, prior to regulatory studies outlined in the ICH S9 guidelines.

PROCEDURE

Initial evaluation of maximum tolerated dose for the investigative agent should be conducted in mice. The maximum tolerated dose (MTD), in respect of cancer pharmaceuticals, being defined as the highest dose of a therapeutic in which the clinical condition of the animal is maintained, and which is independent of the mechanism of drug action (Workman et al. 2010). These tolerability studies must be conducted using the same strain as will be employed for efficacy studies, which in the majority of cases will be an immunocompromised model such as nude or SCID mice. With a few exceptions, these mice must also be non-tumor bearing.

Although these studies are not tightly prescriptive in terms of their design, the consensus across several groups and organizations is the use of two mice per dose level with a doubling dose-escalation or dose-halving de-escalation design (Workman et al. 2010). Such a study design agrees with the suggested guidelines for nonclinical rodent studies and fulfils a major aim of the S9 guidelines to reduce and refine experimental animal numbers (ICH 2010a; Russell and Burch 1959; Workman et al. 2010).

An important criterion when conducting these tolerability studies is to characterize the efficacy and more importantly the safety profile within the therapeutic dose range of the therapeutic, using a schedule which reflects that intended for the clinic, particularly the duration of treatment. The suggested dose schedules, as outlined in the ICH S9 guidelines, are identified in Table 18.1.

In the majority of cases for a new therapeutic, whereby the ideal dosing regimen is not yet identified, the starting point for MTD assessment should be set as a single dosing event. In these cases, escalation to a second dose level requires a minimal interval of 24 h, to allow acute toxic or safety concerns to be identified. For therapeutics intended to be delivered chronically or over a longer time period (e.g., daily for a month, orally or intravenously) the interval is suggested to increase to at least 5 days (Workman et al. 2010).

Table 18.1 Example schedules for oncology therapeutics to support clinical trials, adapted from ICH S9 guidelines (ICH 2010a). These schedules are intended for evaluation of conventional cancer therapeutics and should be modified accordingly for biopharmaceuticals with extended pharmacodynamics or half-lives, and potential for immunogenicity. In addition, the schedules outlined in this table do not identify recovery periods which should be incorporated into study design

Clinical schedule	Nonclinical study schedule
Single dose every 3 weeks	Single dose
Daily for 3 days, Repeated every 3 weeks	Daily for 3 days
Daily for 5 days, Repeated every 3 weeks	Daily for 5 days
Daily for 7 days, Repeat alternating weeks	Daily for 7 days, Repeat week 3
Single dose every 2 weeks	Repeat dose, 14 days apart
Single weekly dose for 3 weeks, 1 week rest	Once a week for 3 weeks
Two doses per week	Two doses per week for 4 weeks
Continuous daily	Daily for 28 days
Continuous weekly	Once a week for 4–5 doses

Additional to the dosing route and treatment schedule, toleration is also dependent upon the composition of the therapeutic solution being administered. Delivery of the therapeutic as an acidic or basic solution commonly is associated with complications due to the irritant nature of the vehicle, limiting the maximum deliverable dose. Therefore, the vehicle in which the therapeutic is delivered should be as close to physiological pH as possible, for example 0.9% saline or 5% dextrose/saline. In cases where the therapeutic is poorly soluble in aqueous solution, a vehicle comprising low concentration organic solvent, not exceeding 10% (or 5 ml kg^{-1}) of the total administered volume, could be used. A common used example being dimethylsulphoxide (DMSO). Chemical composition of the therapeutic may also necessitate inclusion of a detergent (such as Tween), an emulsifier, or a solubilizer, although these shouldn't exceed 20% of the total delivered volume. Additionally, cyclodextrin to a maximum of 45% total volume is also an accepted inclusion for drug administration of poorly soluble therapeutics, although use of this compound necessitates that the animals be rehydrated 2–4 h post-treatment (Workman et al. 2010).

The volume of therapeutic solution to be administered is also of consideration for assessment of tolerability, with the smallest possible volume being used in order to keep solvent related issues and subsequent

toxicity to a minimum. Although no definitive guidelines are available, the following suggestions are widely accepted as standard; oral/intraperitoneal dosing in mice of 10 ml kg^{-1} and intravenous dosing in mice of 5 ml kg^{-1} (equating to 200 and 100 μl for a 20 g mouse, respectively) (Workman et al. 2010).

EVALUATION

Clinical signs of toxicity observed during these nonclinical rodent tolerability and efficacy studies may manifest as either a behavioral, physiological or pathological change. In agreement with the guidelines for the welfare and use of animals in cancer research, all mice should be visually examined twice daily as a minimum (Workman et al. 2010). In addition to the legislative requirements for evaluation of animal physical and psychological distress or suffering, mice should be weighed frequently (i.e., daily) and their behavior observed. A median body weight loss of 15–20% of the weight prior to treatment requires cessation of dosing and monitoring of the mouse for weight recovery. Mice with weight loss exceeding 20% should be sacrificed.

The MTD for the therapeutic is the highest dose of a drug in which the clinical condition of the experimental animal is maintained and no significant drug-induced adverse effects are observed. Specifically, a dose of the drug causing weight loss less than 15% of the weight before treatment, not causing death due to toxicity events, or causing significant detrimental changes in animal wellbeing within 1 week after administration. The dosing schedule should reflect that used for the proposed efficacy studies.

MODIFICATIONS OF THE METHOD

The conventional methodology for evaluation of the MTD is to use healthy non-tumor bearing immunocompromized mice, which in the vast majority of cases is applicable. Despite this, the advent of "targeted molecular therapeutics" will in some cases require the additional use of tumor-bearing or immunocompetent mice for tolerability studies. Studies requiring immunocompetent mice include those involving biotechnologically derived peptides and antibodies. The use of tumor-bearing mice is potentially necessary for determination of the tolerability of tumor-activated prodrugs, although an alternative strategy maybe to evaluate the active moiety in the "standard" tolerability model.

As opposed to evaluation of therapeutic tolerability in non-tumor bearing mice (or for non-oncological conditions), care should be adopted when identifying apparent drug-induced adverse effects in tumor-bearing models as these maybe a result of tumor presence or response rather than a true drug safety concern. Although such events are rare and avoidable by appropriately designed experimental studies they should not be overlooked and should be appraised within all studies as standard. For example, dermal distension and ulceration, and cachexia can both be the result of tumor presence.

CRITICAL ASSESSMENT OF THE METHOD

The MTD in mice provides the foundation for calculating the first in human starting dose for the therapeutic, indicating the significant importance of this approach. The downside to this methodology is that the majority of clinical signs indicative of toxicity do not provide mechanistic information, but simply identify the presence of a toxicity either warranting further investigation or in severe cases animal termination.

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18.3.2 Evaluation of Oral Mucositis: Hamster Cheek Pouch Model

PURPOSE AND RATIONALE

Oral mucostosis is a major toxicity of non-surgical cancer therapy, particularly chemoradiation treatment

of cancers of the head and neck (Sonis 2004). Over the years, the screening of oral mucotoxic therapeutics and the effect of cancer therapeutics has been performed in several rodent models, particularly mice, rats and hamsters. Of these the male golden Syrian hamster model has proved most useful, through use of the buccal cheek pouch, a structure not found in other rodent models (Sonis et al. 1990). Using this model, a five-stage hypothesis of mucositis was developed; initiation, primary tissue response, signal amplification, ulceration and healing (Sonis 2004). Advantages of the hamster cheek over other rodent models are that the check pouch has a similar epithelial structure to the human oral cavity, the oral bacterial flora in the hamster is similar to humans, hamsters demonstrate a similar chemosensitivity profile to humans, and mucositis development in the hamster follows a predictable course. In addition, from an experimental perspective, the check pouch mucosa provides a large mucosal surface accessible for easy examination (Sonis 2004; Sonis et al. 1990). Although the commonest use of the oral mucositis models are to identify and assess agents to treat or prevent this adverse effect of cancer treatment, this model can also be used to evaluate the induction of mucositis in its own right (Bowen et al. 2011; Sonis et al. 1990).

PROCEDURE

The following methodology is based upon that of Sonis and colleagues (1990). Golden Syrian hamsters, aged 5–6 weeks and weighing 80–100 g should be used for this assay. Animals should be housed in small groups, fed standard chow and watered ad libitum. The dose and treatment schedule for administration of the experimental therapeutic should be chosen based on the nonclinical MTD, pharmacokinetic profile and intended clinical treatment regimen. An initial dose of the therapeutic should be administered systemically, through the intended (intravenous or intraperitoneal) route, on day 0 of the study. Since the cheek pouch mucosa is anatomically protected from functional trauma as occurs in humans, superficial irritation of the mucosa (a mechanically induced scratch) on the following day (Day 1) and for up to 3 days is performed. Therapeutic treatment is continued, using the predetermined schedule, and toxicity observed for a minimum of 14 days. The cheek pouch should be inspected on a daily basis for overt toxicity, and histological or morphological changes, such as erythematous changes indicative of mucositis (Sonis et al. 1990).

Table 18.2 Grading scale for mucositis severity in the hamster model

Grade	Mucosal appearance
0.	Pouch completely healthy, No erythema or ulceration
1.	Erythema and vasodilation, but mucosa intact
2.	Severe erythema with superficial mucosal erosion
3.	Mucosal ulceration with a cumulative size of about 25% of the pouch's surface
4.	Ulceration with a cumulative size of about 50% of pouch surface area
5.	Contiguous ulceration involving almost entire surface area of the mucosa

EVALUATION

The following 6-point scale for assessment of the severity of mucositis in the hamster check mucosa has been developed to grade mucositis (Table 18.2) (Sonis et al. 1990). This scale is analogous to that used for clinical scoring (National Cancer Institute-Common Toxicity Criteria).

MODIFICATIONS OF THE METHOD

The hamster cheek model has been used primarily as a tool for assessing anti-mucositis therepeutics, using the methodology described above. If information is required regarding the molecular basis of the toxicity or to further evaluate target cell populations, the check pouch can be readily excised from the hamster and evaluated using other technologies. In the case of histological evaluation, a single layer of tissue is optimal, created following surgical excision from the hamster.

This model is also amenable to the evaluation of chemoradiotherapeutic treatment strategies, using direct focal exposure of the cheek pouch to single dose or fractionated radiation (Alvarez et al. 2003; Watkins et al. 2010).

CRITICAL ASSESSMENT OF THE METHOD

In contrast to the human situation, induction of a "wound" or "irritation" in the hamster cheek pouch is required during the procedure. This can result in complications for toxicity analysis, particularly as result of induction of the wound healing response and consequent alteration in cellular and molecular signaling pathways.

Dose and schedule administered in the nonclinical model must be reflective of those expected clinically with the therapeutic, ideally with doses based on body surface area rather than dose per kilogram. Furthermore, species differences in susceptibility and metabolic capacity must also be borne in mind when translating results of these studies to the clinical situation.

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18.3.3 Evaluation of Gastrointestinal Mucositis: Dark Agouti (DA) Rat Model

PURPOSE AND RATIONALE

Mucositis can affect the whole gastrointestinal (GI) tract from mouth to anus, but differences are observed between the effects observed in the upper GI tract (oral and oesophageal) and lower GI tract (Bowen et al. 2011; Keefe et al. 2004). Whereas the epithelia in the upper tract is developed to resist abrasion and is therefore a squamous mucosa, that of the lower tract is focused on secretion, digestion and absorption and is predominantly columnar epithelia. Consequently, therapeutic exposure, function and kinetics of mucositis are different in the two sites. Mechanistically, mucositis is the same throughout the GI tract, with the specialized function and cellular differentiation status being responsible for the different susceptibilities of mucositis observed (Bowen et al. 2011; Keefe et al. 2004).

The Dark Agouti (DA) rat mammary adenocarcinoma model of gastrointestinal (GI) mucositis was developed by Keefe and colleagues in the mid-90s (Bowen et al. 2011; Gibson et al. 2003; Keefe et al. 2000). This model uses rats bearing a subcutaneously implanted isogenic mammary adenocarcinoma, specific to the DA rat, which arose spontaneously in the 1970s and has been subsequently passaged (Gibson et al. 2003; Keefe et al. 2000). This model is unique as it reflects the human GI tract changes that occur in humans following chemotherapy (Bowen et al. 2011; Keefe et al. 2000).

PROCEDURE

The model and methodology for assessment of mucositis using the DA rat model developed by Keefe and colleagues is described below:

Female dark agouti rats weighing between 150 and 170 g and bearing the mammary adenocarcinoma tumor are assigned into treatment and control groups. Immediately prior to therapeutic dosing, animals should be injected subcutaneously with 0.01 mg kg^{-1} atropine to reduce any resulting cholinergic reaction. The therapeutic to be evaluated should then be administered intraperitonealy (for the reasons outlined later), at a dose based upon nonclinical efficacy, tolerability and pharmacokinetics, and following the intended clinical therapeutic schedule (Table 18.1). Control animals should receive therapeutic vehicle, following the same route and schedule as for the therapeutic.

Animals should be examined at least three times a day for the development of diarrhea and physical signs of mucositis related toxicity. Rats presenting with severe diarrhea or mucositis related symptoms should be terminated, with physical signs being a dull ruffled coat with accompanying dull and sunken eyes, or being cold to the touch with a hunched appearance and no spontaneous movement.

The presence of toxicity and GI mucositis should be evaluated for 7 days (or as dictated by dosing duration) via monitoring of physiological signs of toxicity, and via examination of the GI tract from euthanized animals. The GI tract (from pyloric sphincter to rectum) should be dissected out, flushed with chilled isotonic saline (0.9% w/v) to remove contents, and the wet weights of small and large intestines recorded. Samples (1–2 cm in length) of small intestine (taken at 25% of the length of the small intestine from the pylorus) and the colon (taken at mid-colon position) should be

transferred to either Clarke's fixative (60% w/v ethanol, 40% w/v acetic acid) for subsequent intestinal morphometry analysis, or to 10% neutral buffered formalin for histopathologic assessment. In addition, it is advisable to also excise, weigh, and formalin fix and evaluate accessory organs of the GI tract, such as liver and spleen. Histopathological assessment of the GI tract should be used to evaluate changes in tissue structure and pathology, determine the proliferative and apoptotic index, with TdT-mediated dUTP nick end labelling (TUNEL) commonly used for apoptosis analysis.

For intestinal morphometry, sections (1 cm) of small intestine (jejunum, corresponding to 25% of the jejuno-ileum) and large intestine (colon, 50% of total length) should be opened onto cardboard, fixed in Clarke's fixative for 24 h and then stored in 70% ethanol at room temperature. For analysis, samples are rehydrated through an ethanol gradient, hydrolyzsed in hydrochloric acid (1 M) for 7 min at 60°C, washed, stained using Schiff's reagent, microdissected, and mounted in 45% (v/v) acetic acid prior to microscopic evaluation.

EVALUATION

Therapeutic-induced changes in intestinal pathology and morphometry should be determined by light microscopic analysis of the formalin-fixed and Schiff reagent stained microdissected intestinal samples. Using a calibrated graticule, the length, apical width, and basal width of at least 15 villi (jejunum) in the small intestine should be measured in the microdissected samples. Additionally, the length of at least 15 crypts in both the jejunum and colonic specimens should also be determined. Using these dimensions a trapezoid approximation should be used to calculate the villus area, with the crypt length and villus area correlating with crypt and villus epithelial cell populations, respectively (Gibson et al. 2003; Keefe et al. 2000). Comparisons between treatment and control, as well as temporal effects of the therapeutic can then be determined.

The presence and severity of diarrhea in the rats should be recorded using a four point scale, as described by Gibson et al. (2003) (Table 18.3).

Table 18.3 Grading scale for diarrhea severity in the mucositis model

Grade	Severity	Appearance of staining
0.	None	No diarrhea
1.	Mild	Anus staining
2.	Moderate	Over top of legs and lower abdomen
3.	Severe	Over legs and higher abdomen, often associated with continual oozing

MODIFICATIONS OF THE METHOD

The DA model uses tumor-bearing rats, which can have both advantages and disadvantages in terms of assessing toxicity due to the effects of tumor burden, immunological response and cost of the model. Non-tumor bearing models of mucositis have now been developed, the commonest of which is that developed by Howarth and colleagues (Bowen et al. 2011; Howarth et al. 1996). In this tumor-free model, chemotherapy has been used to induce intestinal damage prior to the assessment of anti-mucositis approaches, indicating its utility for evaluation cancer therapeutics (Bowen et al. 2011; Howarth et al. 1996). The caveat to the tumor-free approach is that response to chemotherapy has been shown to be worse in tumor-bearing compared to tumor-naïve rats, indicating that the tumor adds to the comorbidity following therapy (Gibson et al. 2007).

Molecular targeted therapeutics, such as the tyrosine kinase inhibitors TKIs, have been demonstrated to show some level of GI tract mucositis in the clinic. Bowen et al. have developed a new model for assessment of these agents, using the albino Wistar rat, chosen for the fact that this strain has an appropriate drug metabolising profile for assessing such therapeutics (Bowen et al. 2011). Results to date with this model have appeared to reflect the clinical scenario.

CRITICAL ASSESSMENT OF THE METHOD

As with several other methodologies for assessment of efficacy and toxicity, the route of therapeutic administration has a significant influence on the outcome of the study. In the Dark Agouti rat model, intravenous administration is particularly difficult due to the skin pigmentation of this rodent model. Consequently, therapeutics screened using this model are very rarely given by the intravenous route.

Another disadvantage of rat models of GI tract mucositis is caused by their lack of an emetogenic reflex, known to be a contributing factor to GI mucosal injury. However, it has been suggested that pica can be used a an indirect marker for nausea (Bowen et al. 2011; Vera et al. 2006).

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18.4 Application of ICH Guidelines to Oncology Therapeutics

PURPOSE AND RATIONALE

The ICH S9 guidelines were developed to reduce the attrition rate of cancer drug development with the specific goals of facilitating and accelerating development of new cancer therapeutics, whilst at the same time refining and reducing animal use and protecting patients from unnecessary side effects (ICH 2010a). The focus is on providing guidance on the design of nonclinical testing studies for the development of therapeutics for use in patients with advanced disease and limited therapeutic options. Specifically, nonclinical evaluation studies of therapeutics to identify and interpret the toxicological profile, pharmacological properties of cancer therapeutics, with the ultimate aim of establishing a safe starting dose for Phase I clinical trial (ICH 2010a). These guidelines apply to therapeutics, specifically small chemical chemotherapeutics and biotechnology-derived agents, intended to treat cancer in patients with life-threatening disease and a comparative short life expectancy, as described earlier in the introduction to this chapter.

The guidelines identify the type and timing of nonclinical studies required for progression of these new therapeutic entities into the clinic. The detail provided in the guidelines clearly indicates modifications required to the "standard" nonclinical screening programme in order to specifically address the specific mechanism of the cancer therapeutic or the manner in which it would be administered to patients. The guidelines clearly state the point during the development process when other ICH guidelines should be adopted, specific studies required to support development of cancer therapeutics, and rationale for inclusion or exclusion of specific safety studies. As a consequence of their mechanisms and intended disease, many of the longer-term effects of drugs addressed within general nonclinical evaluation studies are identified as being dispensable, deferrable or not-applicable in this case of cancer therapeutics. As such, it often means that other ICH guidelines are not applicable to development of cancer therapeutics unless specifically referred to in ICH S9. For instance, the guidelines do not include inclusion of healthy volunteers in a trial, indicating that the ICH M3 guidelines (ICH 2010a) should be followed.

Throughout the ICH S9 guidelines there is a clear statement of intent to develop therapeutics with a greater tolerance for risk in terms of cancer treatment, exemplified by modification, elimination or deferral of "standard" nonclinical studies (relative to ICH S6 for biotherapeutics and ICH M3 for non-oncological conditions), and use of nonclinical doses associated with adverse effects as the starting point for first in human clinical trial therapeutic starting dose.

PROCEDURE

Nonclinical safety studies to support clinical progression of an anticancer pharmaceutical should be conducted in accordance with Good Laboratory Practises. The following section identifies the specific study requirements as identified in the ICH S9 guidelines (ICH 2010a). An overview of the requirement for particular studies is indicated in Table 18.4.

18.4.1 Drug Efficacy

Preliminary characterization of mechanism of action, resistance and schedule dependencies in addition to cancer therapeutic efficacy are required as part of the nonclinical package. This should utilize appropriate models based on the target and mechanism of action of the therapeutic, but does not necessarily require evaluation in the same tumor type as intended for the

Table 18.4 Overview of the requirement for particular studies for oncology therapeutics, as described in the ICH S9 guidelines (ICH 2010a)

ICH safety guideline	Requirements for evaluation of oncology therapeutics
S1A-S1C: Carcinogenicity studies	Not required for marketing
S2: Genotoxicity studies	Not required for clinical trials, but data maybe required for marketing
S3A: Toxicokinetic study	S3A: Conducted as appropriate
S3B: Pharmacokinetic study	S3B: Partial requirements prior to clinical trial, with further data required for marketing
S4: Chronic toxicity testing	Shorter duration studies (3 months) are generally sufficient for marketing.
	Assessment of potential to recover from toxicity is required.
S5: Reproductive toxicology studies	No requirement prior to clinical trials, but partial requirements required for marketing
S6: Safety evaluation of biotechnology-derived products	Only required for biotechnology-derived therapeutics
S7A: Safety pharmacology for human pharmaceuticals	Requirement for marketing, but not specifically prior to clinical trial
S7B: The Non-clinical evaluation of the potential for delayed ventricular repolarization	
S8: Immunotoxicity studies	General toxicology studies are considered sufficient, unless immunomodulatory pharmaceutical
S10: Photosafety evaluation	Only required if potential risk is identified
M3: Nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals	Requirement dependent upon route of administration, proposed mechanism of action

clinic. Pharmacodynamic (primary and secondary) studies should also be evaluated, as appropriate.

18.4.2 Nonclinical Drug Safety Evaluation Studies

The screening of cancer pharmaceuticals will usually require evaluation in two species (rodent and non-rodent), as indicated in the ICH S9 guidelines (ICH 2010a). An exception to this being the development of genotoxic therapeutics, targeting rapidly dividing cells, in which safety studies in one rodent species may suffice (Newell et al. 1999). If the anticancer therapeutic is biotechnology-derived then ICH S6 guidelines are referred to with respect the choice and number of species to be screened (ICH 1997). If the initial short-term toxicity studies are comparable between rodent and non-rodent then longer-term studies can be conducted using only the rodent, on the condition that the rodent is a relevant species.

18.4.3 Safety Pharmacology

An assessment of vital organ function, including cardiovascular, respiratory and central nervous systems, is required prior to initiation of clinical trials. In contrast to other therapeutic classes, stand alone safety pharmacology studies are not required to support clinical studies in patients with late stage cancer or advanced disease. Safety parameters should be integrated and included in general toxicology studies.

18.4.4 Pharmacokinetics

A limited set of kinetic parameters identified in nonclinical studies, with the objective of facilitating clinical dose-escalation are required for commencement of clinical studies. These parameters include peak plasma levels, exposure levels and AUC, and therapeutic half-life. Studies in support of drug absorption, distribution, metabolism and excretion, conducted nonclinically, are required for later stage clinical trials and should be progressed in parallel to clinical development.

18.4.5 General Toxicity Studies

Toxicology studies must be conducted using a dosing schedule in agreement with the intended clinical trial design, as identified in Table 18.1. These studies have to be conducted in light of the intended clinical progression of the therapeutic. Accordingly, it must be remembered that the primary goal of Phase I clinical trial is to ascertain safety of the pharmaceutical in

humans, that these agents are generally administered at or close to their maximum tolerable dose, and that administration until dose-limiting toxicity is identified is the norm. Therefore, determination of a no-observable adverse effect level (NOAEL) or no effect level (NOEL) in toxicology studies is unwarranted for clinical trial support.

The assessment and prediction of toxicity recovery and delayed toxicity should also be included within the study to support the clinical trial, although it is not required to demonstrate complete reversibility within these studies before clinical progression.

18.4.6 Reproductive Toxicology

Therapeutics targeting cell division or which belong to a class of agent with known toxic effects upon embryofoetal development do not require embryofoetal toxicity evaluation. Similarly, such studies may not be feasible for biopharmaceuticals, but in principle could be performed during the period of organogenesis, in line with the requirements outlined in the ICH S6 guidelines (ICH 1996, 1997, 2010a).

Assessment of embryofoetal toxicity is only required to identify risks for the developing embryo and to communicate such potential risk to patients who are or may become pregnant. Results from these studies are required prior to marketing, but not for initiating clinical development for patients with cancer. These studies should be conducted in two species, but if found positive for teratogenicity or embryofetal lethality in the first species, a second species is not warranted.

In general, a fertility study is not required to support the treatment of patients with late stage or advanced cancer. Relevant information could be obtained from general toxicity studies, particularly the effect of the therapeutic upon the reproductive organs. Similarly, a peri- and postnatal toxicology assessment is generally not required for evaluation of therapeutics intended for use in cancer patients (ICH 2010a).

18.4.7 Genotoxicity Studies

These studies are not deemed essential to support clinical trials for therapeutics intended to treat patients with late stage or advanced cancer, but should be assessed prior to marketing. In the case of "conventional" cancer therapeutics, in which their mechanism of action is to target DNA replication and repair, a subsequent genotoxicty study is normally not warranted. Additionally, a positive genotoxicity signal from in vitro studies would indicate an in vivo study is not required. For assessment of biopharmaceuticals, the guidance provided in the ICH S6 guidelines should be followed (ICH 1997).

18.4.8 Carcinogenicity Studies

Such studies are normally not required to support either clinical trial or marketing of therapeutics intended to treat patients with late stage or advance cancer. The appropriateness of a carcinogenicity evaluation of an anticancer therapeutic is identified in the ICH S1A guidelines (ICH 1995).

18.4.9 Immunotoxicity Studies

For most therapeutics, no dedicated immunotoxicity study is required, with the design of the general toxicity study being sufficient to assess this toxicity. In the case of therapeutics with known effects in this area, such as immunomodulatory drugs and cytokines, further studies and immunophenotyping evaluation may be required.

18.4.10 Phototoxicity

The photochemical properties of the therapeutic (or chemical class) should be appraised during the nonclinical study period. A potential for phototoxicity would indicate protective measures should be adopted during clinical trial. A specific phototoxicity study is only required if photosafety risk cannot be evaluated from nonclinical or early clinical studies. In these cases, a study as identified in the ICH M3 and ICH S10 guidelines should be followed (ICH 2010b, c).

18.4.11 Assessment of the ICH S9 Guidelines

The ICH S9 guidelines are prescriptive in terms of the "core" studies required, but are limited with regards

their requirements for recovery and reversibility data. The guidelines do not identify a requirement to demonstrate complete recovery, but state "assessment of the potential to recover from toxicity should be provided to understand whether serious adverse effects are reversible" and specific data on reversibility may be expected "if there is severe toxicity at approximate clinical exposure and recovery cannot be predicted by scientific assessment." The primary purpose of inclusion of a recovery group is to ascertain whether any observed toxicity may progress to a more severe state. Irreversibility, or toxicity progression during recovery, may significantly alter clinical trial design due to duration of post-treatment monitoring and reappraisal of drug tolerability and toxicity. The advantage to inclusion of recovery groups are that toxicities developing post-treatment, their severity and clinical impact can be identified. Similarly, reversibility data can be utilized to prevent overestimation of observed toxicities and subsequent under-dosing of patients during clinical trial. Advantages of not including a recovery group would be fulfillment of the ICH S9 objectives to reduce the numbers of animals required per study and accelerate the translation of therapeutics to the clinic. In addition, the study package would be considerably less expensive. Recovery may not be required when the toxicity is predicted from the drugs mechanism, such as conventional DNA-damaging cancer therapeutics, where toxicities and their reversibility are well known. Similarly, a non-reversible toxicity, such as organ necrosis, would not necessitate a recovery group. Consequently, such information would be required on a case by case basis for regulatory submissions, particularly in the situation where no standard for reversibility potential is identified (Ponce 2011).

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Ponce R (2011) ICH S9: developing anticancer drugs, one year later. Toxicol Pathol 39: 913–915

18.5 Extrapolation of Nonclinical Data to Support Clinical Trial Design

PURPOSE AND RATIONALE

The major objective of all preclinical and nonclinical safety studies is to identify the toxicity risks and liabilities, by which to make an informed decision and progress (or not) therapeutics to the clinic.

The relationship between animal and humans with respect to identifying effective and tolerable doses and the subsequent identification of a clinical starting dose has been studied over many decades, with mixed results dependent upon the type of therapeutic being evaluated. Conventionally, the safe starting dose for clinical trial of cytotoxic agents has been defined as one tenth the lethal dose to 10% of mice (LD10) (Newell et al. 1999). However, if the toxicology studies in the non-rodent species demonstrate significant toxicity, it is suggested that the starting dose be defined as one sixth to one third of the lowest dose that results in toxicity in the more sensitive species (Le Tourneau et al. 2010; Newell et al. 1999; Rozencweig et al. 1981). In the case of "molecular targeted therapeutics" whereby optimal therapeutic effects occur at doses below the maximum tolerated dose, an option is to use inhibition of the subcellular target and plasma levels rather than toxicity as an endpoint. Although appealing, this approach is difficult to adopt because of a need for serial tissue samples and larger patient cohorts, and consequently the "conventional" toxicity approach is used to guide clinical dosing. For biopharmaceuticals, a safe starting dose depends upon good understanding of the therapeutics pharmacology and anticipated safety profile, a choice frequently complicated by the specificity of the therapeutic for a human protein (with rats, mice and non-primate species often unsuitable as test systems).

Accordingly, the ICH S9 guidelines state that the starting dose should be scientifically justified from all the available nonclinical study data and its selection based on the proposed clinical study design and treatment duration (ICH 2010a). In this context, data obtained from nonclinical pharmacokinetic studies of cancer therapeutics is now reliably used to extrapolate toxic effects from animal to humans, by relating drug exposure and peak plasma levels to the occurrence and degree of systemic toxicity. These processes and strategies are embedded in the ICH S9 guidelines, particularly in terms of the objective to decrease the attrition rate of new cancer therapeutics.

PROCEDURE

Retrospective analysis of animal and human data by Freireich, Rozencweig and colleagues demonstrated that maximum tolerated dose in animals and humans was very close when normalised to body surface area (mg/m^2) but differential when normalized on a body weight (mg/kg) basis, requiring a species specific conversion factor in the latter case (Freireich et al. 1966; Rozencweig et al. 1981). The ICH S9 guidelines state that for most small molecule therapeutics the clinical dose should be determined by allometric scaling of the animal doses to the equivalent human dose, based on normalization of body surface area (ICH 2010a). These principles are also applicable for biopharmaceuticals which are either antagonists of the intended target/ligand or are without agnostic activity. In the case of therapeutics where allometric scaling is inappropriate or inadequate, particularly biopharmaceuticals, the starting dose maybe calculated by extrapolation from animals to humans based on other parameters, such as body weight. However, an exception to this approach are protein therapeutics with agonistic properties, whereby selection of the starting dose using an identified, minimally anticipated biological effect level (MABEL) should be considered (ICH 2010a). Facilitation of faster progression to the clinic necessitates that the design of nonclinical studies should mirror the route and dosing schedule proposed for clinical trial. The suggested preclinical study schedules to support clinical trials, as outlined in the ICH S9 guidelines, are shown in Table 18.1.

In the clinic, treatment for the majority of patients and cancer types involves combination of several therapeutics with different mechanisms of action. The pharmaceuticals involved in such combinations will of course have undergone individual toxicological investigations in their own right. Therefore, an informed decision should be made as to the requirement for further toxicology studies of the therapeutic combination prior to commencement of clinical trial. This decision must be based on both the toxicity profiles of the therapeutics in question and there being a pharmacological rationale for such combination therapy, supported by previous preclinical studies. Generally, toxicology studies to ascertain the safety profile of combination therapies intended to treat patients with advanced or aggressive cancer are not warranted.

MODIFICATIONS OF THE METHOD

In line with the objectives to increase the clinical dose escalation rate toward an active dose, attain the most therapeutically active dose, and reduce the time taken for new therapeutics to enter the clinic, the ICH S9 guidelines specify modifications to clinical trial of oncology therapeutics. Primarily, the guidelines state that dose-escalation or the highest dose investigated in an oncology clinical trial should not be limited by the highest dose tested in the nonclinical studies. The majority of cancer therapeutics will show comparability between toxicity response in nonclinical and clinical studies and thus adopt a standard process for dose escalation. In situations whereby a steep dose-response curve is obtained in nonclinical toxicology studies and no preceding marker of toxicity is available, particularly with regards biopharmaceuticals and therapeutics with a novel mechanism of action, consideration should be given to a slower than normal dose-escalation.

In contrast with other types of therapeutic, the ICH guidelines state that treatment should not cease whilst the patient is showing a response and can continue beyond the proposed end of the trial. This continuation beyond the duration of the completed toxicology studies would not require a new toxicology study. An exception to this being if the continuation of treatment is associated with a major change in the clinical schedule, such as alteration in treatment to a more intense clinical schedule (e.g., from one to three treatments per week), which is beyond the scope of the completed preclinical toxicology study. Such a situation would require further toxicological evaluation, with a single species study being deemed sufficient (ICH 2010a).

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Transgenic Animals

19

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19.1 Introduction

Safety pharmacology studies are defined as:

> "Those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above." (ICH S7A International Guidelines on Safety Pharmacology Studies; Anon 2001)

The ICH S7A document also lists the principal aims of safety pharmacology as being:

1. To identify undesirable pharmacodynamic properties of a substance that may have relevance to its human safety.
2. To evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies.
3. To investigate the mechanism of the adverse pharmacodynamic effects observed and/or suspected.

Safety pharmacology is distinct from general toxicology in many ways, primarily in being focused on adverse functional *responses* to drugs, rather than on pathological cellular damage. Assessing functional responses involves measuring biochemical, physiological, and behavioral changes, using both in vitro and in vivo approaches. Safety pharmacology tends to focus on five organ functions for which functional responses are readily observable and quantifiable, namely, the cardiovascular system, nervous system, respiratory system, gastrointestinal system, and renal system. Compromising the function of any of the first three of these could be immediately life threatening, and these are classed as "vital organ functions" by ICH S7A.

Safety pharmacology is a dynamic discipline, with new technologies, new approaches, and new

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regulatory guidelines arriving in quick succession (Redfern et al. 2002; Redfern and Wakefield 2006; Valentin and Hammond 2008; Bass et al. 2011). Rather than being viewed as merely a regulatory requirement, an increasingly broader range of techniques is being deployed in safety pharmacology to de-risk compounds early, thereby contributing to reducing candidate drug attrition at later, more expensive stages of drug development (Bass et al. 2011). However, one technique that has not been widely adopted is that of genetically modified animals, which are applied to differing extents in other areas of drug discovery, including toxicology (Wolf and Henderson 1998; Törnell and Snaith 2002; Bolon 2004; Lin 2008). This chapter explores why there has been limited uptake of transgenic animals into safety pharmacology, how they are being used and could be used, and recent technological changes that will facilitate their use to address safety pharmacology problems.

19.2 Reasons Limiting Use of Genetically Modified Animals in Safety Pharmacology Thus Far

The primary reason is that the rodent species of choice in safety pharmacology investigations is the rat, whereas the vast majority of genetically modified rodents are mice. Specifically:

1. Regulatory repeat-dose toxicity studies require evaluation of the candidate drug in a rodent and a non-rodent mammalian species (Anon 2009); the default rodent species is the rat, and the default non-rodent species for conventional pharmaceuticals (i.e., small molecules) is the dog (Keller and Banks 2006). To facilitate comparison between safety pharmacology and toxicology studies, including dose selection, systemic exposure, and maximal tolerated dose, it makes sense to confine the species used in the majority of safety pharmacology studies to the same two species used in toxicology studies: rat and dog (Redfern et al. 2002).
2. Several types of rodent studies conducted in safety pharmacology are more suited to the rat (or guinea pig) than to the mouse. These include cardiovascular assessments in anesthetized animals (guinea pig and rat), cardiovascular telemetry (rat and guinea pig), and certain operant behavioral techniques (rat). Nonetheless, mice are commonly used in safety pharmacology in earlier stages of drug discovery, specifically as this reduces compound requirements by approximately tenfold. Each mainstream safety pharmacology technique used to address the five organ functions in rats has been scaled down for use in mice (Redfern and Wakefield 2006). Even telemetry technology to record physiological variables (e.g., blood pressure, heart rate, body temperature, EEG), miniaturized so as to be suitable for use in freely moving mice, has been available for over a decade (Kramer et al. 2001).

Figure 19.1 shows a plot of posters arising from work on mice and rats as a percentage of the total across all species, as presented at the annual meeting of the Safety Pharmacology Society (SPS) over a 10-year period since its inaugural meeting in 2001 (Redfern and Valentin 2011), compared to the published literature across the whole of bioscience over the same period. Although posters and publications may not reflect actual usage, nonetheless the proportion of mice as the rodent species in SPS posters is low and static, compared to an increase in their appearance in bioscience publications, generally over the same time period. In terms of the number of SPS posters on transgenic animals, there were only 5 out of a total of 1,180 posters; all of them since 2007 (Odening et al. 2008; Sun et al. 2008; Hassanain 2009a, b; Banfor et al. 2011; Redfern and Valentin 2011).

The development of techniques for genetically modified rats (Tesson et al. 2005, 2011 Geurts et al. 2009; Tong et al. 2010; Normile 2010), and their increasing availability (e.g., SAGE™ Labs; www.sageresearchmodels.com), is likely to result in an increased deployment in safety pharmacology. Safety pharmacologists are already utilizing genetic modifications in other species, including rabbit (Odening et al. 2008) and mini-pig (Forster et al. 2010).

19.3 Potential Applications of Genetically Modified Animals in Safety Pharmacology

These fall under six main themes:

1. *Target safety risk assessment*: Knockout (or conditional knockout) of the target of interest to study the impact on each of the five organ functions studied in safety pharmacology (cardiovascular, nervous system, respiratory, gastrointestinal, and renal).

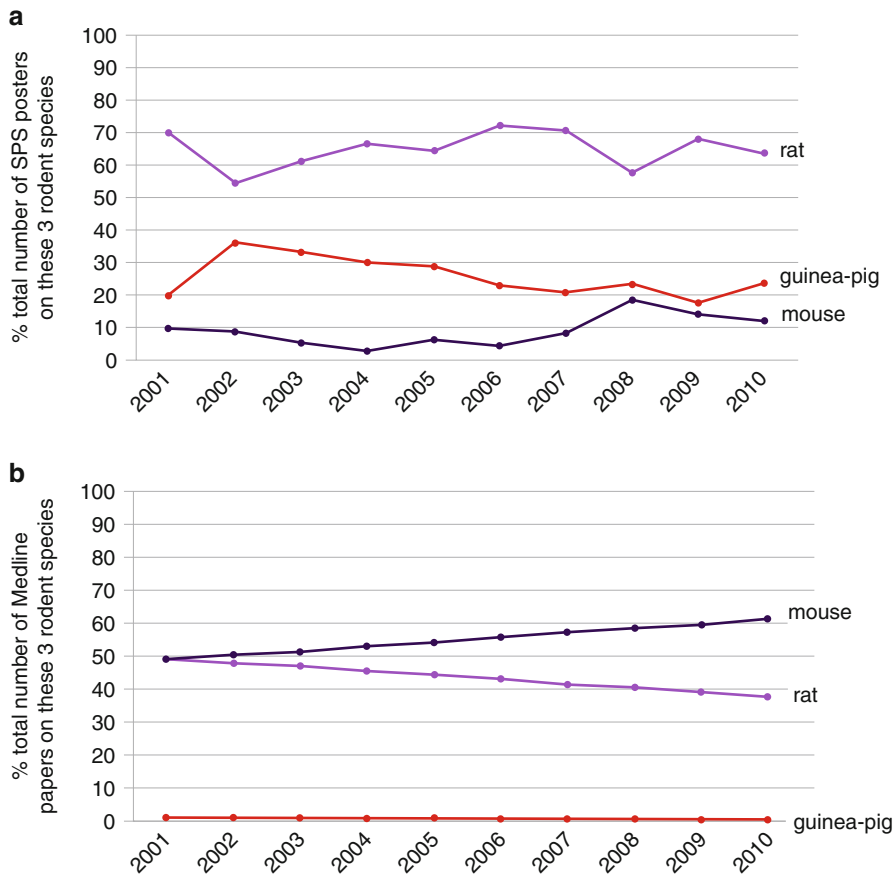

Fig. 19.1 (**a**) Posters on three rodent species presented at the annual meetings of the Safety Pharmacology Society since its inauguration in 2001. Plotted as % total posters involving rat, guinea pig, or mouse. Includes both in vivo and in vitro methodology. Various other species not plotted. Note the relative lack of research on mice presented throughout this 10-year period. Total SPS posters in 2010: mouse 11; rat 56; guinea pig 21) (Adapted from Redfem & Valentin (2011)). (**b**) Publications on the same three rodent species in the wider bioscience literature since 2001. Includes both in vivo and in vitro methodology. Note the steady rise in the publications on mice over the decade, with a corresponding reduction in publications on rats. Total publications in 2010: mouse 59, 607; rat 36, 756; guinea pig 575 (Source: Medline)

2. *Pharmacodynamic modification*: Knockout of the primary target receptor (or an off-target receptor) to determine the mechanism of an observed adverse effect with a compound or series of compounds.
3. *Pharmacokinetic modification*: Knockout of a transport mechanism/P450 enzyme to assess the modification of an observed adverse effect with a compound or series of compounds.
4. *Humanization*: Insertion of a human receptor to assess any potential adverse effects of a compound or series of compounds.
5. *Altered physiological/pathophysiological state*: Genetic modification to mimic aspects of a human disease, to enable the safety pharmacology evaluation in a "disease model."
6. *Bioluminescent/fluorescent reporter genes*: Insertion to indicate molecular mechanisms/pathways activated by an adverse effect of a compound or series of compounds.

19.3.1 Target Safety Risk Assessment

For a new molecular target, this can begin before any probe compounds have been synthesized. The purpose is to assess what happens to each of the five organ functions when the molecular target is disabled (by gene knockout), thereby mimicking what might happen if the same target is inhibited pharmacologically (by a receptor antagonist in the case of a cell surface or nuclear receptor; an inhibitor in the case of an ion channel, enzyme, or transporter). This approach is valid for the majority of drug discovery programs, as only a small proportion are designing agonists or activators. The three main problems with this approach are that some gene deletions are embryolethal, secondly, that the observed phenotypes may be due to developmental effects and not due to the acute absence of the receptor (Stark et al. 2007), and thirdly, that adaptive changes during development can provide

a "workaround" to circumvent the missing gene. One way round these problems is to use conditional knockouts. In these animals, a specific drug is administered at the time of the experiment, which causes inactivation of the targeted gene by inhibiting binding of an inserted transactivator to a gene promoter (e.g., the Cre-lox system), thereby switching off expression of the targeted gene. This can also be restricted to specific organs or tissues, for example, the brain (Aiba and Nakao 2007), by placing the transgene under a tissue-specific promotor. The first drug trigger used in the production of conditional knockouts was tetracycline (Gossen and Bujard 1992; Gossen et al. 1993; Aiba and Nakao 2007; Stark et al. 2007). Other approaches have included the use of the estrogen receptor antagonist tamoxifen, for example (Aiba and Nakao 2007; Lau et al. 2011; Friedel et al. 2011).

Currently, if knockout animals are not available for the gene of interest, there can be a significant delay until they are produced (e.g., >12 months), by which time probe compounds may become available within a drug discovery project. However, the availability of receptor knockouts is likely to increase from various sources, for example, the Mutant Mouse Regional Resource Centers (www.mmrrc.org) and the Wellcome Trust Sanger Institute (www.sanger.ac.uk); the latter is aiming to produce a knockout strain for every gene in the mouse genome. This will greatly facilitate target safety risk assessment by phenotyping, and provides an opportunity for safety pharmacologists to become more involved at this early stage of drug discovery projects, by applying more focused, safety pharmacology-related techniques than are currently used in phenotyping test batteries (Bailey et al. 2006; Crawley 2008).

19.3.2 Pharmacodynamic Modification

Table 19.1 shows the main causes of adverse effects in safety pharmacology investigations.

When such adverse effects occur, at any stage of drug discovery, a key question is whether this is mediated by the primary target. For the cardiovascular and central nervous system, adverse effects in humans are related to the primary target for ~50% of candidate drugs; for gastrointestinal adverse effects this is slightly lower (Olson et al. 2000). In early stages of drug discovery, evidence against an involvement of the primary target in the adverse safety pharmacology finding would be if the effect was not observed in all compounds interacting with the primary target that were tested. However, this is not always definitive. For example, the different ligands could interact with the primary target in subtly different ways; their cellular uptake could differ; and in the case of adverse effects mediated by the central nervous system, the different compounds could vary in their penetration of the blood–brain barrier. Using a knockout animal can help settle the argument. It is important to include a reference compound causing the same (or closely similar) adverse effect by a different mechanism, and to demonstrate that the gene knockout does not affect either the baseline control level or the response to the reference compound. If it does, the outcome is difficult to deconvolute, as shown in Fig. 19.2.

Published examples of this approach in safety pharmacology-related areas include elucidation of target- and off-target-mediated adverse effects of selective glucocorticoid receptor agonists (Kleiman and Tuckermann 2007). However, it is likely that its use is more widespread than this within the pharmaceutical industry; the paucity of publications may reflect commercial sensitivity, and if so, publications may ensue in due course.

Table 19.1 Causes of adverse effects in safety pharmacology studies and of Type A adverse drug reactions (ADRs) in humans. Type A effects are dose-dependent, pharmacology-related effects, comprising approximately 75% of human ADRs (Redfern et al. 2002).

Mechanism	Example
Augmented ("supratherapeutic") effect of interaction with the primary target	Pronounced bradycardia with a beta-blocker
Interactions with the primary target present in nontarget tissues	Sedation caused by antihistamines
Interactions with nontarget receptors	QT interval prolongation by blocking hERG channel
Inhibition of synthesis/ trafficking of receptors	Inhibition of hERG channel trafficking by pentamidine, leading to QT interval prolongation
Nonspecific effects	Gastric irritation leading to emesis
Pharmacologically active metabolites	Seizurogenic properties of bupropion metabolite

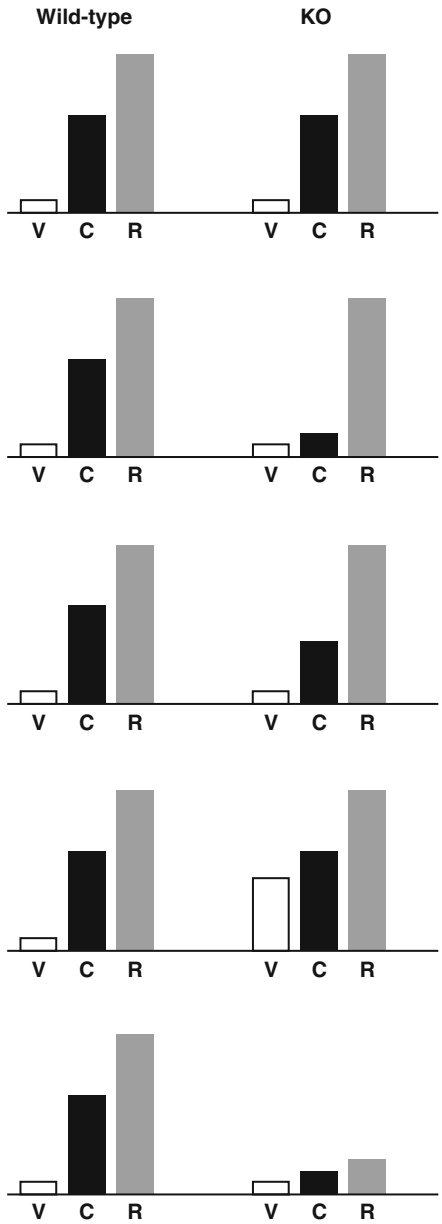

Fig 19.2 Five potential outcomes of safety pharmacology evaluations of the effects of a gene knockout on the adverse effects of a test compound (in vivo or in vitro). *V* vehicle control (baseline), *C* test compound, *R* reference compound acting via an unrelated mechanism

19.3.3 Pharmacokinetic Modification

This refers to a genetic modification to a protein involved in drug absorption, metabolism, distribution, or elimination. Such approaches can help to define where and how an adverse effect is occurring (Wolf and Henderson 1998). For example, deletion/reduction of a key cytochrome P450 enzyme can help determine whether an adverse effect is due to the parent compound or its metabolite; deletion/reduction of key drug transporters can point out which organ is involved in mediating an adverse effect, or whether such an effect is due to direct access of the compound to that organ. An example includes reduction of p-glycoprotein, an important efflux carrier protein in the blood–brain barrier. This has been used to elevate brain concentrations of a compound under evaluation, to determine whether its cardiovascular adverse effects were centrally mediated (Banfor et al. 2011).

19.3.4 Humanization

This category refers to the insertion of a human transgene into a laboratory animal species. This approach has been widely used in studying disease pathophysiology; however, its use in safety pharmacology has been minimal thus far. It would be particularly useful for safety pharmacology applications where the laboratory species differ significantly from humans in expression, homology, or ligand-binding characteristics of a particular target protein of interest, be it pharmacodynamic or pharmacokinetic (Cheung and Gonzalez 2008; Stanley et al. 2009; Strom et al. 2010; Jiang et al. 2011). Examples include mice expressing a humanized CYP3A4 enzyme, which is a key enzyme in the metabolism of drugs (Cheng et al. 2011). This approach has also been used to explain the carcinogenic effect of PPARα ligands in rodents (an effect not observed in humans), by generating a PPARα-humanized mouse line. A species-specific gene expression profile was observed with PPARα ligands between the transgenic mice and their wild-type counterparts, which explained the carcinogenic proclivity of rodents to this class of drug (Gonzalez 2007).

In safety pharmacology applications, this technique of "humanised" transgenic animals probably has most benefit in the area of biotechnology-derived therapeutics (biopharmaceuticals), especially human-specific proteins such as monoclonal antibodies. Transgenic rodents expressing the human form of the antibody's target receptor could have value in assessing target-related adverse effects in safety pharmacology studies for this class of biopharmaceuticals.

19.3.5 Altered Physiological/ Pathophysiological State

The primary purpose of safety pharmacology evaluations is to protect healthy human volunteers in Phase I clinical trials from harm due to adverse effects of new medical entities. Therefore, it is obvious that we would conduct the safety pharmacology evaluations in healthy animals. However, an aspiration of safety pharmacologists is to predict Type A adverse effects (i.e., adverse effects related to the primary or secondary pharmacology; Table 19.1) in the larger patient population. Arguments have been proffered in support of running safety pharmacology studies in animal models of the targeted disease. Counter arguments include the fact that disease models in animals only model one aspect of the human disease; the time course of the pathophysiology in the disease model (for some chronic diseases) is usually much faster; and that anatomical, biochemical, physiological, and behavioral differences between the test species and humans degrade the translation from the aspirational 1:1 relationship. Moreover, the translation of safety pharmacology findings (in healthy animals) to Phase I readouts (in healthy humans) is less than perfect (Valentin et al. 2009), so factoring in the above caveats as well would further diminish the confidence in translation of safety pharmacology findings in an animal disease model.

Notwithstanding these caveats, there may be specific situations where a strategy of using a model of the targeted disease would be relevant to the safety pharmacology evaluation (e.g., Hamlin and Kijtawornrat 2008). Also, there are other situations where the adverse effect of a test compound in normal animals is far worse (even dose limiting) than in a disease model. An example of this would be in the case of hypoglycemic agents, which may lower blood glucose to levels resulting in adverse physiological or behavioral effects in normal animals, whereas in rodent models of type 2 diabetes they merely restore the elevated blood glucose to normoglycemic levels. In both these areas, transgenic rodent models of human diseases may be appropriate for safety pharmacology applications.

19.3.6 Bioluminescent/Fluorescent Reporter Genes

The predominant fluorescent marker used in transgenic animals is green fluorescent protein (GFP), a naturally occurring protein found in *Aequorea victoria*, a species of jellyfish (Chalfie 2009). It can be coupled to proteins of interest by inserting tags on the transgene. This enables real-time monitoring of the localization of the protein of interest (e.g., a drug receptor) in live tissue (Spergel et al. 2001). It can also be used to identify individual cells (expressing a protein of interest) within a cell culture or tissue slice for

electrophysiological or biochemical assessments (Spergel et al. 2001).

It has potential applications for in vivo, ex vivo, and in vitro techniques in safety pharmacology, and GFP transgenic rats have been developed as well as mice (Remy et al. 2010; Ueta et al. 2011; Moore et al. 2012). As there are no published examples of its use in safety pharmacology applications, examples of analogous applications include cochlear hair cell loss following ototoxic treatment in vitro (Yorgason et al. 2010), ex vivo measurement of the hypothalamic vasopressin response to various physiological stimuli (Ueta et al. 2011), and in vivo assessment of nerve injury and regeneration (Moore et al. 2012). Realistically, for safety pharmacology applications, visualization (or imaging) of GFP expression in mammalian species in vivo would probably be too invasive for longitudinal studies in any of the five organ functions studied, and would therefore generally require terminal anesthesia. An exception to this could be in vivo retinal imaging by confocal scanning laser ophthalmoscopy in GFP-tagged mice (Seeliger et al. 2005). However, for in vitro applications in safety pharmacology, this technique could be quite useful, which is also the case for larval zebrafish, which are transparent, and potentially valuable as frontloaded assays for some aspects of safety pharmacology (Redfern et al. 2008).

19.4 Caveats and Pitfalls of Using Transgenic Animals for Safety Pharmacology Applications

Limitations on the application of genetically engineered animals in toxicology (Bolon 2004; Lin 2008) also apply to safety pharmacology. Both the genotype and phenotype of the transgenic model need to be well characterized. The genetic background of the animal strain used can greatly affect phenotypic expression, and some strains develop spontaneous conditions that could be misinterpreted as relating to the altered gene function. Compensatory changes in other biochemical/physiological pathways can lead to misinterpretation of the role of the altered gene function (although the use of conditional knockouts should mitigate against this). Conversely, gene functions may be missed because specific challenge conditions were required, and were not applied.

19.5 Concluding Remarks

Applications of genetically modified animals in safety pharmacology currently lags behind other areas within drug discovery, especially target validation, disease biology, drug metabolism and pharmacokinetics, and (to a lesser extent) toxicology. Part of the reason for this is the reduction in quality of physiological and behavioral data obtained from the mouse compared to the rat. The increasing availability of transgenic rats may help overcome the prevailing view in safety pharmacology that, for most of our requirements, this is still a solution awaiting a problem.

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Zebrafish in Drug Discovery: Safety Assessment

20

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20.1 General Overview

20.1.1 Need for Alternative Models for Safety Assessment

Nonclinical safety and toxicity assessment of new chemical entities can be a labor intensive and expensive endeavor which can lead to the loss of large numbers of drug candidates. However, any toxic pharmaceutical agent not successfully identified can prove much more costly as the potential monetary loss caused by a failure associated with any major human safety concern escalates as the drug development process continues. Late-stage clinical failures account for a significant proportion of costs incurred by the drug discovery industry (Schachter and Ramoni 2007), and alongside efficacy, toxicity and safety liability remain the most likely causes of candidate drug failure (Kola and Landis 2004). Discovery and development costs required to successfully introduce a single new pharmaceutical agent to the market have previously been cited at over $1 billion USD (DiMasi et al. 2003) and, given the recent lack of productivity in the pharmaceutical industry in the last decade, these projected development costs are undoubtedly much higher.

Currently, a multitude of possibilities exist for today's safety pharmacologists to help identify compounds with the least potential liabilities, but nevertheless, drugs still enter the market with lethal consequences. This demonstrates the battery of screens and regulated studies commonly in use are insufficient for determining all possible risks; although one could argue that with the complexity of drug–drug interactions, genetic diversity and various environmental factors, no drug can be considered 100% safe even

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after "extensive" clinical trials. What can be concluded is that all models have their limitations and that predictivity, speed, and correct positioning of relevant screening assays can be of utmost importance to limit expenditure and prioritize the best quality candidates.

On the whole, in vitro assays are less likely to be representative when compared to in vivo models but have the advantage of being low cost, rapid to perform, and require small amounts of compound. To help demonstrate this point, the cardiovascular system can be used as an example. Manual and automated patch clamp assays are now well established for evaluating the hERG channel, but without the combined interactions with other cardiac ion channels, the induction of QT-prolongation and potentially TdP cannot be fully predicted (Lawrence et al. 2005). Ex vivo assays, such as the Langendorff preparation (perfused rabbit heart model), fair a little better with regard to predictivity but is certainly more costly and time consuming than cellular assays. At the other end of the scale, mammalian models utilizing noninvasive external telemetry to monitor, collect, and analyze a number of physiological parameters including ECG, respiration, body temperature, and activity, are the most reliable option prior to commencing human trials. But again, these come at a premium price; the typical animals (dogs) are highly regulated and generally limited in subjected number, thereby making data analysis problematic (Aylott et al. 2011), and can be the target for animal activists. Therefore, an urgent need exists for a relatively cheap, medium-high throughput, in vivo, predictive vertebrate model to help drive candidate selection earlier in drug discovery.

20.1.2 Introduction to Zebrafish and Positioning of Larval Screening in Drug Discovery

The pioneers of zebrafish research mainly focused their attention on genetics, neurobiology, and development and soon discovered a high degree of conservation compared to man, especially with regard to the embryonic vertebrate body plan (Kimmel et al. 1995; Postlethwait et al. 2000; Goldsmith 2004; Zon and Peterson 2005). Over a period of four decades, the number of academic groups working in this field grew rapidly and further similarities to mammals were established including mechanisms of toxicity and disease. As such, a series of zebrafish models were created for both efficacy and safety assessment (Grunwald and Eisen 2002; Hill et al. 2005; Zon and Peterson 2005; Lieschke and Currie 2007; Barros et al. 2008; Hill 2008a). Today, the zebrafish has become one of the most funded models by agencies such as the NIH (National Institute of Health), and over the past 5 years, an average of 3,600 zebrafish peer reviewed publications have been released annually.

As a consequence of zebrafish becoming a recognized research tool, they have also recently gained attention from the pharmaceutical sector as models for evaluating novel drug candidates. In particular, the use of larval zebrafish for assessing compounds for toxicity and safety liabilities early in the drug discovery process has been actively investigated. When treated with different toxicants, the zebrafish has comparable benefits as well as various advantages over alternative cell-based toxicity and safety assays. Measuring a mere 1–4 mm in length, a full dose-response study using embryonic or larval forms of the zebrafish can be performed in microtiter plates with only single milligrams of compound (Hill 2008a). These assays therefore share the key benefits of an in vitro assay platform, namely, the ability to screen novel compounds, usually in limited supply, before significant compound scale-up is required, and potentially in an automated fashion. In contrast, as an in vivo vertebrate model, physiological, morphological, and histological similarities to mammals and man can be observed, potentially making extrapolation of data of greater value. In addition, as a whole organism, the zebrafish larva also enables adverse physiological effects and toxicity associated with toxic metabolites to be captured (Hill 2008a), which are unlikely to be synthesized in basic in vitro screens lacking metabolic activation and necessary cell–cell interactions. However, although these zebrafish assays are very predictive in their own right, the greatest utility of such a model is as part of a front-loaded battery of assays, ideally positioned alongside or immediately after classic cell-based screens, to take full advantage of throughput, cost, and focused readouts for each assay, prior to making a fully informed decision.

Many zebrafish safety assays are relatively quick to prepare and assess, both with respect to animal sourcing and compound screening. In contrast to other vertebrates, embryogenesis is complete in just a few days and organ dysfunction can be evaluated from between 3 and 5 days postfertilization (dpf) (Westerfield 1995).

Also, with the addition of enhanced automated platforms, including liquid handling, fish recognition software, and image acquisition, data can now be compiled more efficiently. Endpoints typically assessed by monitoring changes in movement such as heartbeat (contraction of the atrium and ventricle), eye flicker (vision), and locomotion (swimming behavior, convulsion/seizure, and sedation) have been gaining support (Barros et al. 2008).

Whether assessed in a manual or fully automated assay, the zebrafish larva lends itself to being the first in vivo vertebrate model in drug discovery for studying safety pharmacology and toxicity and has the potential to bridge the gap between traditional cell assays and regulated mammalian screens. Adult zebrafish screens can also be used for focused CNS readouts and to add support to larval evaluations. Fulfilling the 3 R's principle (replace, reduce, refine) for animal testing, the use of zebrafish may help decrease the numbers of rodents needlessly treated with toxic compounds which have a safe in vitro profile and also has the potential to decrease the cost of drug development to the pharmaceutical industry by reducing late-stage attrition commonly related to drug toxicity and safety liabilities. As such, although predominantly still in a validation phase for most companies, the pharmaceutical industry is beginning to embrace the zebrafish to help advance their drug discovery programs in a cost-efficient manner.

20.1.3 Common Assay Design and Larval Maintenance

Fertilized eggs, typically obtained from paired or group breeding of AB or Tübingen strain adult zebrafish, are staged for uniform development and reared in a suitable salt-buffered media such as E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$; Westerfield 1995) or 0.3× Danieau's solution (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, 1.5 mM HEPES, pH 7.1) in a humidified incubator at 28.5°C ± 0.5°C. Embryos or larvae can be arrayed to 384-, 96-, or 24-well microtiter plates until the desired age is reached, when test compound is added to the supporting medium in a suitable vehicle like DMSO (use of 0.5–2% final concentration has been reported). Each assay is optimized for duration and timing of exposure, although for safety-related assays, these typically begin between 2 and 10 days postfertilization (dpf) and last for 1–48 h.

Larvae can be raised in commercially available aquaria, fed on brine shrimp and common flake food, and removed as juveniles or adults as required for other kinds of safety assessment, such as ECG recording or behavioral (CNS) readouts.

All procedures must be performed in accordance with local legislation such as the UK Home Office Animals Scientific Procedures Act (1986), although certain early embryonic and larval stages of development are not currently subject to Directive 86/609/EEC which regulates the use of animals in scientific experiments.

20.1.4 How Toxicity May Impact Safety Evaluation

One of the major advantages of this model is that the zebrafish larvae are virtually transparent and develop ex utero, thereby allowing assessments of both exterior features (including the eye, fins, and jaw extension) and internal organs and tissues. Many zebrafish studies have hence concentrated on creating medium throughput assays based on phenotypic morphological aberrations which are assessable using transmitted light without the need for dissection, such as for screening hepatotoxicity (Jones et al. 2009; Hill et al. 2012) and developmental toxicity (Janaitis et al. 2007; Chapin et al. 2008; Brannen et al. 2010; Van den Bulck et al. 2011). Although toxicity models are beyond the scope of this review, chemically induced alteration to tissues or organs can have deleterious effects on various physiological processes, induce stress and secondary toxicity, and in particular can adversely affect neurological and cardiovascular function, so any evidence of necrosis, abnormal development, and acute toxicity must also be considered when evaluating more functional safety-related endpoints.

20.1.5 Compound Absorption and Metabolism

A possible complication to the zebrafish model is the typical route of exposure, namely, passive diffusion through the skin via the surrounding medium (aka aqueous exposure). Although effective levels are

commonly reached in target tissues, this will be dictated by the physicochemical properties of each compound and may result in either poor absorption and a false-negative result, or potentially rapid absorption to "overdose levels" and false-positive effects (Doshna et al. 2009). Microinjection of compound into the yolk or cardiovasculature is feasible and can be performed in a reasonably high-throughput manner in experienced hands to help control the level of exposure to larvae and adults, but this would not be as efficient as using the aqueous exposure method and without knowing how readily the compounds are circulated and deposited around the body, or metabolized and excreted, could lead to misleading results. Bioanalysis, an LCMS-MS approach, has been utilized effectively alongside aqueous exposure to evaluate the uptake of each test compound by the larvae and help rule out such complications, although this too has some limitations, especially if metabolites of test compounds are unknown. Nevertheless, this has become a valuable addition to zebrafish screening, especially as it also helps to rank compounds for toxicity and safety liabilities. A further advantage of bioanalysis is that it allows zebrafish metabolism to be investigated. This is a key factor to understand as extensive metabolism of parent compound could lead to reduced activity whereas the creation of active metabolites or metabolites with a different pharmacological spectrum of activity could lead to increased or competing/off-target pharmacology, respectively (Alderton et al. 2010). Determining the amount of compound in both dosing medium and zebrafish tissue is also important, as for some compounds metabolism can be significant and therefore mass balance needs to be established. For instance, in one study, only 35% of the testosterone exposed to larvae was recovered as unchanged parent compound after just 4 h (Alderton et al. 2010).

20.1.6 Conservation of Metabolic Pathways

One of the best known detoxification mechanisms in zebrafish is the aryl hydrocarbon receptor (AHR) pathway, mainly attributed to extensive research on dioxins and PCBs (Hill et al. 2005), but much is also known about a number of drug metabolizing enzymes, transcription factors, and transporters. For example, pregnane X receptor (PXR)-mediated induction of CYP3A and multiple drug resistance 1 (MDR1) has been demonstrated in zebrafish treated with clotrimazole, nifedipine, and synthetic steroid pregnenolone 16alpha-carboninitrile (Bresolin et al. 2005), and liver X receptors (LXRs), transcription factors activated by oxysterols with essential roles in lipid and glucose metabolism, have been characterized (Archer et al. 2008). ATP-binding cassette (ABC) protein subfamilies found in zebrafish were also found to correspond to the human subfamilies, although a single ABCH subfamily gene was unique to zebrafish (Annilo et al. 2006). Essential for the activation or inactivation of many endogenous and exogenous chemicals, with roles also associated with development and disease, cytochrome P450 (CYP) enzymes are diverse. Many of the 94 zebrafish CYP genes are direct orthologs of their human counterpart, especially those belonging to CYP families 5–51, as well as some in CYP 1, 2, and 3 with specific relevance for drug and pollutant detoxification (Mattingly et al. 2001; Tseng et al. 2005; Goldstone et al. 2010). However, only two of the 47 CYP2 genes (CYP2R1 and CYP2U1) are considered orthologs when sequences were compared to the 16 in humans (Goldstone et al. 2010). Using a variety of substrates, it has been clearly demonstrated that zebrafish embryos have the ability to perform both phase I (oxidation, *n*-demethylation, *o*-demethylation, and *n*-dealkylation) and phase II (sulfation and glucuronidation) drug metabolism reactions, thereby indicating the strong potential for this model in drug testing. Specifically, acetyltransferases (Zok et al. 1991), aldehyde and alcohol dehydrogenases (Lassen et al. 2005; Dasmahapatra et al. 2001; Reimers et al. 2004; Song et al. 2006), epoxide hydrolase (Thompson et al. 2010), glucuronyltransferases (Iannelli et al. 1994; George and Taylor 2002), glutathione *S*-transferases (Suzuki et al. 2005; Thompson et al. 2010), sulfotransferases (Liu et al. 2010), and monoamine oxidase (Anichtchik et al. 2006) have all been reported in zebrafish. However, although in many cases zebrafish metabolic profiles may be similar to mammals, such as for verapamil (Alderton et al. 2010), they may not be identical. As some atypical metabolites have also been identified, these subtle differences in metabolic pathways may therefore need further evaluation. For example, although conjugation of cisapride with glucuronic acid in mammals gives rise to cisapride *N*-glucuronide (rat, dog, and man: Meuldermans et al. 1988a, b) or cisapride *N*-sulfate

(dog: Meuldermans et al. 1988b), in 7-day-old zebrafish, only cisapride *N*-sulfate was detected following a 3-h incubation and no typical phase I metabolites (norcisapride and hydroxycisapride) were identified (Alderton et al. 2010).

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20.2 Core Zebrafish Safety Assays

Included in this review are a series of the more common larval and adult zebrafish safety screens that have been developed over the past decade. As such, this is not a comprehensive list but aims to highlight benefits, advances, and pitfalls of key screens related solely to the cardiovascular system and CNS, as well as provide comparisons to earlier models and alternative in vitro and in vivo screens.

20.2.1 Cardiac Function

Various techniques, biomarkers, and models are currently available or under evaluation as surrogates for the assessment of proarrhythmia risk in humans (Joshi et al. 2004), but initially, insight into potential cardiac risk can be gained from automated patch clamp screening and concentration-response assays, especially when a comprehensive survey of cardiac channels, potentially including but not exclusive to hERG, calcium (Cav1.2, Cav3.2), potassium (HCN2, Kir2.1, Kv1.5, Kv4.3, KvLQT1/mink), and sodium (Nav1.5) channels, is undertaken. These are typically performed with mammalian cells, but studies with zebrafish myocytes have demonstrated that zebrafish also possess the majority of the known cardiac currents (Baker et al. 1997). However, these high-throughput in vitro assays are generally focused on determining the effects of compounds on specific rather than multiple cardiac channels, potentially using a single concentration instead of establishing an $IC_{50.}$ In particular, identification of potent hERG (human ether-a-go-go-related gene) blockers has typically been prioritized (Redfern et al. 2008), due to the association of hERG with some headlining withdrawals of marketed drugs in recent years, but this overdependence on the hERG assay as a preclinical cardiac risk indicator could lead to the loss of potentially useful compounds that nonselectively block hERG and other compensatory channels (e.g. voltage-gated Na^+ and Ca^{2+}). In contrast, compounds that prolong repolarization via interactions with non-hERG cardiac channels may be advanced that pose a safety risk. It is hence important to understand that the heart requires a delicate balance of multiple factors, including numerous ion channels, and therefore additional ex vivo and in vivo methods may need to be employed to verify any finding, even though these are typically expensive and time consuming (Yang et al. 2001). However, in order to predict safety concerns in man, these too need interpretation and have been difficult to develop due to complexity arising from heterogeneity within the myocardium

(Antzelevitch 2004), failure to identify effects induced only after drug–drug interaction, interspecies variation, and any individual genetic variation that potentially alters the predisposition to developing such arrhythmias (Yang et al. 2002). Nevertheless, the effect of any new drug candidate must been screened for hERG activity in vitro and for causing QT-prolongation in vivo prior to initiating human trials (FDA 2005).

In order to help identify some of these liabilities and potentially bridge the gap between in vitro and in vivo testing, various zebrafish in vivo assays have been designed and validated based on different parameters.

20.2.1.1 Cardiac Imaging and Heartbeat Assessment

PURPOSE AND RATIONALE

The potential for a drug intended for noncardiovascular indications to cause drug-induced QT interval prolongations or fatal arrhythmias, known as Torsades de Pointes (TdP), is a significant public health issue (Friedrichs et al. 2005) and remains a leading concern when screening compounds for safety pharmacology liability, especially as these adverse reactions can occur with drugs from a variety of therapeutic areas including antihistamines (Woosley 1996; Yap and Cammet 1999), antipsychotics (Glassman and Bigger 2001), and antimicrobials (Darpö 2001) as well as antiarrhythmics (Raehl et al. 1985).

hERG potassium channels conduct the rapid component of the delayed rectifier potassium current, IK_r, which is crucial for repolarization of cardiac action potentials. hERG and its zebrafish homolog (zERG) show a high degree of amino acid conservation in the S6 and pore domain (99% identity) suggesting an evolutionary conserved role (Langheinrich et al. 2003). *zERG* is expressed specifically in both heart chambers of zebrafish embryos, and when the *zERG* gene is knocked down, a distinct atrioventricular (AV) decoupling or "block" is observed (Langheinrich et al. 2003). AV decoupling is also induced when larvae are treated with compounds (hERG blockers) known to prolong QT (Langheinrich et al. 2003; Milan et al. 2003; Hill et al. 2007; Berghmans et al. 2008). A relatively simple measurement of heart rate in zebrafish larvae can hence be a rapid in vivo method to identify potential hERG liabilities as well as compounds that cause other arrhythmias.

PROCEDURE

Staged zebrafish larvae are arrayed to 96- or 24-well microtiter plates and allowed to develop in a suitable salt-buffered media until either 2 or 3 dpf, depending on the desired protocol. One to five larvae per well are treated in a dose-response format with each compound for a relatively short period, typically 1, 3, or 24 h, after which they are imaged in lateral view with a charge coupled device (CCD) or video camera, capable of a fast rate of image acquisition. For large-scale assessments of multiple blinded compounds, larvae can be screened using an automated platform.

EVALUATION

Measurements of heartbeat (number of atrial and ventricular contractions) over a period of 15–20 s are determined either manually (by eye) (Berghmans et al. 2008) or using customized software applications. In the latter scenario, video imaging using transmitted light with wild-type larvae (Langheinrich et al. 2003; Hill et al. 2009) or fluorescence imaging with transgenic larvae expressing green fluorescent protein in the myocardium (Burns et al. 2005) has been reported. Both software applications offer relatively high-throughput readouts by identifying changes in average pixel density in the region of the atrium and ventricle, and when plotted over time, the number of contractions for each chamber and average heart rate is automatically determined.

CRITICAL ASSESSMENT OF THE METHOD

The 2:1 atrial (A) to ventricular (V) contraction ratio is a surrogate for QT-prolongation and has been successfully reproduced in 2–3-day-old zebrafish larvae when exposed for 1–24 h to a variety of compounds known to prolong QT (Langheinrich et al. 2003; Milan et al. 2003; Hill et al. 2007; Berghmans et al. 2008). In addition to adverse effects on heart rate caused by hERG blockers, zebrafish also respond similarly to man when treated with classic cardioactive drugs that cause bradycardia and tachycardia (Fig. 20.1) including adrenergic or muscarinic receptor agonists and antagonist, calcium and sodium channel blockers, NO donors, and beta blockers. Classic cardiac drug–drug interactions resulting in impaired metabolic detoxification, such as with erythromycin and cisapride caused via CYP3A4 inhibition, have also been demonstrated (Milan et al. 2003).

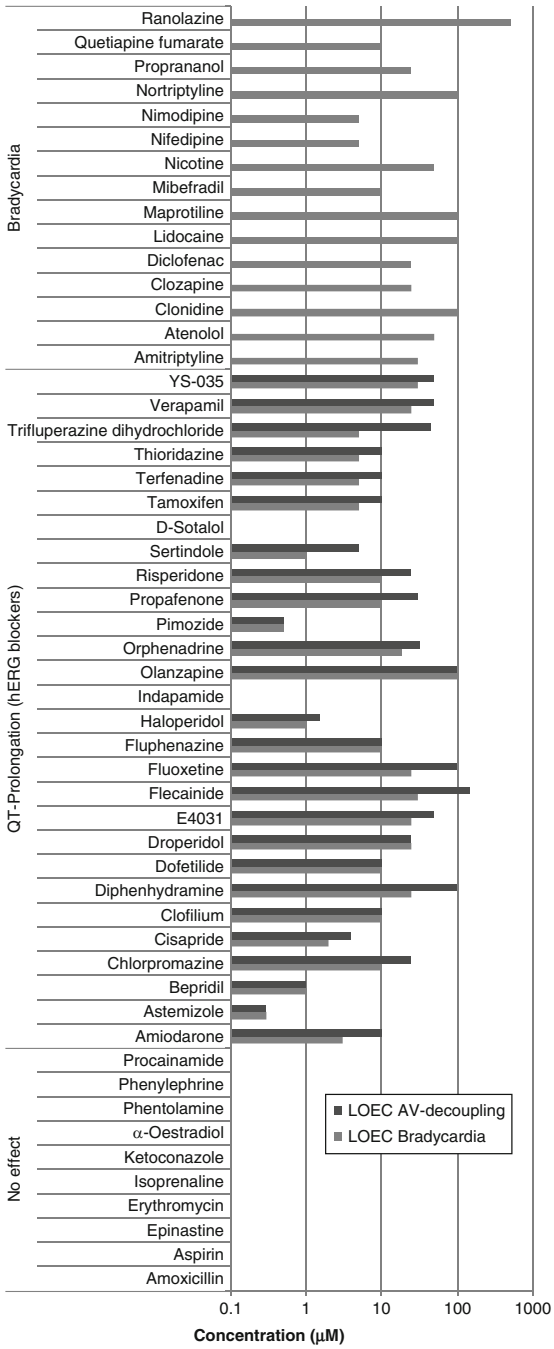

Fig. 20.1 Lowest observed effect concentrations (LOEC) in zebrafish larvae at 3 dpf for compounds causing altered cardiac function in humans. Larvae were treated in a dose-response assay for 3 h prior to imaging the heart. Compounds causing a decrease in heart rate (HR) (0–85% compared to the mean control HR) were classified as inducing bradycardia. Compounds known to cause QT-prolongation in humans elicited a greater effect on the ventricle compared to the atrium, atrioventricular (AV) decoupling, typically observed as a 2:1 AV ratio

As a whole organism, zebrafish may offer certain advantages over existing cell-based safety pharmacology assays such as the patch clamp, as in vitro screens are not considered fully predictive since they do not allow for affects on trafficking of hERG channels or effects on other/multiple ion channels to be taken into account (Kinter et al. 2004). However, more studies are required before this is fully understood in zebrafish, especially with regard to hERG trafficking as compounds such as Chromanol 293B, a specific IK_s blocker, failed to cause an adverse effect (Hill et al. 2009) whereas linopiridine and XE991, reasonably selective IK_M blockers (associated with modulating neuronal excitability) but also with activity against KCNQ1 (KCNQ1 forms the IK_s channel with KCNE1), induce bradycardia, and bepridil and clofilium, both with activity against IK_s and IK_r, cause AV decoupling (unpublished).

MODIFICATION OF THE METHOD

Correct classification of toxic compounds based purely on heart rate is generally reproducible regardless of the exact methodology, age of larvae, and duration of exposure, but in order to achieve comparable results, such as the lowest observed effect concentration, and to make the screen more widely accepted by the pharmaceutical industry, a standardized protocol should be agreed. Firstly, the use of 3-day-old larvae for these assays currently appears more common and should have the most supporting validation data. Also, as larvae can respond to certain compounds like adrenalin within just 3 min and the majority of active compounds cause an effect within 1–3 h, a 3-h period of exposure should be suitable for most cardiac assessments in this context.

As mentioned previously, a possible complication to the zebrafish model is the typical route of exposure. To investigate this possibility, Milan and colleagues (2003) microinjected hERG blockers previously classified as false-negatives with the aqueous exposure method and reported AV decoupling, and in later studies, bioanalysis, an LCMS-MS approach, has been utilized to evaluate the uptake of each test compound by the larvae and help rule out such complications (Berghmans et al. 2007a, 2008; Hill 2008a, b; Hill et al. 2009, 2012). In one such cardiac study undertaken by Novartis, three hERG blockers and one negative control compound remained unclassified due to poor uptake, thereby helping to explain the

false-negative rate. Nevertheless, of the 20 blind compounds tested, all five compounds that cause bradycardia were correctly identified along with the majority of those inducing QT-prolongation, resulting in an overall predictivity of 94% (Hill et al. 2009). In addition, with the exception of haloperidol, bioanalysis demonstrated that compounds could potentially be ranked for potency using this model with the zebrafish AV LOEC (lowest observed effect concentration) for E4031, astemizole, haloperidol, terfenadine, thioridazine, amiodarone determined as 1.0, 8.8, 50.8, 12.7, 50.6, and 182.0 ng/larva, respectively, versus the mammalian plasma LOEC for these compounds that correspondingly ranged between 0.01 and 1.8 μg/mL.

In conclusion, although this zebrafish heartbeat screen can be used to rank compounds for potency and identify those that can affect the hERG channel, as a basic readout, heart rate lacks the complexity of other methods of assessment like the ECG, and thereby the potential value of the assay is diminished when compared like with like. However, in the context of a high-throughput assay intended for use early in the drug discovery process, this in vivo model may be used to help prioritize drug candidates when in vitro assays have identified potential liabilities for hERG and other cardiac channels, or especially when liabilities have been identified for structurally similar compounds in animal models but missed previously in vitro. Notwithstanding this point, further applications have also been devised to optimize the value of such digital recordings of the beating heart.

By implementing a series of additional analytical tools, researchers have successfully assessed cardiovascular physiology in a quantitative manner by means of applying measurements for cardiac size and myocardial function (Shin et al. 2010). One such parameter, the diameter of the ventricle, can be determined at end systole and end diastole, and thereby, based on the assumption that the ventricle is a prolate spheroid, the ventricular area and volume can be calculated. Finally, by subtracting the volume of the blood in the ventricle at the end of a beat (end-systolic volume) from the volume of blood just prior to the beat (end-diastolic volume), the stroke volume can be determined, calculated for wild-type larvae as 0.16 nL (Shin et al. 2010). In addition, the potential exists with enhanced imaging capabilities for identifying drug-induced cardiac valve anomalies and regurgitation without the need for an echocardiogram as with mammals, such as demonstrated with dioxin exposure (Mehta et al. 2008). However, this has not been investigated in depth, and the translatable readout when compared to the human four-chambered heart may be somewhat limited by the embryonic larval morphology; a two chambered heart adjoined to the bulbus arteriosus and sinus venosus.

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20.2.1.2 Flow Velocity

PURPOSE AND RATIONALE

In addition to measurements focused specifically on the heart, zebrafish are also amenable to imaging the cardiovascular system. As reviewed previously by Hove (2004), numerous methods of in vivo biofluid dynamic imaging have been reported. For example, by tracking the movement of blood cells with specialized video image capture systems and confocal microscopy, the blood vessel diameter can be established and blood flow (cell velocity) determined (Schwerte and Pelster 2000). These parameters are useful as they allow the calculation of cardiac output, vascular resistance, and contractility. However, the use of Doppler ultrasound with adult zebrafish (Ho et al. 2002) marked a significant advancement in imaging the heart and blood vessels, especially as ultrasound biomicroscopy (UBM) systems provided an estimation of blood flow in the microcirculation at a sensitivity of a few millimeters per second (Foster et al. 2002). This was later developed further for the assessment of adults as a high-frequency ultrasonic system (Sun et al. 2008) offering valuable insight into the cardiovascular system.

PROCEDURE

According to Sun et al. (2008), wild-type adult zebrafish of similar size (1 year old) were anesthetized by submersion in 0.08% MS-222 for 30 s before scales were gently removed at the ventral side between the gills. While under anesthetic (0.04% MS222) and maintained at 27.5°C, fish were positioned for precise imaging of the ventricle and bulbus arteriosus with an ultrasound probe. The probe was lowered into the MS222 solution until it reached a distance of 3 mm from the skin and rotated and fixed, as required, to obtain Doppler waveform images along various planes in real time. Fish were euthanized in a 1% MS222 (submersion for 15 min) after completing the assessment of cardiac size and blood flow measurements.

During imaging, an area (4 mm by 4 mm) was scanned at a frame rate up to 200 images per second. A 115-μm sample volume length was chosen, and a pulse repetition frequency of 11 KHz was utilized.

EVALUATION

Doppler waveforms were estimated using a method previously described (Jensen 1996). Further detailed information regarding the equipment settings can be obtained from Sun et al. (2008).

CRITICAL ASSESSMENT OF THE METHOD

Using this method, Sun et al. (2008) determined the dimensions of the atrium and ventricle as 0.81 × 0.58 mm and 1.19 × 0.62 mm, respectively, and found the cardiac cycle and corresponding heart rate to be 650 ± 175 ms and 93 ± 25 beats per minute (mean ± SD). The peak E and A velocities, where (E) represents early diastolic filling and (A) late diastolic filling, were 3.6 cm/s and 14.4 cm/s, respectively. In agreement with Ho et al. (2002), the E/A ratio was calculated as 0.25. UBM was therefore validated as a suitable method for real-time assessments of the heart, allowing dimensions of each chamber to be taken and measurements for cardiac function and blood flow to be established in vivo (Sun et al. 2008).

Dual-beam Fourier domain optical Doppler tomography (FD-ODT) has also been used to analyze blood velocity in zebrafish, but in contrast to the previous studies, this was undertaken in larvae. In addition to the typical advantages of using larvae instead of adults, in comparison to UBM, this generated high-resolution three-dimensional images and accurately measured absolute flow rates in major vessels of the larvae (Iftimia et al. 2008).

However, other ways of assessing cardiac function and blood flow velocity have also been made possible through technological advancements in the field of microscopy. For example, cell velocity has also been evaluated by laser-scanning velocimetry (Malone et al. 2007). In addition, as analytical and imaging tools have recently been developed that can identify and track individual blood cells, frame by frame, in a series of sequential still images with a high-speed camera at high temporal resolution (Shin et al. 2010), this may offer a significant advantage over Doppler imaging in terms of throughput and the decreased need for specialized equipment. As such, custom algorithms using the coordinates of these cells over a set time, determined by the number of frames, can be processed and normalized to establish erythrocyte flow velocity (FV). In one study, image analysis was undertaken from at least ten frames prior to the onset of systole and continued until at least ten frames into the second systolic acceleration, thereby encompassing an entire cardiac cycle (Shin et al. 2010). They determined a mean FV value of 685 μm/s (peak systolic and diastolic FV, 1,644 and 206 μm/s, respectively), which is comparable to a mean FV of between 291 and 766 μm/s previously reported (Bagatto et al. 2006).

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20.2.1.3 ECG

PURPOSE AND RATIONALE

As the electrocardiogram (ECG) is a common diagnostic tool for humans, any model species that can successfully reproduce or mimic the ECG trace would be considered in preference to other safety cardiac assays by the pharmaceutical industry and regulatory bodies. A typical ECG of the human heart has five deflections, arbitrarily named “P” to “T” waves. The Q, R, and S waves occur in rapid

succession and appear centrally as the most prominent part of the ECG trace. This QRS complex corresponds to the depolarization of the right and left ventricles whereas the T wave represents the repolarization. Therefore, the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle, known as the QT interval, represents electrical depolarization and repolarization of the left and right ventricles. The P wave corresponds to the depolarization of the atria.

In zebrafish studies, two electrodes can be placed either side of the heart to detect and amplify tiny rises and falls in the voltage during each contraction. Depolarization can therefore be traced as it is initiated in the sinoatrial node, progresses as a wave through the atrium, before passing through into and over the ventricles. Therefore, in addition to being a sensitive device for monitoring the overall rhythm of the heart, the zebrafish ECG can also help identify weaknesses in different parts of the heart muscle.

PROCEDURE

Electrocardiograms on adult zebrafish have previously been undertaken (Milan et al. 2006). In brief, zebrafish of the same strain were selected with similar mass and heart size. Two needle electrodes were inserted through the ventral epidermis of each paralyzed adult, one between the pectoral fins and the other at two thirds of the body length away from the head. Zebrafish were orally perfused with 10 mM HEPES (pH 7.5) in E3 medium for 10 min before taking baseline recordings and exposing the fish with test compound diluted from DMSO stocks in E3. To help match the traces with each contraction, the beating heart was simultaneously imaged with a CCD camera. This was synchronized with the electrical recording by pulsing a light-emitting diode in the optical field and recording the electrical stimulus artifact.

EVALUATION

All recordings were processed offline and were accepted only if they met a defined criteria including achieving a minimum amplitude and maintaining signal stability (Milan et al. 2006). Unpaired two-tailed t-tests were used to compare the means of normally distributed continuous variables (P values ≤ 0.05). The observed QT interval was also corrected for variations in heart rate by applying a mathematical calculation.

CRITICAL ASSESSMENT OF THE METHOD

Two distinct issues discussed by the authors were overcome for this study. Firstly, as hypoxia was shown to cause bradycardia and more severe phenotypes within 30 min, the use of a perfusion system to maintain fish hydration and oxygenation was implemented to allow sufficient time for stable ECG recordings. Secondly, as identified for other ECG studies (Egorouchkina et al. 2005), due to the nature of these kinds of readouts, extensive computerized processing of the electrical recordings was necessary, especially with regard to filtering out noise from the signal. Appropriate screening criteria and algorithms developed for offline data processing were hence devised. Signal averaging helped reduce electromyographic noise, although Milan and colleagues demonstrated that skeletal muscle paralysis with an intraperitoneal injection of CTX was necessary to achieve optimal noise reduction. Finally, it was noted that subtle differences in the settings for the ECG, such as reducing the cut-off frequency, and precise electrode placement and heart orientation was required to achieve successful and reproducible recordings.

MODIFICATION OF THE METHOD

No distinct modifications are considered necessary to this assay, although the need for data processing, correct use of the equipment, and likelihood for further adjustments on a case-by-case basis may indicate this assay may be better suited for researchers experienced in this field.

The adult zebrafish ECG has been shown by Milan and colleagues (2006) to be an efficient way to investigate drug-induced adverse effects on heart function, including QT-prolongation, and can add value to supporting the zebrafish as a comparable model species to other vertebrates. In addition, based on the similarity of the mean QTc for adult wild-type zebrafish (416 ± 8 ms) compared to the normal value for humans (typical range 300–450 ms) (Arnaout et al. 2007), these readouts may also be easily translatable to man. However, in terms of throughput and difficulty, one should consider whether small savings in cost and time would warrant the use of zebrafish instead of proceeding directly to well-established mammalian ECG models. Larval ECGs have also been attempted (Forouhar et al. 2004), but contrary to other larval-based screens, this method too has some limitations associated with assay throughput. In addition, although

these studies have successfully recapitulated the adverse effects of certain hERG blockers shown in man, these studies have only assessed a relatively small set of compounds, so it remains to be determined whether other compounds known to affect the heart can be identified.

Finally, as specific electrocardiographic approaches are required for humans in order to diagnose different conditions, for example, ST-elevation myocardial infarctions can be detected using the resting ECG, whereas exercise ECG is better suited for the diagnosis of stable coronary artery disease (Huebner et al. 2010), the zebrafish ECG in its current form may or may not be a suitable model for studying or modeling other cardiac disorders. However, in the future, there may be alternative uses for the zebrafish ECG for investigating heart phenotypes related to toxicity or disease, possibly in concert with transgenic or mutant lines of zebrafish.

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20.2.1.4 High Resolution Voltage Detection: Action Potential Evaluation

PURPOSE AND RATIONALE

In contrast to ECG, more technical and cutting-edge molecular technologies have also been utilized in order to investigate the intrinsic electrical conduction of the embryonic and larval heart. These tend to utilize either transgenic larvae or fluorescent markers and can visualize electrical activity in certain regions of the heart or characterize the temporal-spatial pattern of activity throughout the whole atrium and ventricle. One such example is high-resolution optical mapping with voltage-sensitive dyes such as di-4 ANEPPS, a fluorescent reporter of transmembrane potential, that allowed a comprehensive assessment of cardiac repolarization throughout the heart (Milan et al. 2009; Peal et al. 2011).

PROCEDURE

In the study described by Milan et al. (2009), the hearts from embryos obtained from pairwise crosses of AB zebrafish were excised at 48 hpf by microdissection in modified Tyrode's solution (136 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, 2% BSA). Those exhibiting spontaneous beating were treated with 15–17 mM 2,3-butanedione monoxime, a myofibrillar ATPase inhibitor, in order to stop cardiac motion and then bathed in a 7-μM solution of di-4-ANEPPS for 10 min immediately prior to imaging. Hearts were field paced at 60 min^{-1}, and electrical activity of the isolated hearts was captured in a customized chamber using a CCD camera with appropriate fluorescence illumination.

EVALUATION

The authors used Cardioplex software to perform analyses including exponential subtraction of baseline photobleaching. Action potential durations (APDs) were determined from an average of three or more successive heartbeats (calculated at 90% repolarization). Signal averaging of five successive action potentials was performed using Clampfit to reduce signal noise.

CRITICAL ASSESSMENT OF THE METHOD

This method was used to help investigate the underlying biology of AV-decoupling and a network of genes that regulate cardiac repolarization. For instance, it was determined that wild-type embryos exposed to the hERG blocker dofetilide and homozygous breakdance mutants (possessing a KCNH2 mutation that phenocopies long QT (LQT) type 2 syndrome, the life-threatening disorder in humans associated with prolongation of cardiac repolarization) exhibited the typical 2:1 AV block due to marked prolongation of ventricular refractory periods (Milan et al. 2009). Furthermore, as part of a pharmacogenetic screen

investigating myocardial repolarization, Milan and colleagues used this method to identify 15 out of nearly 300 insertional mutants not exhibiting AV block after being sensitized with 12 μM dofetilide, thereby successfully identifying novel genes that modify the cardiac response to IK_r blockade. Also, using this approach, a small-molecule screen of 1,200 compounds was undertaken with the breakdance mutant zebrafish, resulting in the identification of two molecules that rescued the LQT type 2 phenotype by shortening the ventricular action potential duration (Peal et al. 2011). These included flurandrenolide that was claimed to function via the glucocorticoid receptor-mediated pathway. In addition, compounds like verapamil that act on hERG and the Ca^{2+} channel tended not to have any effects on arrhythmia risk even if QT was prolonged. This method thereby offered the next level of complexity to zebrafish cardiac readouts at higher resolution than ECG and led to several scientific achievements.

As this model requires hearts to be excised prior to staining and assessment, this adds an additional level of technical expertise and processing time to the assay. A potential alternative noninvasive method was previously reported by Arnaout et al. (2007) who demonstrated how monitoring Ca^{2+} release as a surrogate in vivo readout could be used to assess atrial and ventricular conduction. In this study, calcium cycling was captured using a cardiac-specific transgenic line of zebrafish ($Tg(cmlc2:gCaMP)^{s878}$) that expressed a voltage-sensitive fluorescent calcium indicator know as gCaMP (Nakai et al. 2001). At 48 hpf, systolic Ca^{2+} release, recorded as repetitive waves of fluorescence, could be observed transferring from atrium into the ventricle via the atrioventricular junction (Arnaout et al. 2007). However, as selective plane illumination microscopy (SPIM) (Huisken et al. 2004) was used to image the conduction waves, these were only visible in the chosen plane of view (a thin slice of the heart chambers), not the whole heart. It was also necessary to prevent contraction of the heart by injecting a silent heart cardiac troponin (tnnt2) morpholino at the 1-cell stage. Therefore, in conclusion, the potential benefits of the ex vivo optical mapping method should outweigh that of the in vivo calcium indicator model.

MODIFICATION OF THE METHOD

No modifications are as yet identified.

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20.3 CNS

Zebrafish possess comparable brain morphology and signaling pathways as with other vertebrates and have therefore been a key model for investigating both neurobiology and neurological disease (Guo 2004; Mathur and Guo 2010; Rinkwitz et al. 2011). Importantly, dopaminergic, noradrenergic, cholinergic, and serotonergic neurotransmitter pathways have been mapped (Rink and Wullimann 2004; Wullimann and Mueller 2004; Maximino and Herculano 2010; Lillesaar 2011 in press), and a blood-brain barrier can be observed in larvae between 3 and 10 days postfertilization (dpf) (Goldsmith and Fleming 2007). In particular, endothelial cells exhibiting tight junctions in cerebral microvessels can be observed from 3 dpf by implementing permeability studies with horseradish peroxidase and fluorescent tracers (Jeong et al. 2008). Zebrafish should therefore be suitable as an alternative in vivo vertebrate CNS model for assessing the safety and efficacy of new neurological drug candidates.

20.3.1 Locomotor Assays

PURPOSE AND RATIONALE

Zebrafish are amenable to relatively high throughput neurobehavioral screening via locomotor-based

tracking assays from 96 hpf when they are free swimming (Drapeau et al. 2002), and as such can be used to monitor various drug-induced effects on activity including sedation, stimulation, and convulsion (Zhdanova et al. 2001; Barros et al. 2008; Bate et al. 2010), as well as sleep patterns, learning, and potential drug addiction (Zhdanova et al. 2001; Cahill 2002; Guo 2004; Orger et al. 2004; Ninkovic et al. 2006; Mathur and Guo 2010; Stewart et al. 2011). These semiautomated screens can simultaneously quantify parameters such as distance moved, speed, number of movements over time, and even response to startle stimuli, similar to those used in rodent safety pharmacology assessments.

PROCEDURE

One of the first automated locomotor assays was used to investigate the activity of larval zebrafish on a circadian time scale (Cahill et al. 1998). They demonstrated that time-lapse video image analysis could be performed simultaneously on 100 larvae using a single recording system with custom specimen plates, temperature controlled imaging system, and analytical software. In this study, larvae from the AB strain of zebrafish were arrayed 1 per well and assessed for 130 h from 10 dpf at 28.5°C. A series of 60 images was collected every 4–5 min at a rate of 1 per second. No food was provided during the recording period. In order to enhance the contrast of the fish for imaging with a CCD camera, the larvae were backlit by placing a mirror beneath the specimen plate. This allowed a pixel value threshold to be used to distinguish the fish from the lighter background and monitor movement frame by frame. To allow movement to be tracked throughout the day and night, an infrared (IR) camera was used to record the images.

A few years later, this method was modified by Zhdanova et al. (2001) who assessed Tubingen strain larval zebrafish between 7 and 14 days old in constant darkness, also using an infrared camera. In this case, larvae were backlit by an 880-nm IR source, and a custom software program designated "FishWatch" captured the movements of 60 fish. After a 2-h basal recording, larvae were treated with different compounds (melatonin, diazepam, and pentobarbital) and then imaged for a further 2 h in consecutive 15-s intervals. It was noted that the onset of behavioral effects caused by exposure to the test compounds typically occurred within 20 min after treatment (Zhdanova et al. 2001).

Since these early studies, imaging hardware and software based on these methods has become commercially available for the purpose of tracking small organisms such as zebrafish larvae. For example, Berghmans et al. (2007) captured and assessed the locomotor activity of larvae from the WIK zebrafish strain from 6 dpf using EthoVision 3.1 locomotion tracking software (Noldus, Wageningen, The Netherlands) and imaging apparatus containing a high-resolution digital video camera and IR light source for image capture in the dark. In this study, larvae were arrayed in 96-well plates, 1 per well, 12 larvae per treatment group, and co-treated for 24 h with defined concentrations of test compound and pentylenetetrazole (PTZ, 20 mM). Concentrations of test compound were determined from a dose-ranging assay in which the maximum tolerated concentration (MTC) was established. The main parameter used in this locomotor assessment was the mean total distance moved for each treatment group, based on a 60-min recording. As PTZ treatment was shown to induce a distinct series of movements resembling tonic-clonic seizures (Baraban et al. 2005), the aim of this study was to identify antiepileptic drugs, those that ameliorated the PTZ phenotype (Berghmans et al. 2007).

EVALUATION

When evaluating the adverse effects of compounds on zebrafish larvae, any significant increase or decrease in locomotor activity was determined by making comparisons with positive control, sham, and vehicle control larvae.

Compounds co-treated with PTZ were considered to have potential antiepileptic activity if treatment groups exhibited a statistically significant reduction in the total distance moved in comparison to PTZ-only control treatment groups. For the purpose of quality control, the PTZ treatment group had to show a significant increase in locomotion in comparison to the vehicle controls and no significant increase of locomotion had to occur for larvae treated with the test drug alone.

Data is typically analyzed by Student's *t*-test or one-way analysis of variance (ANOVA), followed by post-hoc comparisons between the experimental groups, such as using Tukey' test (significance $P = 0.05$).

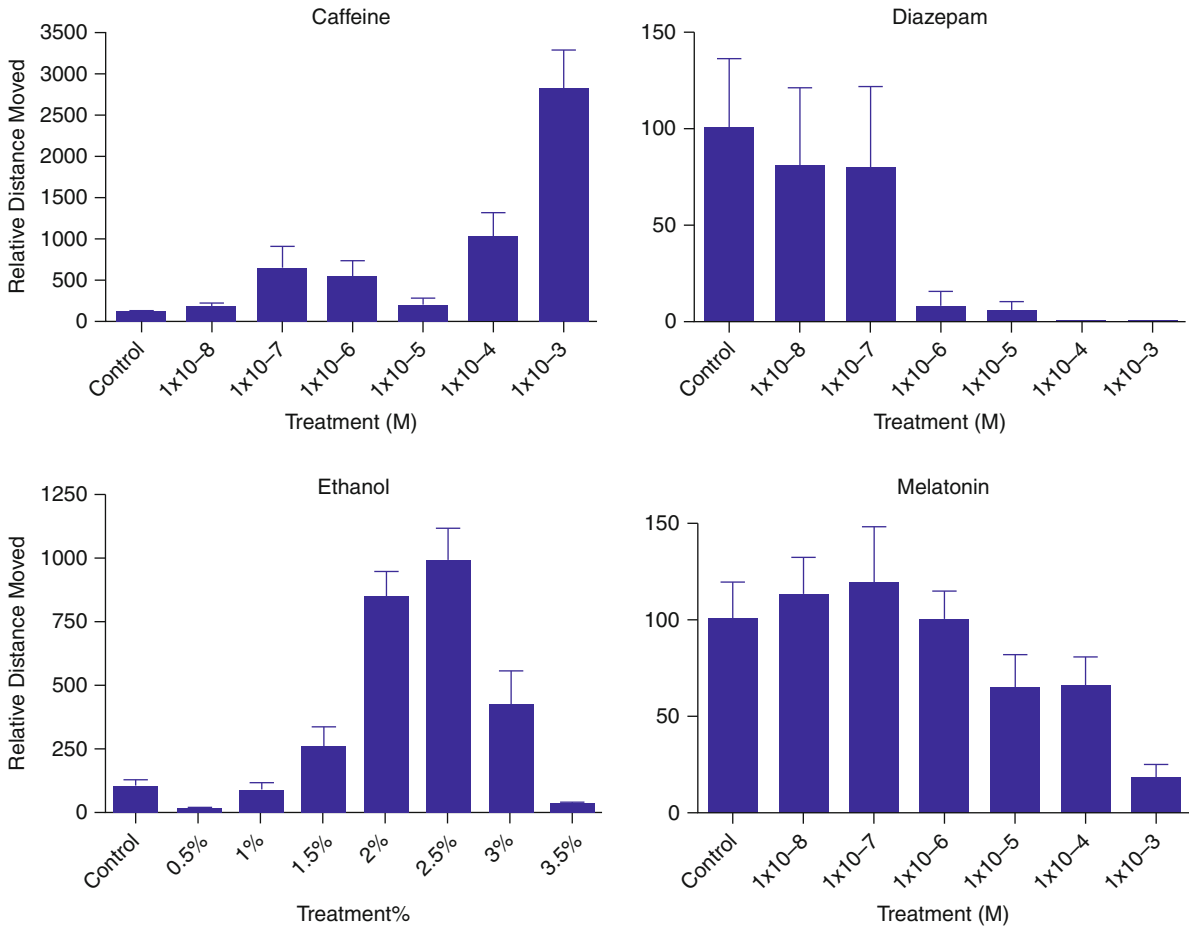

Fig. 20.2 Effect of stimulants and sedatives on zebrafish larvae at 7 dpf assessed via locomotor activity. Exposure to caffeine and ethanol causes increased movement (hyperactivity) whereas diazepam and melatonin induce a sedative effect. Concentrations of ethanol greater than 3% lead to toxicity

CRITICAL ASSESSMENT OF THE METHOD

During the early studies, problems were commonly associated with the software failing to identify the larvae. Cahill et al. (1998) reported that, in some cases, often when fish were inactive near the bottom of the well, a fish was not recognized because too few pixels crossed the given threshold. This therefore led to a possible underestimation for the locomotor activity as these instances were recorded as if the larva had not moved. They also noted that on occasion the software misinterpreted the background as an object or recorded two or more separate locations for the larvae, again due to subtle changes in pixel value. In contrast, as software became more advanced, new ways to identify and monitor the larvae were implemented, such as the use of different reference points on a larva including the eyes and swim bladder, to distinguish fish from other potential artifacts like air bubbles or shadows.

Nevertheless, the locomotor platform in various forms has successfully demonstrated that zebrafish larvae respond to certain compounds in a similar fashion as exhibited by man. For example, sedatives such as melatonin, diazepam, and pentobarbital have caused hypomotility (Fig. 20.2; Zhdanova et al. 2001) whereas stimulants including caffeine and low-medium doses of ethanol (Fig. 20.2; Lockwood et al. 2004) have caused hyperactivity. Although at high concentration (>3–4%), ethanol reduced locomotor activity, leading to toxicity and ultimately lethality. In addition, as noted previously, exposure to PTZ caused a dramatic increase in swimming speed in

combination with rapid circling and the induction of a series of postures and movements, culminating in generalized clonus-like seizures. This is a reproducible phenotype that has been used for large-scale screening for antiepileptic drugs which ameliorate the response (Berghmans et al. 2007; Bate et al. 2010). However, larvae have not always responded as expected after exposure to certain compounds. In fact, as a larval model for seizure liability assessment, this video-tracking method for identifying characteristic chemically induced changes in locomotion only achieved a predictivity of 72% in one study consisting of 25 blinded compounds, including the convulsants 4-aminopyridine, amoxapine, bemegride, maprotiline, picrotoxin, semicarbazide, and strychnine (Winter et al. 2008). As such, in the context of a high-throughput in vivo screen aimed at determining potential liability very early in the drug discovery process, this level of predictivity as a standalone assay may or may not be considered acceptable, and the future use of the assay may be dictated by how zebrafish respond to specific classes of compounds and whether these compounds are typically misclassified in vitro, in which case, the zebrafish assay could prove more advantageous. However, when applied in a cascade of assays alongside in vitro screens like brain (hippocampal) slice electrophysiology, the combined approach would be more valuable, offering increased specificity and sensitivity when compared to the outcome of each individual assay (Easter et al. 2009). This should therefore help prioritize candidate drugs before testing in the more expensive, yet more predictive, rodent behavioral seizure assays or the gold standard electroencephalogram (EEG).

MODIFICATION OF THE METHOD

As these methods are still evolving and are now predominantly commercialized with proprietary software and hardware, it is difficult to focus on specific modifications. On the whole, the key requirements that will enable this method to be widely accepted is the assessment of more validation compounds using a variety of parameters for analysis and a greater understanding and optimization of the protocol used to treat the larvae with compounds. Altered locomotion in response to subtle changes in concentration, confusion between a toxic and sedative response, and different results depending on the duration of compound exposure all need to be investigated further.

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20.3.2 Behavioral Analysis: Cognitive Flexibility

PURPOSE AND RATIONALE

Some of the most complex and important CNS models involve the study and evaluation of behavior. Similar to established mammalian models, software has been developed to monitor the swimming behavior of fish in tanks as well more classical maze designs. To date, this has been attempted with both larval and adult zebrafish using a variety of platforms involving learning and memory tests, essential for determining the underlying molecular mechanisms of cognitive function.

Although more studies are required before the cognitive ability of zebrafish is fully understood, certain molecular factors have already been implicated (Pradel et al. 1999, 2000). Support for the use of fish cognition models is also available from other species. Goldfish for instance were one of the first fish to be studied and have been documented showing habituation to a fear response (Brookshire and Hognander 1968; Laming and Ennis 1982), short-term memory (Ohnishi 1997), and appetitive learning enhancement (Spieler et al. 1999). Toxicological studies were also used to demonstrate that compounds like atrazine can affect basic social behaviors such as grouping (Saglio and Trijasse 1998), fear responses can be altered by endorphins (Olson et al. 1978), and dizocilpine can impair escape learning (Xu and Davis 1992; Davis and Klinger 1994; Xu 1997).

Similarly, zebrafish have been observed responding to a manner of basic stimuli. In particular, early research utilized a defensive behavior linked to an antipredatory alarm response. For example, Hall and Suboski (1995) demonstrated that fish can be trained to react to harmless olfactory and visual stimuli in the form of an escape response if first presented to fish together with an alarm substance, a pheromone released under stress or injury. In contrast, in a later study, fish were trained within 3 weeks to swim to one side of the tank after an acoustic signal (tapping on the side of the tank) that represented the imminent delivery of food (Williams and Messer 1998). These kinds of investigations therefore set the scene for more complex learning paradigms.

PROCEDURE

In order to monitor several adult fish in a single assay, it may be necessary to permanently label them with a marker. In one example, zebrafish were anesthetized with MS-222 (84.5 mg/L) and subcutaneously injected with a fluorescent elastomer (Arthur and Levin 2001).

Depending on the hypothesis being tested, various designs of maze can be utilized. These can involve customized aquaria, such as a classic Y- or T-maze, or can be relatively simple apparatus consisting of static walls or moveable doorways intended to partition a standard tank. In the latter case, the most basic method is to separate the tank into two, thereby designating one half to be the "start" chamber and the other as an "escape" chamber (Fig. 20.3). Multiple partitions can then be added to provide alternative choices for escape, each with a differentiator (e.g. color, light/shade, chemical, etc.). Various stimuli can then be applied to test the immediate escape and/or avoidance response and repeated several times throughout each week to evaluate reinforcement of learning.

Threat avoidance: In the preliminary study by Arthur and Levin (2001), the aversive stimulus was in the form of a fishnet. After a period of acclimation, the net was lowered into the tank and a door was opened to an escape chamber. For one test group (fish tested individually), the net was then moved back and forth, whereas for a second group, the net remained stationary. The escape or avoidance latency was recorded over six repeat assays, after which the situation was reversed, so the fish used to the stationary net were observed how they responded to a moving net and vice versa.

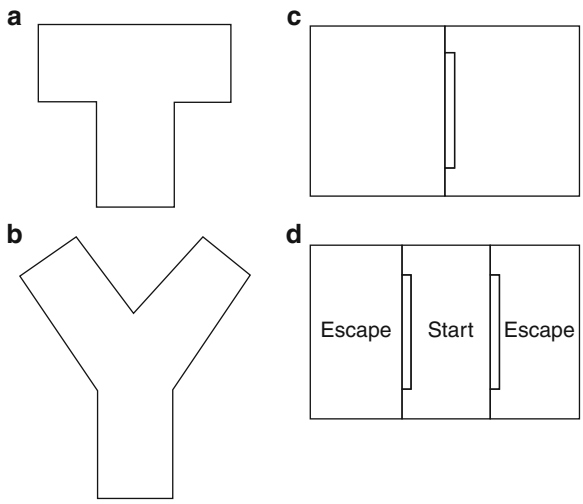

Fig. 20.3 Examples of classic maze designs for studying zebrafish behavior. (**a**) T-maze, (**b**) Y-maze, (**c**) two-compartment design with a single sliding door separating the chambers, and (**d**) three-compartment design with a central start chamber flanked either side by left and right escape chambers

Discrimination: Arthur and Levin (2001) continued to describe a similar assay, but in this study, they used a three-compartment maze with a central start chamber and two choices for an escape chamber (left and right): one predesignated as a "safe" chamber and the other as a "punishment" chamber (Fig. 20.3d). After a period of acclimation, the aversive stimulus was again lowered into the tank and moved back and forth. The net was removed if the fish entered the safe chamber, whereas those in the punishment chamber were subjected to further stimulation by the net. Two sets of fish were trained, one with the safe chamber on the left and the other on the right side of the tank. This exercise was repeated, 3 trials per session for 10 sessions. As performed previously, the assay was also repeated, but the punishment and safe chambers were reversed partway through the exercise (3 sessions prior to switching and 18 sessions afterwards).

EVALUATION

Zebrafish were scored for avoidance or escape learning depending on whether they reacted before (avoidance) or after (escape) the start of the stimulus. Latency was measured in seconds (mean ± SEM). The duration of the acclimation, stimulus, and punishment (where applicable) was strictly controlled, and no fish were touched with the net. The readouts from two sessions were averaged to assess performance.

CRITICAL ASSESSMENT OF THE METHOD

While the larval capacity for learning has been investigated via assessing altered responses and habituation to acoustic, touch, or visual stimuli (Best et al. 2008; Aizenberg and Schuman 2011), adult models are generally accepted to be more robust. This may be because the neural circuitry (Kastenhuber et al. 2010), endocrine systems (Kimmel et al. 1995), and neuromuscular systems (Dou et al. 2008) are not fully established at the earlier stages of development and thereby larvae may not always display the complex behavior of their adult counterparts, or may be attributable to certain screening parameters which can be used and optimized in such adult studies.

Due to the adaptability of the basic model that relies on assessing spatial and temporal tracking of the zebrafish, different test conditions and assay parameters can be applied to examine different hypotheses. The outcome of the initial threat avoidance study revealed that only fish regularly exposed to the stimulus (moving net) learned to escape the threat by swimming into the adjacent compartment, and when exposed to a stationary net, upon reversal of the test, these fish suppressed the escape response (Arthur and Levin 2001). The discrimination study demonstrated that fish could be trained within six sessions to avoid the punishment chamber and after the chambers were reversed, rapidly adapted. In addition, discrimination based on color was also demonstrated in another more complicated version of this study which used red and blue chambers to dictate which one was the punishment chamber. Once again, the fish learned to identify the safe chamber, even though it had to be determined from a visual versus a spatial cue (Arthur and Levin 2001).

In addition to conditioning the fish to display these behaviors when discrimination is related to a spatial or nonspatial, visual, or olfactory-based response, evidence of reversal and extinction have also been demonstrated when the assay parameters were changed, thereby indicating zebrafish possess cognitive flexibility.

MODIFICATION OF THE METHOD

Further modifications to these models have demonstrated that adults can respond to alternating feeding zones (Williams et al. 2002) or associating a food

reward with certain colors placed in the tank (Colwill et al. 2005) and reliably exhibit avoidance behavior when exposed to other adverse stimuli such electric shock (Xu et al. 2007). Also, as zebrafish have responded well when treated during development to compounds that enhance learning such as nicotine (Arthur and Levin 2001) or impede learning and cause memory deficits like lead and ethanol (Carvan et al. 2004), these studies add further support to the use of zebrafish in neurobehavioral screening.

Finally, a conditioned place preference (CPP) model can be used to evaluate the reward reinforcement properties of a test compound. In these studies, the zebrafish are allowed to choose a preferred location or compartment, after which a compound is added to the least preferred compartment and the zebrafish are observed to establish whether their preference in location changes, as demonstrated with cocaine and D-amphetamine (Darland and Dowling 2001; Ninkovic et al. 2006). In addition, as discontinuation of a nonanesthetic dose of cocaine leads to anxiety-like state consisting of hyperactive behavior and this increase in motility can be subsided by either treatment with diazepam or reinstatement of cocaine exposure, the adult zebrafish may also be a potential model for testing drug withdrawal (Lopez-Patino et al. 2008). These findings are supported by Cachat et al. (2010) who demonstrated that withdrawal of diazepam, ethanol, and morphine also produced anxiogenic-like behavioral responses as well as changes to the stress marker cortisol. Likewise, microarray analysis of ethanol- and nicotine-treated zebrafish, also showing the reward CPP response, confirmed that neuroadaptation pathways implicated in drug dependence were conserved between zebrafish and mammals (Kily et al. 2008). However, when performing assessments for anxiety-related phenotypes, the choice of zebrafish strain is important as certain mutants, including albino and leopard, have a higher baseline anxiety compared to wild-type strains and hence may be better suited for testing anxiolytics (Egan et al. 2009).

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Chronobiology and the Implications for Safety Pharmacology

21

Björn Lemmer and Maxim Soloviev

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21.1 Introduction

In order to get an insight into the mechanisms of action, efficacy, the pharmacokinetics, side effects, and toxicity of a compound for clinical use, it is essential to perform studies in animal models. In preclinical studies, rodents such as rats and mice serve as an important tool for drug development. In addition, dogs, monkeys, pigs, and some other large animals are of great importance in preclinical safety evaluation. Safety pharmacology is an essential part of this process. This review will concentrate on published data within these groups of experimental animals under the special focus whether and to what extent biological rhythms are involved in studies using these animal models.

When going into detail concerning the published data, it is obvious that by far, the most data were obtained in rats and mice. Much less studies under a chronobiologic design were performed in large animals. This situation will be mirrored by composition of the review.

It is interesting to note that already in 1838, Kreutzer mentioned in his Handbook of Veterinary Medicine that daily variations exist in drug effects in animals (Kreutzer 1838).

21.2 The Biological Clock

Rhythmicity is the most ubiquitous feature of nature. Rhythms are found from unicellular to complex multicellular organisms both in plants, animals, and men. Living organisms are continuously influenced by external stimuli, many of which have rhythmic patterns. Environmental rhythms in daily and seasonal

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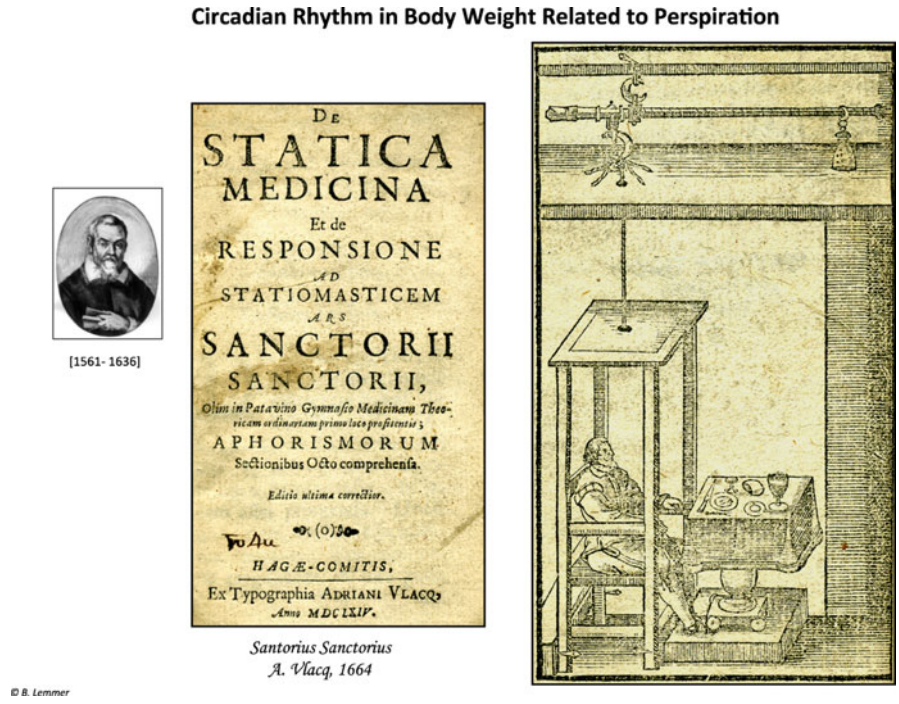

Fig. 21.1 The weighing chair (sella sedento) of Sanctorius Sanctorius shown in his book *De Statica Medicina*, with which he determined the perspiration insensibilis and performed around-the-clock experiments on himself for 30 years Sanctorius (1644)

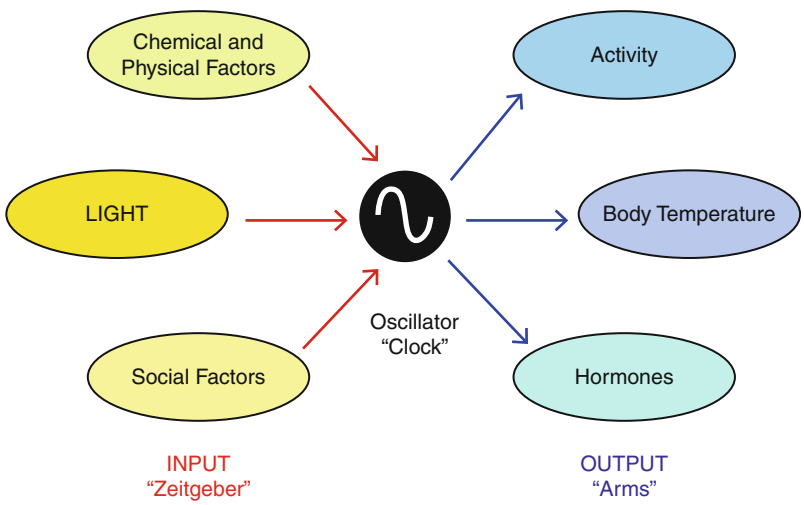

Fig. 21.2 Interaction between the internal clock, zeitgeber, and the influence on bodily functions which are the arms of the clock

patterns of light, food availability, temperature, etc., are predictable, and animals – including humans – have the ability to anticipate these environmental events with periodically and predictably changing internal conditions. These rhythmic patterns of anticipation have clear advantages and survival value (Strubbe and Woods 2004). The frequencies of rhythms in nature cover nearly every division of time. There are rhythms which oscillate once per second (e.g., in the electroencephalogram), once per several seconds (respiratory rhythm, heart rate), up to rhythms which oscillate once per year (circannual rhythm).

Table 21.1 Major hemodynamic parameters and duration of ECG intervals (mean ± SD) obtained by telemetry from freely moving beagle dogs and cynomolgus monkeys collected around 10–11 a.m. (adapted from Soloviev et al. (2007)), minipigs during daytime (adapted from Stubhan et al. (2008)), and CD(SD) rats collected around 9–11 a.m. (Adapted from Atterson P., WIL Research Laboratories - Historical control summary of hemodynamic values for male CD(SD) Rats (Body weight range: 239 – 453g), Personal communication 2011)

	Beagle dogs	Cynomolgus monkeys	Göttingen minipig	CD(SD) rat
Heart rate (bpm)	94 ± 26	141 ± 31	56 ± 7	376 ± 37
Systolic blood pressure (mmHg)	141 ± 15	100 ± 16	122 ± 15	129 ± 10
Diastolic blood pressure (mmHg)	83 ± 11	67 ± 14,	86 ± 10	89 ± 7
Mean blood pressure (mmHg)	104 ± 12	83 ± 15	–	107 ± 8
Core body temperature (°C)	38.0 ± 0.4	38.2 ± 0.3	37.0 ± 0.8	37.1 ± 0.3
PR interval (ms)	117 ± 12	101 ± 16	125 ± 21	–
QRS complex (ms)	32 ± 3	35 ± 9	56 ± 9	–
QT interval (ms)	215 ± 21	221 ± 31	320 ± 38	–

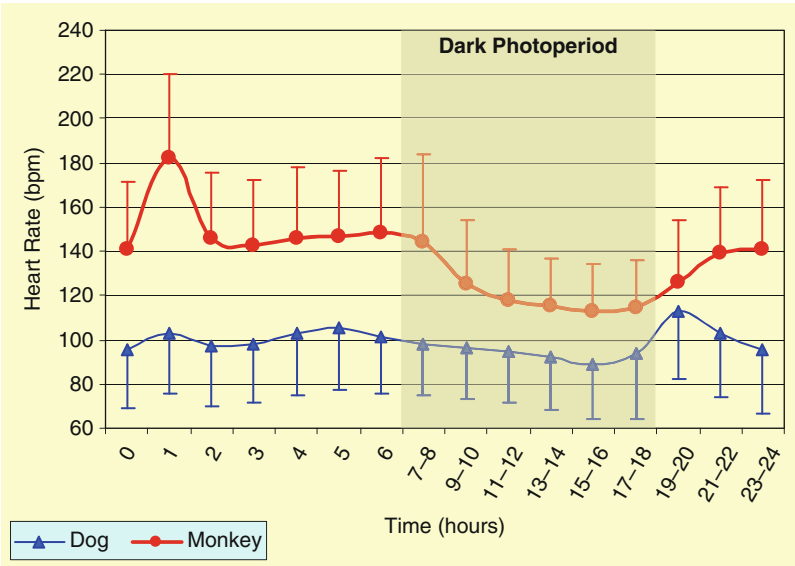

Fig. 21.3 Daily variations (mean ± SD) of the heart rate in dogs and monkeys (Adapted from (Soloviev et al. 2006c))

The most evident environmental change which results from the regular spin of the earth around its central axis and resulting in the alternation between day and night seems to have induced the predominant oscillation, the circadian rhythm (the about-24-h rhythm; circa = about, dies = day, as proposed by (Halberg 1959, 1969)). There is sound evidence that living systems including humans are not only organized in space but are also highly organized in time. One of the first observations on a rhythmic pattern in man was presented by the famous physiologist Sanctorius Sanctorius in 1664 when he described in a self-experiment daily variations in body weight due to transpiration, his famous experimental setting is shown in Fig. 21.1 Sanctorius (1644).

Circadian rhythms have been documented throughout the plant and animal kingdom at every level of eukaryotic organization. Circadian rhythms by definition are endogenous in nature, driven by oscillators or clocks (Aschoff 1954, 1963a, b, 1965), and persist under

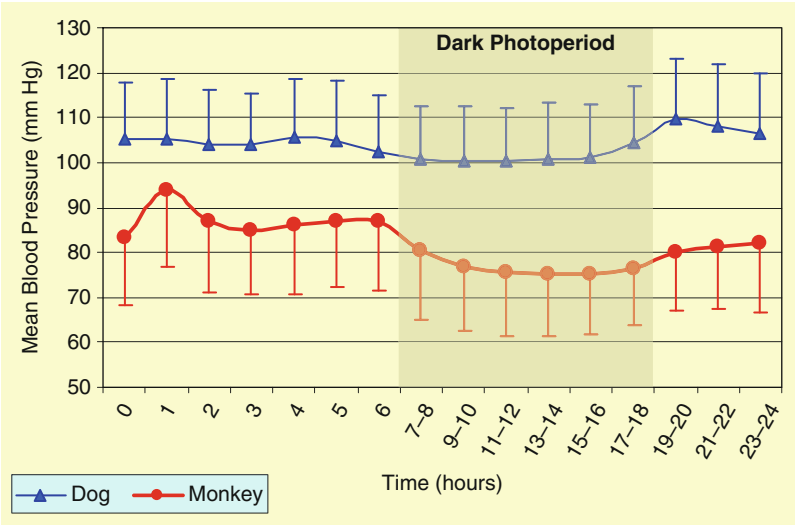

Fig. 21.4 Daily variations of the mean blood pressure (mean ± SD) in dogs and monkeys (Adapted from (Soloviev et al. 2006c))

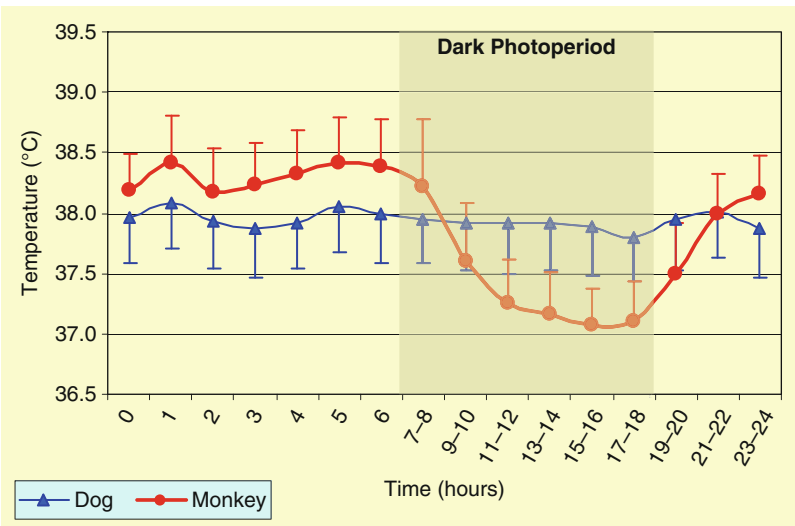

Fig. 21.5 Daily variations of the core body temperature (mean ± SD) in dogs and monkeys (Adapted from (Soloviev et al. 2006c))

free-running conditions. In various species (e.g., *Drosophila melanogaster, Neurospora,* mouse, golden hamster, rhesus macaque, man), the genes controlling circadian rhythms have been identified (genes: *per, frq, clock, tau, Rev-erb alpha*) (Hall 1998; Takahashi 1992; Sitzmann et al. 2008; Hastings 1998; Reppert 2000; Albrecht 2002). In 1971, Konopka and Benzer (1971) were able to identify on the X chromosome of *Drosophila* a region which controlled the period in the eclosion rhythm of three mutants (*per* clock gene). In 1984, Bargiello et al. demonstrated that a fragment of the *per* gene injected into embryos of an arrhythmic mutant of *Drosophila* could restore rhythmicity in eclosion (Bargiello et al. 1984). This data provided the first evidence that the biological clock is genetically determined and can even be transplanted from one animal into another, thereby inducing the rhythmicity of the donor into the recipient.

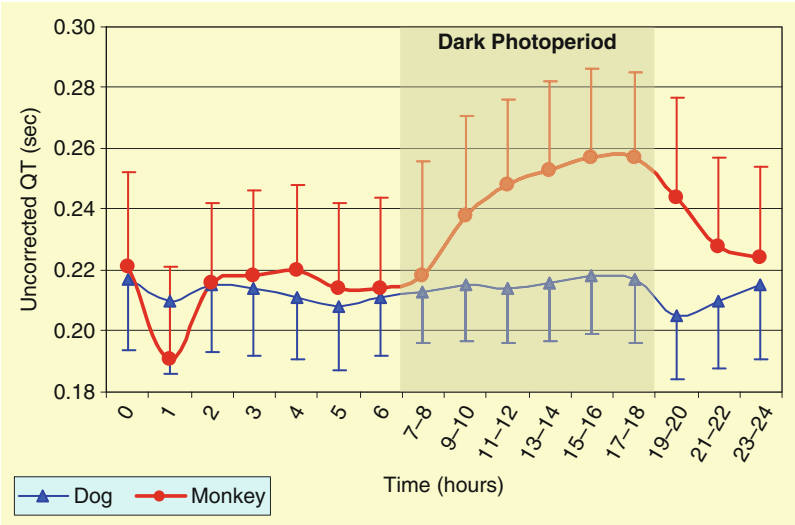

Fig. 21.6 Daily variations of the uncorrected QT interval (mean ± SD) in dogs and monkeys (Adapted from (Soloviev et al. 2006c))

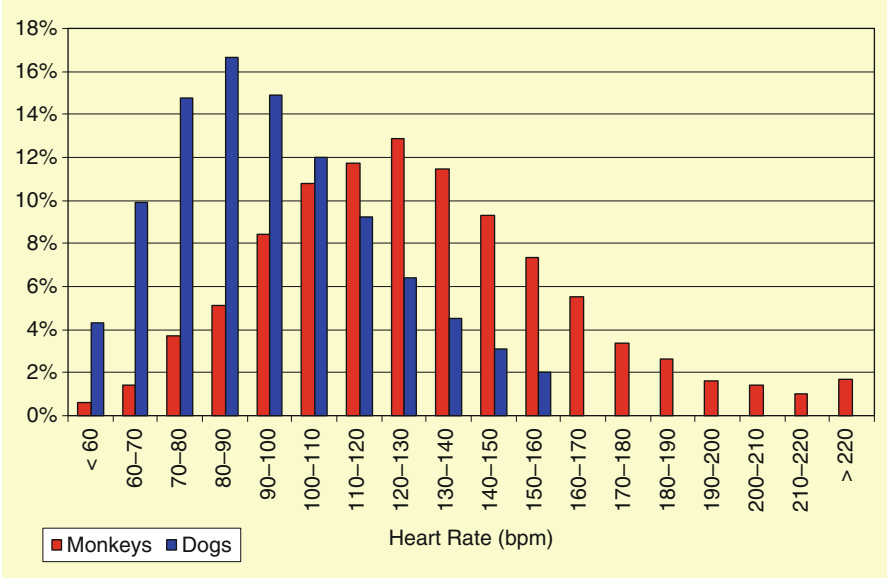

Fig. 21.7 Heart rate distribution in dogs and monkeys (Adapted from (Soloviev et al. 2007))

Circadian clocks are believed to have evolved in parallel with the geological history of the earth and have undergone selection pressures imposed by cyclic factors in the environment (Kyriacou et al. 2008). These clocks regulate a wide variety of behavioral and metabolic processes in many life forms (Hastings 1997; Edmunds 1997; Almon et al. 2008; Tsinkalovsky et al. 2007). They enhance the fitness of organisms by improving their ability to efficiently anticipate periodic events in their external environments, especially periodic changes in light, temperature, and humidity.

The mammalian circadian clocks, located in the neurons of suprachiasmatic nuclei (SCN) in the brain and in cells of peripheral tissues, are driven by a self-sustained molecular oscillator, which generates rhythmic gene expression with a periodicity of about 24 h (Reppert and Weaver 2002; Hastings and Herzog 2004). This molecular oscillator is composed of interacting positive

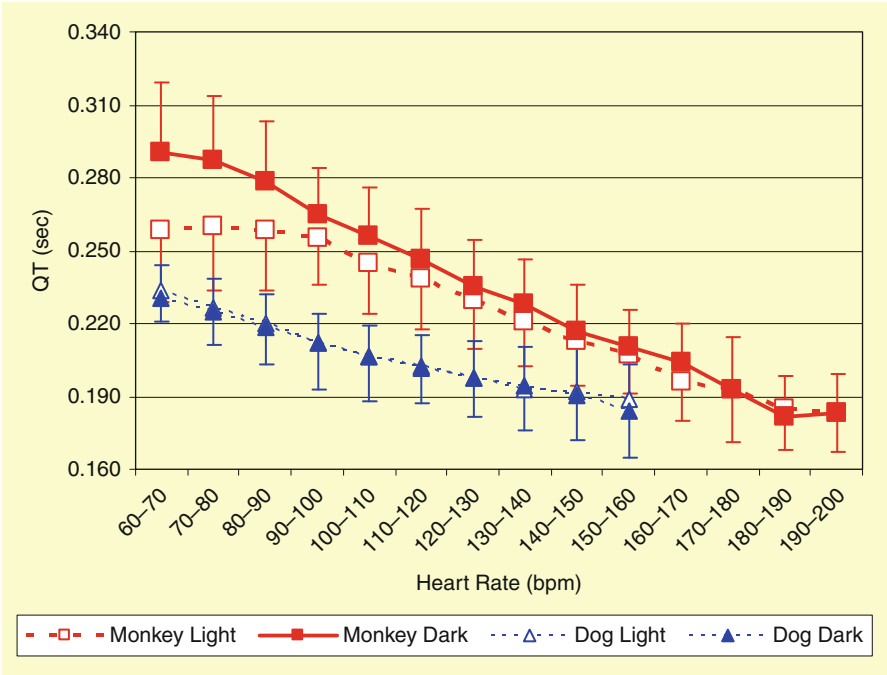

Fig. 21.8 Comparison of QT intervals (mean ± SD) collected during light cycle to those collected during dark cycle at certain heart rates in dogs and monkeys (Adapted from (Soloviev et al. 2006c))

Table 21.2 Seasonal variations of heart rate, core temperature, and blood pressure (data presented as mean ± SD for 66 beagle dogs) (Adapted from Soloviev et al. (2006b))

	Winter		Spring		Summer		Autumn	
	M	F	M	F	M	F	M	F
Number of measurements	2870	*	2160	2564	9195	4055	5142	1581
Heart rate(bpm)	88.8 ± 27.2	*	109.2 ± 26.7	112.4 ± 27.7	96.6 ± 25.6	96.8 ± 25.2	98.4 ± 29.2	98.0 ± 26.7
Mean pressure (mmHg)	104.1 ± 12.5	*	109.1 ± 12.3	108.5 ± 13.2	103.6 ± 13.0	101.9 ± 13.1	103.8 ± 12.3	100.2 ± 13.6
Systolic pressure (mmHg)	146.4 ± 14.6	*	145.8 ± 15.3	143.8 ± 14.3	141.2 ± 14.4	137.3 ± 15.9	141.5 ± 14.6	133.2 ± 15.8
Diastolic pressure (mmHg)	82.7 ± 11.6	*	88.5 ± 11.5	87.6 ± 11.4	83.2 ± 12.1	81.4 ± 11.6	83.6 ± 11.7	80.5 ± 12.3
Core body temperature (°C)	37.89 ± 0.38	*	38.00 ± 0.30	37.98 ± 0.32	37.91 ± 0.38	37.97 ± 0.29	37.96 ± 0.54	37.94 ± 0.34

and negative transcription/translation feedback loops (Hastings 2003; Hardin 2004; Rensing 1997) in which the heterodimeric transcription activator CLOCK/Bmal1 promotes the transcription of E-box containing cryptochrome (Cry1 and Cry2) and period (Per1 and Per2) genes, as well as clock-controlled output genes. After being synthesized in the cytoplasm, CRY and PER proteins feedback in the nucleus to inhibit the transactivation mediated by positive regulators (Harms et al. 2004). The mPER2 protein acts at the interphase between positive and negative feedback loops by indirectly promoting the circadian transcription of the Bmal1 gene and by interacting with mCRY proteins (see Hardin 2004; Lowrey and Takahashi 2004).

It is interesting to note that clock genes have now been found in single cells of human skin

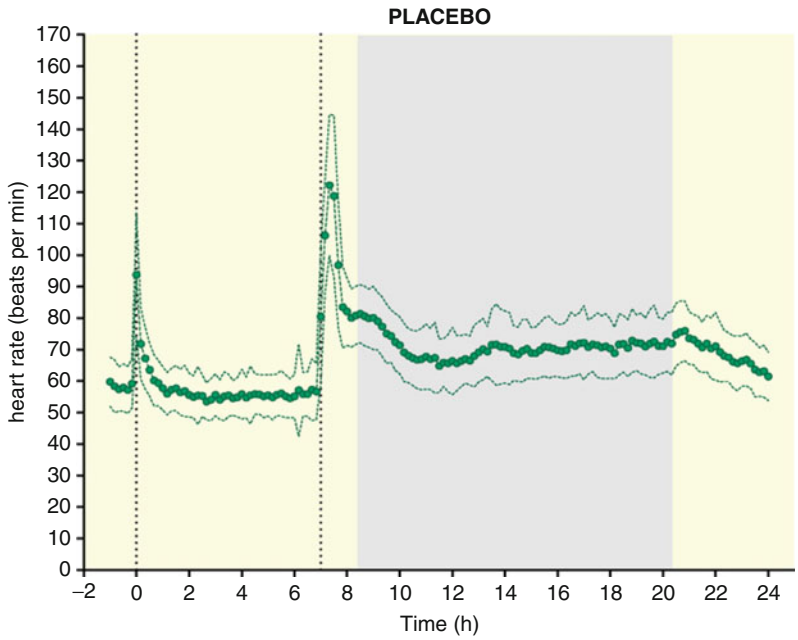

Fig. 21.9 Heart rate (mean ± SD) measured over a 24-h monitoring period by implantable telemetric device in freely moving Göttingen minipigs. The dotted line at 0 h indicates oral administration of placebo, whereas the dotted line at 7 h indicates feeding. The gray-shaded area represents the dark period (Adapted from Markert M (2011))

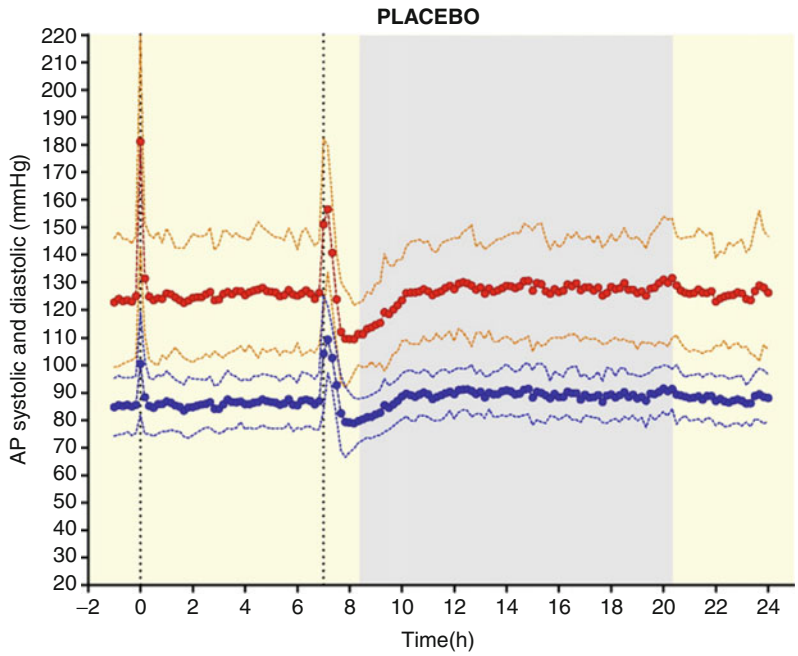

Fig. 21.10 Systolic and diastolic arterial blood pressures (mean ± SD) measured over a 24-h monitoring period by implantable telemetric device in freely moving Göttingen minipigs. The dotted line at 0 h indicates oral administration of placebo, whereas the dotted line at 7 h indicates feeding. The gray-shaded area represents the dark period (Adapted from Markert M (2011))

and mucosa (Bjarnason et al. 2001); furthermore, it has been shown that about 8–10% of all genes are regulated in a circadian fashion (Storch et al. 2002).

In general, the human endogenous clock does not run at a frequency of exactly 24 h but somewhat slower. The rhythm in human body temperature which is timed by the biological clock has a period of

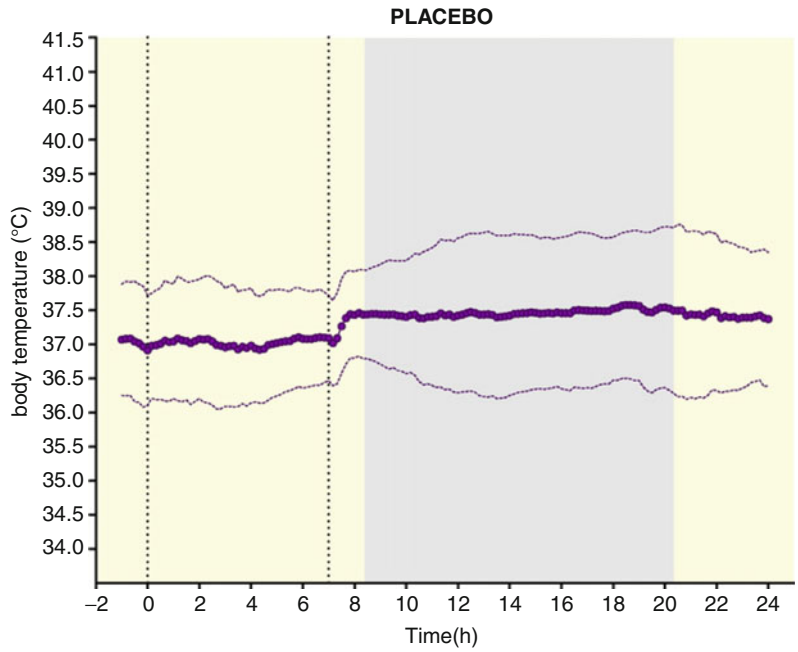

Fig. 21.11 Body temperature (mean ± SD) measured over a 24-h monitoring period by implantable telemetric device in freely moving Göttingen minipigs. The dotted line at 0 h indicates oral administration of placebo, whereas the dotted line at 7 h indicates feeding. The gray-shaded area represents the dark period (Adapted from Markert M (2011))

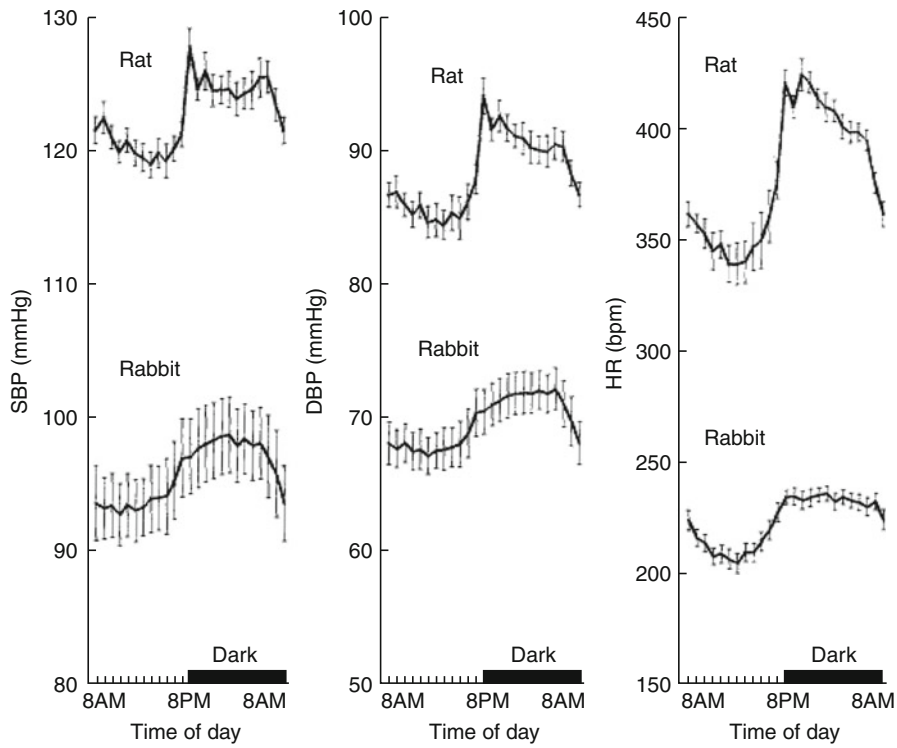

Fig. 21.12 Comparison of circadian rhythms of systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR in rabbits (n = 17) and rats (n = 10). Hourly means are averages for 4 consecutive days (Adapted from Sato et al. (1995))

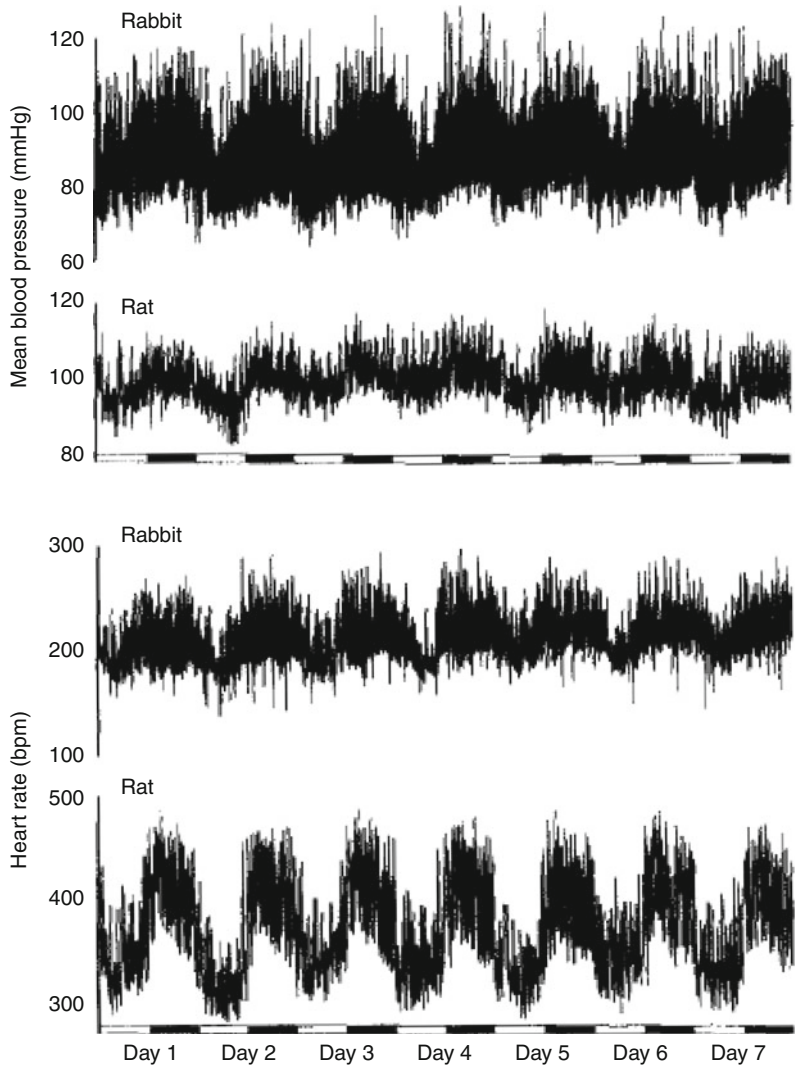

Fig. 21.13 Continuous recordings of mean blood pressure and heart rate plotted every 2 min for 7 days in one rabbit and one rat. Closed bar, dark phase; open bar, light phase (Adapted from Sato et al. (1995))

about 24.5 h under free-running conditions, i.e., without environmental time cues or zeitgebers (e.g., light, temperature) (Fig. 21.2).

The term "zeitgeber" introduced by Jürgen Aschoff (1954, 1965) is now part of the international scientific language. Mammals such as rodents or humans can entrain their activity to regular light cycles not shorter than 22 or longer than 26 h (Aschoff and Pöhl 1978). Zeitgebers entrain the circadian rhythm to a precise 24-h period. Zeitgebers are, therefore, necessary to entrain a living subject to a "normal" period of 24 h!

In experimental animals and in humans, however, most rhythmic fluctuations still cannot be studied under free-running conditions, leaving the answer open to what degree they are really "circadian." Purely exogenous rhythms are better termed as "24-h" or "daily" rhythms. Thus, an overt 24-h rhythm in a given parameter can be endogenous or predominately exogenous in nature. Within the published clinical literature, however, the term "circadian" is not always used in the above-mentioned correct sense (as used by chronobiologists); the broader term will be used here, too. Though seasonal rhythms in cardiovascular functions were also described, the present review will focus on circadian rhythms since much more data were accumulated over a 24-h scale and the underlying mechanisms studied.

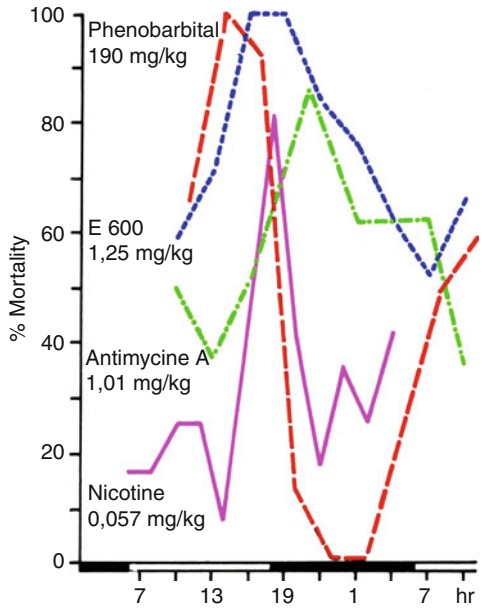

Fig. 21.14 Chronotoxicity of drugs in mice (Acc. Mayersbach (1976))

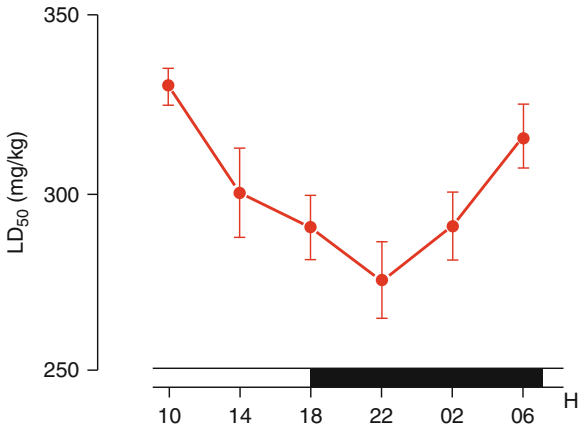

Fig. 21.15 LD_{50} value of procainamide in mice (Acc. Bruguerolle (1984))

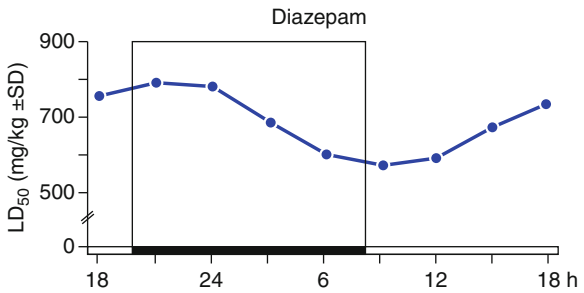

Fig. 21.16 Chronotoxicity of diazepam in mice (Acc. Ross et al. (1981))

21.3 Safety Pharmacology Assessments

A battery of safety pharmacology assessments (cardiovascular, respiratory, and CNS evaluation) is required by ICH S7A Guideline "Safety Pharmacology Studies for Human Pharmaceuticals" (Food and Drug Administration and H.H.S 2001) prior to first administration of a new chemical entity or biopharmaceutical to man. Major systems are evaluated in these assessments in order to identify any significant physiological effects around or above the intended clinical exposures. While respiratory and CNS studies can be conducted in rodents as well as in large nonrodent species, the lack of I_{kr} channels in rat and mice (Pond et al. 2000) dictates that cardiovascular evaluation be conducted in nonrodents. Use of modern telemetry systems with automated data collection from cardiovascular studies allows collection of information for a prolonged period of time – usually at least 24 h after dose administration (and in chronopharmacological research up to months, see below), thus covering the whole circadian cycle. Recent advances in technology allow the use of telemetry techniques on respiratory and CNS studies (Atterson et al. 2010; Authier et al. 2010; DeBoer and Friedrichs 2009; Pugsley et al. 2010), thus also making interpretation of those studies dependent on circadian rhythms. These safety pharmacology assessments are also used by toxicologists to increase confidence in safety and inform colleagues in clinical development, regulators, physicians, and ultimately patients about potential side effects of new drugs. All these require well-defined, robust assay systems that are well understood (Leishman et al. 2011).

21.3.1 Large Animals Used in Safety Pharmacology Studies

21.3.1.1 Most Common Large Species: Beagle Dogs and Cynomolgus Monkeys

Cardiovascular safety pharmacology studies in nonrodent species are usually conducted in nonhuman

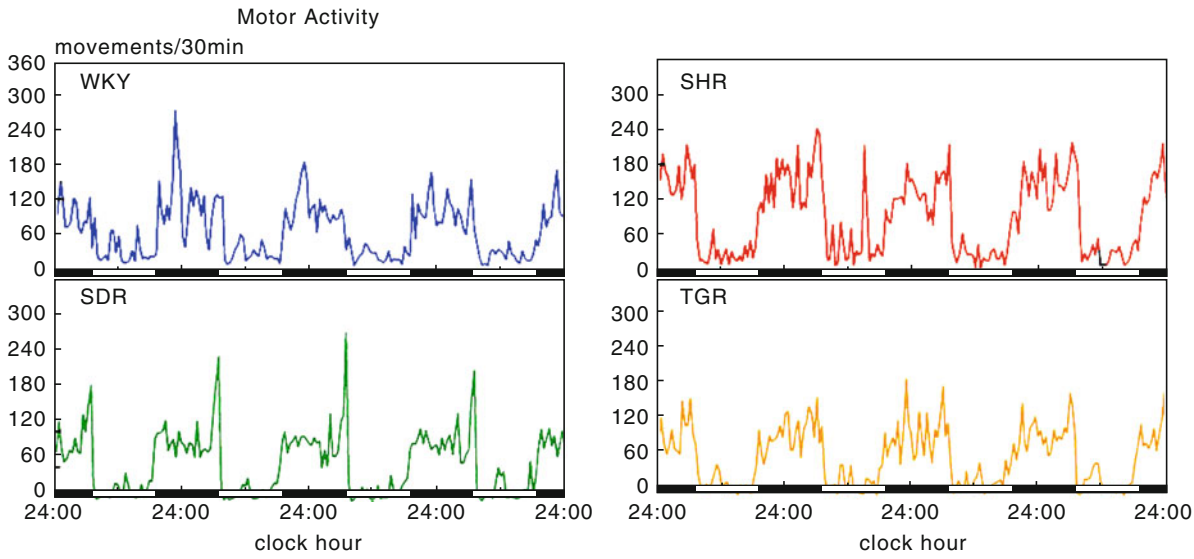

Fig. 21.17 Motor activity in four strains of rats as determined by radiotelemetry (Acc. Lemmer et al. (1993a))

primates and dogs (Bass et al. 2005; Davis 1998). In a 2003 survey of international pharmaceutical companies, 98% and 56% of responders used dogs and monkeys, respectively, in their assessment of QT interval prolongation (Friedrichs et al. 2005). Canine models are favored, though no model reliably correlates with human electrophysiologic results (Gralinski 2003). Small molecules are usually evaluated using a Latin square design, so each animal receives all dose levels including vehicle and serves as its own control. It is vital to recognize behavioral patterns of different species – their expected physiological reaction to the presence of technical personnel, dosing, bleeding, and other activities. Appreciation of the diurnal fluctuations in blood pressure (BP), heart rate (HR), core body temperature, and duration of ECG intervals that occur in animals is extremely important. Accounting for normal physiological reactions is crucial for accurate interpretation of study results and compliance with regulatory requirements. Section 3.1.3 "In vivo electrophysiology studies" of ICH S7B Guideline "Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals" (Food and Drug Administration H 2005) specifically requests the evaluation of the QT interval in an in vivo model. Several time-dependent factors may considerably alter results of the study, e.g., a remarkable difference in the duration of QT interval collected during the dark cycle versus the light cycle at the same HR has been documented in primates (Soloviev et al. 2006a). Although the reason is not fully understood, the hypothesis is that diurnal changes of vagal tone influence the duration of ECG intervals.

As mentioned above, dogs and monkeys are widely used to evaluate effects of new chemical entities on the hemodynamics and electrical activity (repolarization, conductivity, etc.) of the heart. Several publications with detailed description of dogs' (Soloviev et al. 2006a, b; Gauvin et al. 2006a, b) or monkeys' (Authier et al. 2007a, b) hemodynamic parameters obtained via radiotelemetry are available. There are significant differences in cardiovascular profile as well as physiological response to study-related activities in these two species. To illustrate this, a comparison of summarized data obtained during 2003–2006 after oral vehicle dosing from cardiovascular studies was conducted at a contract research organization (Soloviev et al. 2006c, 2007).

Data were collected from healthy animals: 22 male and 18 female cynomolgus monkeys and 41 male and 25 female beagle dogs. Overall, 64 monkey and 166 dog episodes of at least 25-h telemetry data collection in control group animals were summarized (Soloviev et al. 2007). Monkeys were between 2 and 5 years old. Body weight varied from 2.0 to 6.4 kg, with the majority between 2.5 and 4.5 kg. Dogs were between 6 and

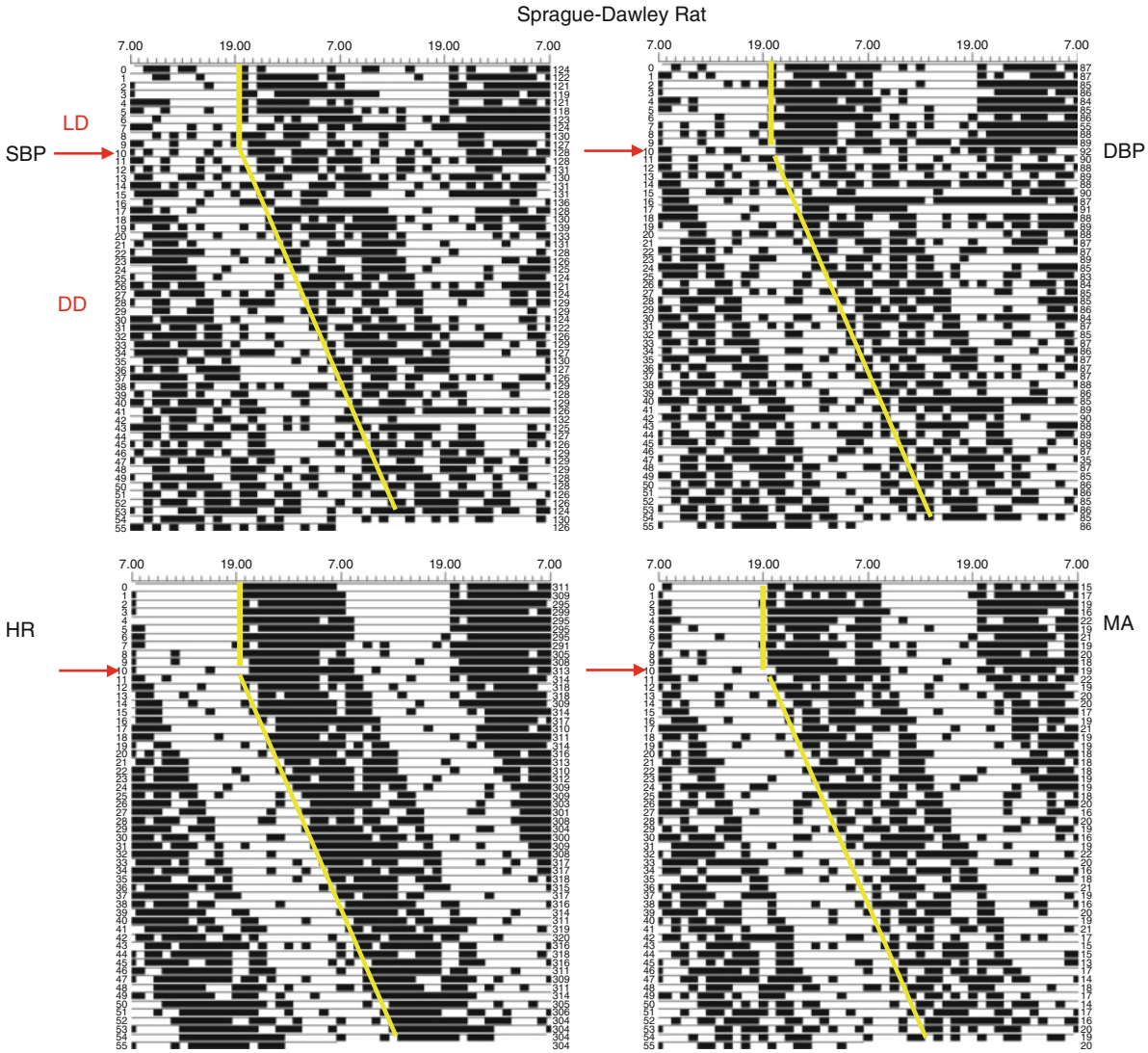

Fig. 21.18 Circadian rhythms in systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and motor activity (MA) in a normotensive rat. Data are represented as double plots with values above the 24-h mean marked black. Experiments were performed under a 12:12 light–dark cycle as well as under free-run conditions in total darkness (DD). In: Lemmer (2011))

29 months old. Body weight varied from 6.3 to 14.8 kg, with the majority between 8 and 11 kg. Detailed physical examinations were performed prior to each data collection. Animals identified with abnormalities did not participate in the studies. The room temperature and humidity controls were set to maintain daily averages of 71 ± 5°F (22 ± 3°C) and 50 ± 20% relative humidity for CM and 68 ± 5°F (20 ± 3°C) and 50 ± 20% relative humidity for BD. Room temperature and relative humidity were monitored and recorded approximately hourly. A light–dark cycle of 12:12 h was used with lights on between 6:00 and 18:00. On the day of dosing, 1-h baseline recordings were obtained from all animals, starting approximately between 10:00 and 11:00 (at least 1 h after feeding). Dosing usually occurred between 11:00 and noon. After dosing, telemetry signals, which included ECG waveforms, were recorded for a 30-s interval

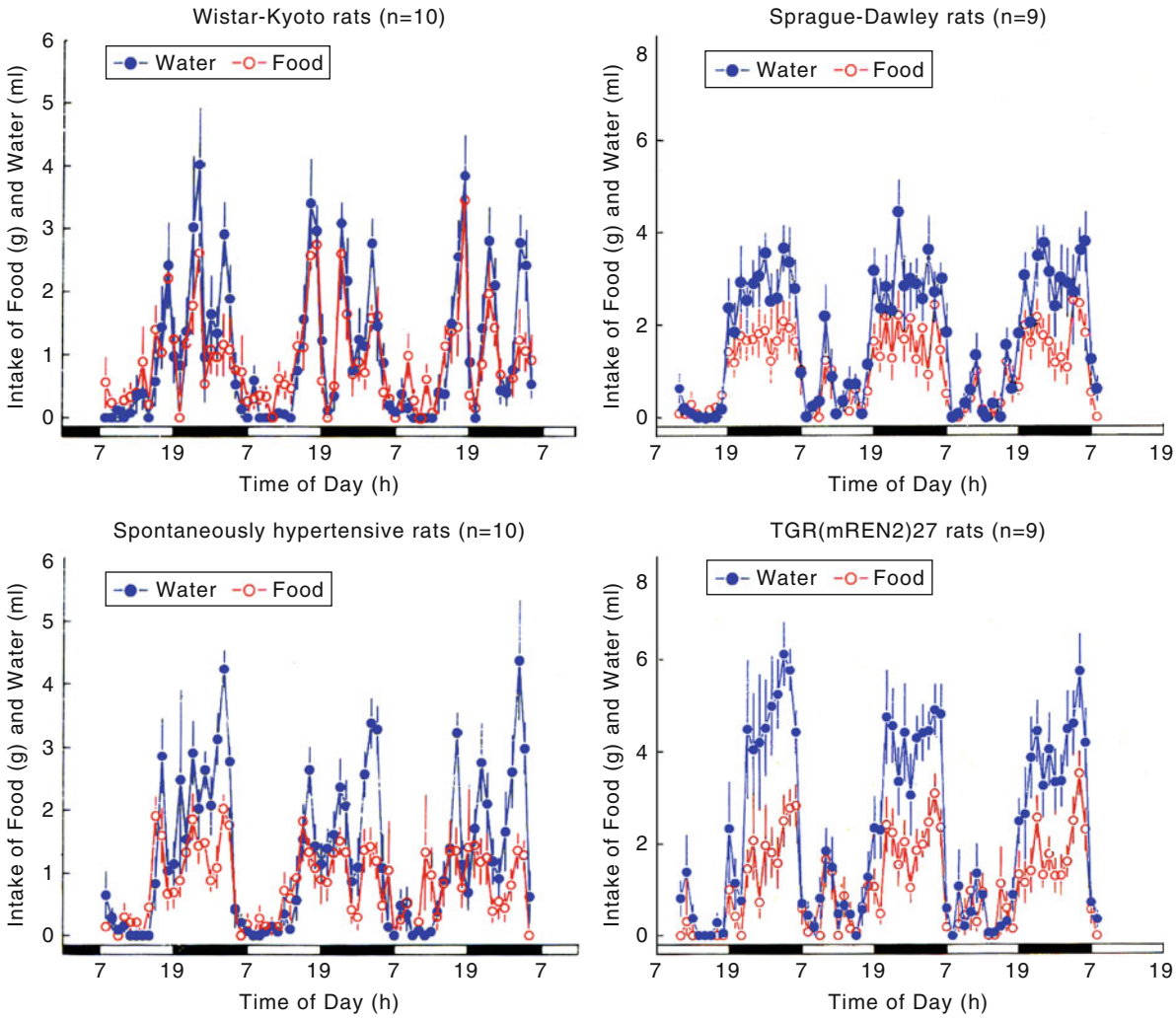

Fig. 21.19 Rhythms in drinking and feeding behavior in four strains of rats (Acc. Witte and Lemmer (1999) and unpublished)

every 10 min for at least 24 h. DSI Physiostat ECG analysis software (version 4.0 and higher) summarized all ECG complexes recorded during an acquisition period (30 s) into the derived reference complex. The software then compared the derived reference complex based on statistical correlation to all actual complexes and identified and eliminated artifacts and/or abnormal complexes. Mean HR for each 30-s interval was used for QT correction to compensate for cardiac "memory phenomenon" (Pueyo et al. 2004). One-hour averages for hemodynamic parameters and ECG intervals were analyzed for the first 6 h after dosing and then 2-h averages were analyzed.

Major hemodynamic parameters and duration of ECG intervals for both species prior to dose administration (approximately between 10:00 and 11:00) are presented in the Table 21.1.

Cynomolgus monkeys had stronger hemodynamic response to dosing: the average HR for the hour following dosing increased 30% in monkeys versus 8% in dogs. The average mean BP for the same interval increased 12% in monkeys versus 1% in dogs. Monkeys had a pronounced diurnal pattern with night

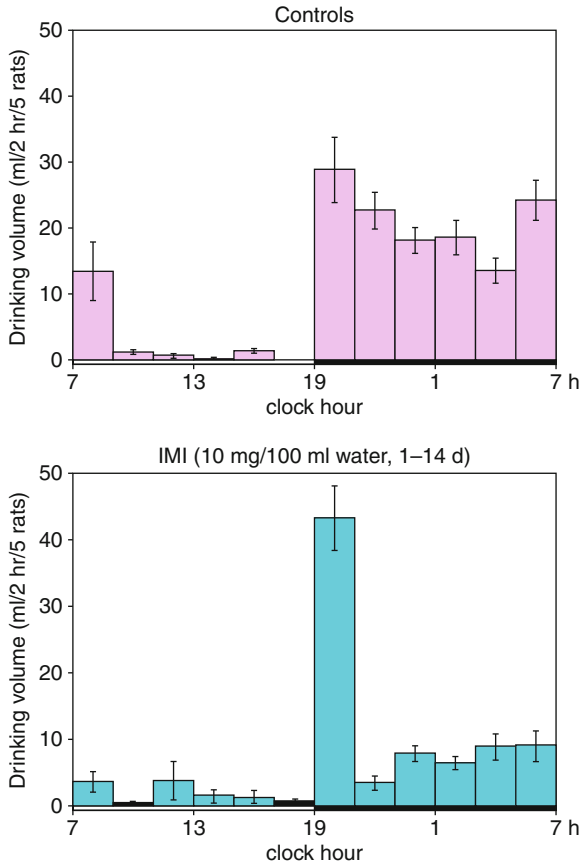

Fig. 21.20 Effect of imipramine on drinking behavior in rats (Acc. Lemmer and Holle (1991))

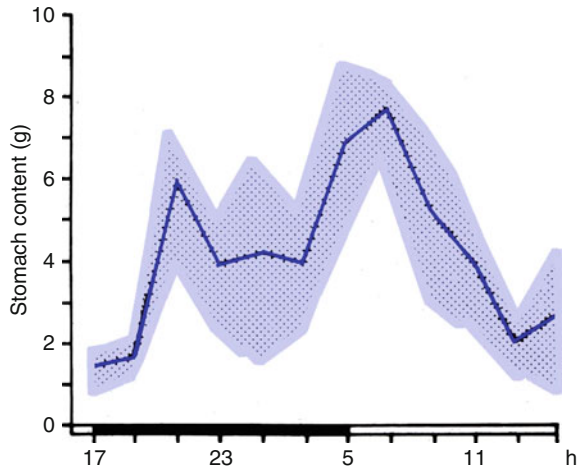

Fig. 21.21 Rhythm in gastric emptying in rats (Acc. Armstrong et al. (1978))

decreases in HR (20%), mean BP (10%), and core body temperature (1.1°C); in dogs, these changes were smaller – 10%, 4%, and 0.3°C, respectively. In both species, daily variations of the HR, mean BP, core body temperature, and uncorrected QT interval are presented on Figs. 21.3, 21.4, 21.5, and 21.6.

All recordings for both species (10,761 in monkeys; 24,882 in dogs) were arbitrarily divided into divisions ("bins"), each covering a range of 10 bpm, except for the first and the last bins (Fig. 21.7).

Ninety-five percent of all HR collected from monkeys were between 70 and 210 bpm, and 93% of all HR collected from dogs were between 60 and 160 bpm. The duration of the absolute QT interval depended upon the HR in both species, but different species required different QT correction formulas. Van de Water's correction formula (Van de Water et al. 1989) provides optimal results for evaluation of the QT interval duration in dogs, while Bazett's correction formula (Bazett 1920) provides optimal results in monkeys. QT interval duration measured during the light cycle (mostly active, awake) was compared to those collected during the dark cycle (sleeping) at the same HR (Fig. 21.8).

To avoid the influence of the transition period from the light cycle to the dark cycle and vice versa, only 8 h in the middle of the cycles were analyzed (2 h on each side of the cycle were not included in the analysis). In contrast to the dogs, monkeys demonstrated a remarkable difference – up to 32 ms prolongation (12.7%) of the QT interval measured during the dark cycle compared QT interval measured during the light cycle at the same HR (60–70 bpm). This difference became less prominent with the increase of the HR: QT was prolonged 27 ms (10.4%) at HR from 70 to 80 bpm and 20 ms (7.8%) at HR from 80 to 90 bpm. A less pronounced prolongation (4.2 ± 2.1%) of QT during the night at HR of 60 bpm was also described in healthy humans (Murakawa et al. 1992). A similar degree of nighttime QT prolongation (4.7%) was reported by Bexton et al. (Bexton et al. 1986). It is interesting that both of the aforementioned studies in addition to those conducted by (Ishida et al. 1997; Bilan et al. 2005) reported a decrease in the diurnal QT difference in diseased patients with sympathovagal balance shifted in the sympathetic direction. These results indicate that although

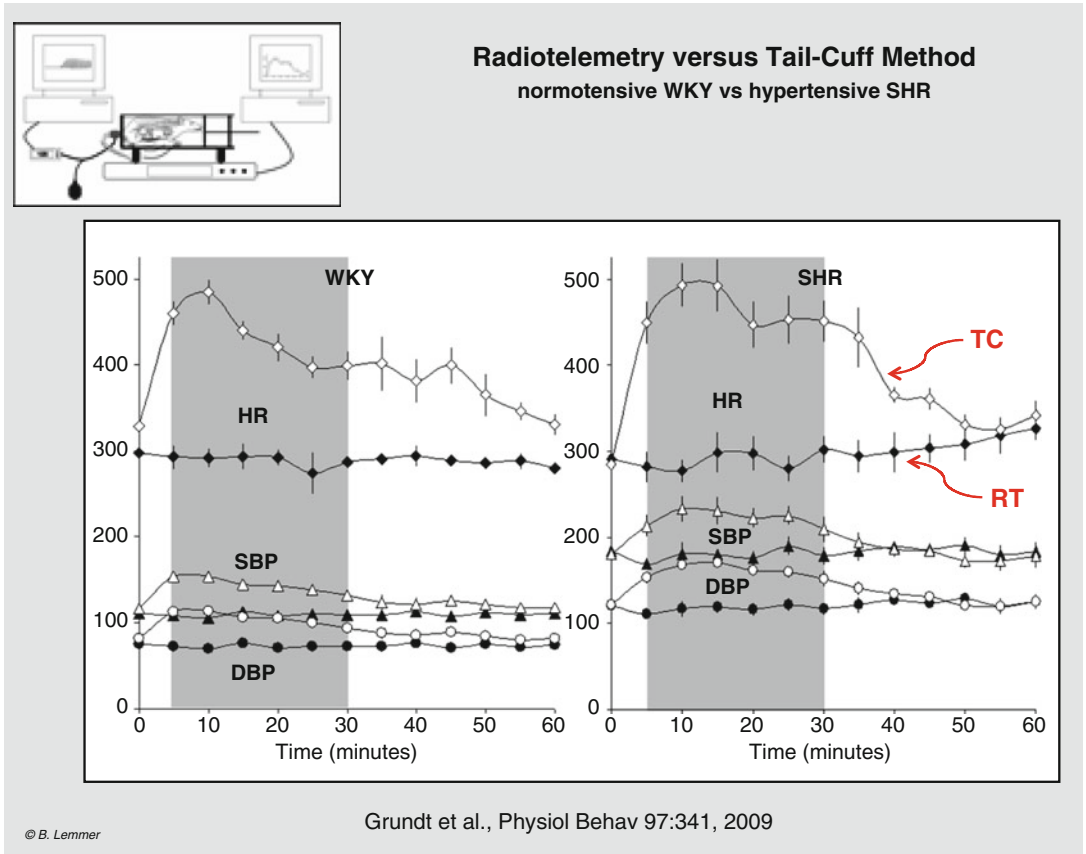

Fig. 21.22 Comparison of radiotelemetric data (RT) on systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) with those obtained by the tail-cuff method (TC) in normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats, dark part represents time spend under tail-cuff (Acc. Grundt et al. (2009))

a change in sympathovagal balance was responsible for the diurnal variation in QT interval, the enhanced sympathetic activity in the day was a major determinant of this phenomenon. The absence of a similar effect on QT duration in dogs can be explained by their sleeping pattern – frequent "naps" during light cycle of the day (Takeuchi and Harada 2002).

It is of great interest that RR and QT intervals lengthen comparably at lower HR during the dark period (nonactive sleeping hours) in both monkeys and dogs, but the lengthening of QT in monkeys could not be attributed only – or even primarily – to lengthening of RR interval. That is, when QT was measured in monkeys at equivalent HR during daytime and nighttime, QT was longer at nighttime in monkeys but not longer in dogs. Thus, although lengthening of QT interval in dogs can be attributed to a reduction in HR, it cannot be attributed solely to the slowing of HR in monkeys. The changes in QT were attributable only to changes in ST-T interval because QRS did not manifest diurnal fluctuations (Gauvin et al. 2006b; Holzgrefe et al. 2007) and is known to be independent of HR. There are many determinants of the duration of ST-T interval, several of which are, themselves, interdependent. For example, it is well known that altering HR by changing autonomic efferent traffic to the sinoatrial node changes QT via the effects on I_{Ks}. However, drugs may both alter HR and still affect other channels important for ventricular repolarization (e.g., I_{Kr}, I_{Kto}, I_{Na}, I_{Ca}). In addition, there are other less investigated effects of ST-T interval. Among these nonautonomic effects are serum electrolyte

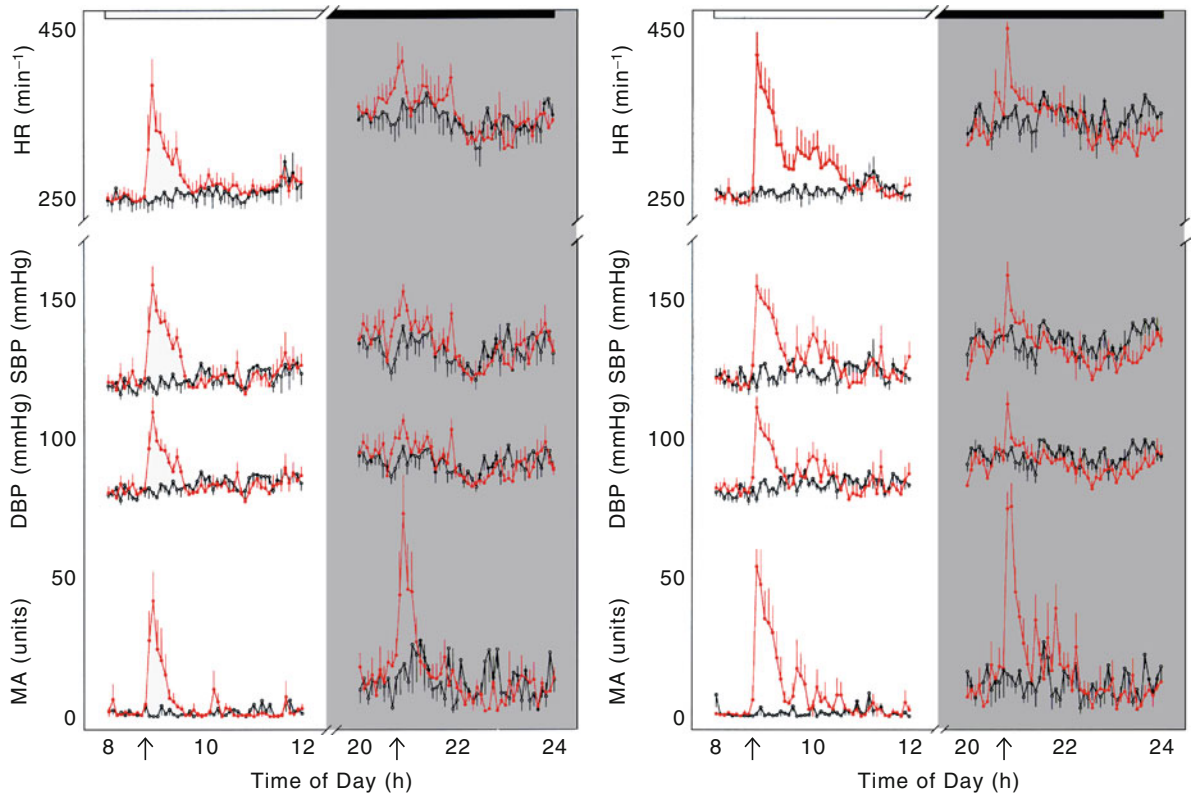

Fig. 21.23 Routine procedures on cardiovascular functions in rats. *Left*: i.p. injection of 0.5 ml NaCl solution. *Right*: clean cages and fresh bedding (Acc. Schnecko et al. (1996))

concentration (Lepeschkin 1951), neuroendocrine changes (Davey and Bateman 1999), gender (Bortolan et al. 2004; Nakagawa et al. 2005; Schulze-Bahr et al. 2005), aging (Bonnemeier et al. 2003), temperature (Mattu et al. 2002; van der Linde et al. 2008; Shellhammer et al. 2009), heart size, and heart failure (Bortolan et al. 2004).

In contrast to monkeys, who did not demonstrate differences in the duration of PR interval during light cycles, PR interval in dogs was slightly shortened during the night hours. The duration of the PR interval was prolonged at night by 3.3% in male dogs and 1.7% in female dogs despite the fact that HR was lower in both sexes during the dark cycle. HR usually slows at night because of an increase in parasympathetic (vagal) efferent activity and/or a decrease in sympathetic efferent activity to the sinus node. Because these factors also should decrease velocity of propagation across the atrioventricular conduction system, there should be a concomitant lengthening of the PR interval. However, the paradoxical abbreviation of the PR interval at night when HR is slower may be explained by the decreased rate at which the atrioventricular node is bombarded, which provides more time for the cells to complete their period of relative refractoriness. This implies that even at relatively slow HR during the day – but not as slow as at night – the atrioventricular node did not have sufficient time between inputs from the sinus node to completely recover from its relative refractoriness; thus, the PR interval was longer than in the nighttime when HR was still slower.

In addition to interspecies diversity, a possible reason for these dramatic differences in diurnal pattern as well as reaction to study-related activities may be due to autonomic regulation based on behavioral distinctions. While the beagle dog is a domesticated animal, the cynomolgus monkey is not. This fundamental behavioral difference may have implications for how

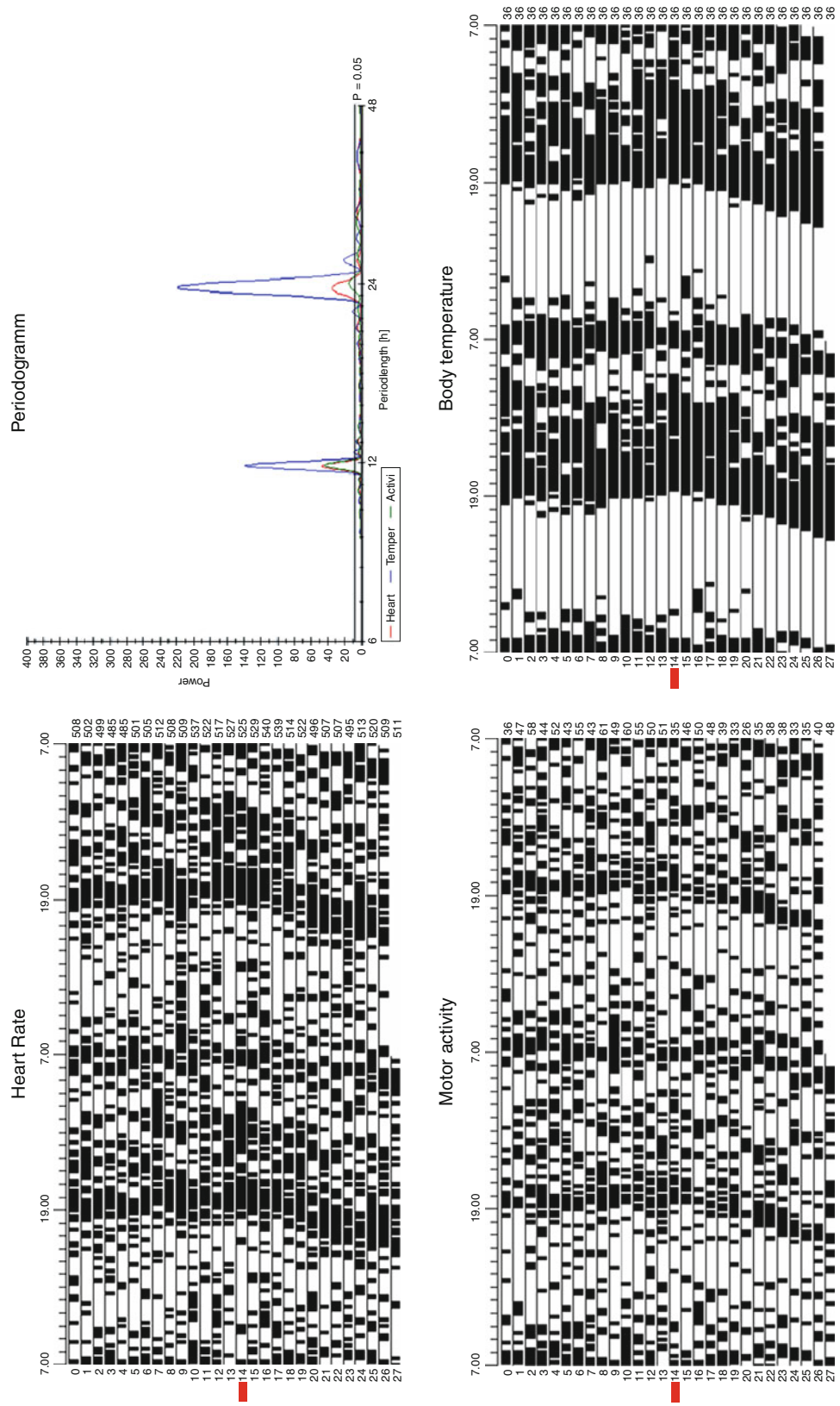

Fig. 21.24 Circadian rhythms in heart rate, motor activity, and body temperature in normotensive mice; under LD (day 0-13) and after free run in total darkness (DD) from day 14, a periodogram (Zuther and Lemmer 2004a) is shown for the data monitored (Acc. Arraj and Lemmer (2006))

the two species interact with their environment, particularly with regard to the presence of humans (response to dosing), and the corresponding changes in HR with changes in their respective environments. For example, in captivity, which is an ordinary condition to the domesticated animals, the dogs' HR is under parasympathetic control, whereas in captivity, the monkeys' HR is under strong sympathetic control.

Another important deliverable of cardiovascular studies is a determination of any qualitative ECG abnormalities. A thorough evaluation of qualitative ECG findings in dogs was done by (Cools et al. 2011). The authors analyzed ECG obtained by external telemetry for a 22-h period and concluded that a high percentage of clinically normal beagle dogs showed different types of arrhythmias: 49% of the animals had episodes of AV block second degree; 59%, single atrial premature complexes; 17.6%, junctional tachycardia; 13.7%, ventricular escape complexes; 21.6%, ventricular premature complexes; 3.9%, runs of ventricular complexes; and 3.9%, runs of ventricular escape complexes. The chronic implantation of a ventricular probe through the apex of the heart resulted in temporary (up to 8 weeks) higher incidences and frequencies of ventricular episodes. Interestingly, atrial and junctional arrhythmia incidence was higher during the night period, but the incidence of ventricular events during day and night was comparable. Similar work in primates is still to be done.

While circadian rhythms in animals have been elucidated in the literature, notably less information on seasonal variability of the hemodynamic parameters is available, and it remains controversial possibly because of the lack of thoroughly controlled environmental factors (Bícego-Nahas and Branco 1999; Dunlap et al. 2007; Martins et al. 2006). Table 21.2 gives data obtained from controlled environment of the experimental laboratory by analyzing cardiovascular studies conducted at different seasons of the year in the same facility in dogs of the same age (Soloviev et al. 2006b) (Table 21.2). To exclude the influence of circadian rhythms, the analyzed baseline data were collected approximately at the same time of the day. Seasons were defined by the study starting date (date on which the first telemetry data were collected) as follows: winter, December through February; spring, March through May; summer, June through August; and autumn, September through November. Although sufficient data for evaluation of females tested during the winter months were not available, both sexes demonstrated statistically significant higher HR and BP in spring when compared with other seasons. In males, the HR differed by 11–22 bpm (up to 23%) between the seasons. A smaller difference was noted in mean BP – 4–5 mmHg (~5%).

21.3.1.2 Minipigs

The minipig has gained an increased acceptance as an alternative for large animal toxicology and safety pharmacology assessments (van der Laan et al. 2010). ICH S7B Guideline (Food and Drug Administration H 2005) includes swine as a potential species for in vivo electrophysiology studies. The minipig becomes the species of choice for cardiovascular safety pharmacology studies when pivotal toxicology work is conducted in this species (Bode et al. 2010). Anatomical, physiological, and biochemical similarities between pigs and humans (Bollen and Ellegaard 1997; Douglas 1972; Hiebl et al. 2010; Hughes 1986) support the relevance of minipigs for regulatory safety pharmacology. Minor difference in ion channels, absence of 4-aminopyridine-sensitive transient outward K current I_{to1} (Li et al. 2003), and thus inability to identify QT-prolongation-specific I_{to1} blockade does not disqualify minipigs from cardiac safety pharmacology (Authier et al. 2011; Laursen et al. 2011; Pugsley et al. 2008). Today, several different minipig breeds are available and suitable for laboratory use. The Göttingen minipig is a special breed for medical research, the smallest of the minipig breeds available for research; it reaches a weight of only 20–35 kg when it is fully grown (Köhn et al. 2007).

Limited information on hemodynamic and ECG parameters in conscious minipigs is available, and below we present a brief summary of the most thorough studies available for us to date (Stubhan et al. 2008; Markert et al. 2009; Markert 2011). HR in freely moving minipigs during daylight hours averages about 56 bpm (Fig. 21.11). Kano et al. (2005a) reported values of 72–76 bpm in 17 kg, freely moving miniature pigs (but not Göttingen minipigs). The HR in resting miniature pigs was 80 ± 3.5 bpm (Kuwahara et al. 1999). Beglinger and Becker reported HR of 103 ± 14 bpm in sling-restrained Göttingen minipigs (~20 kg) (Beglinger et al. 1975a).

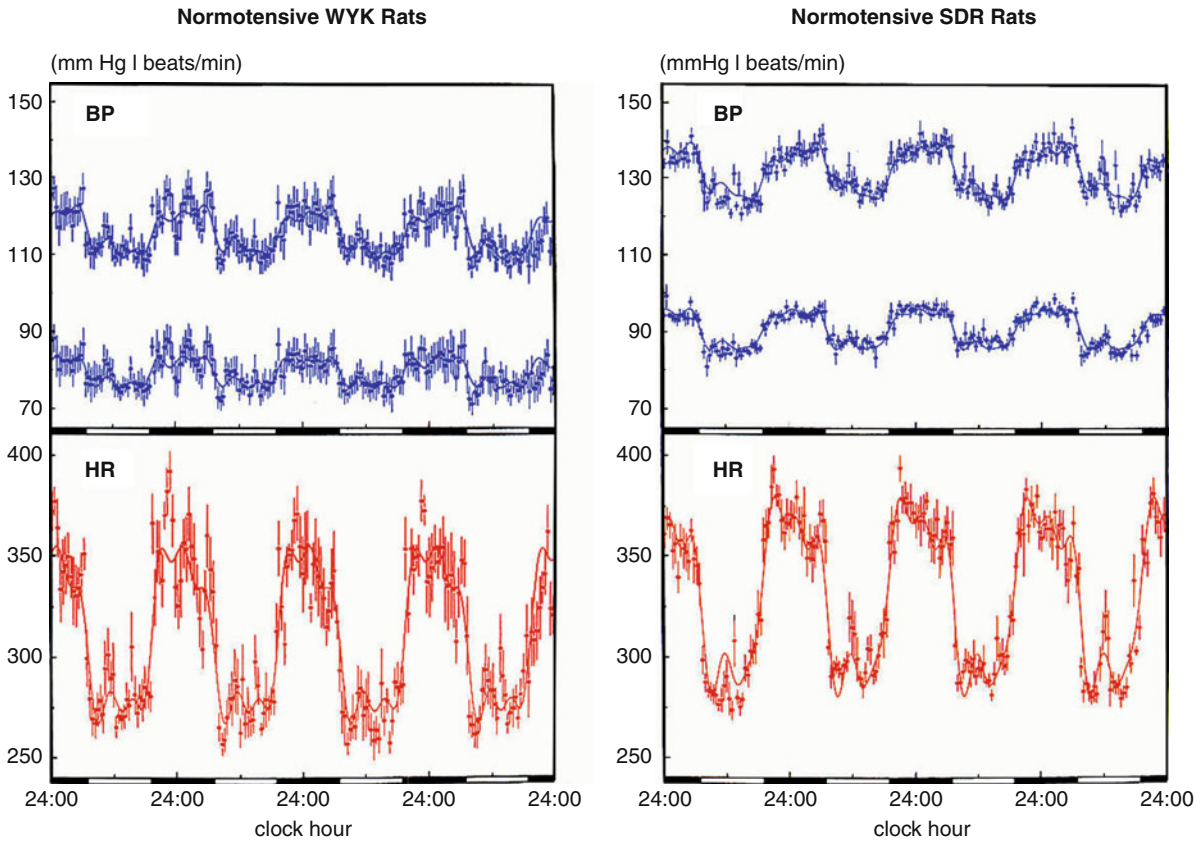

Fig. 21.25 Circadian rhythms in blood pressure (BP) and heart rate (HR) in two normotensive strains of rats (Acc. Lemmer et al. (1993a))

Comparing to the currently available data in minipigs obtained with invasive measurements, it may seem low. Markert (Stubhan et al. 2008; Markert et al. 2009; Markert 2011) attributed the very low HR to the low stress level achieved using well-trained animals in a laboratory environment where special measures have been taken to reduce unnecessary stress factors.

The diurnal rhythms of minipigs' HR have been investigated by Kuwahara et al. (1999). They found that HR in the daylight phase was higher than in the dark phase when the animals were housed singly, but minipigs housed in pairs had no diurnal variation. Another group (Kano et al. 2005b) reported no marked changes in HR between periods of daylight and darkness. Recent data from Market's group (Stubhan et al. 2008; Markert et al. 2009; Markert 2011) differ from earlier findings: they saw a significant increase in HR at night when the animals were fed shortly before the start of the dark phase (Fig. 21.9). Examination of videos taken at night indicated that the minipigs have been sleeping most of the time (main sleeping period 11 p.m.–5 a.m.), with only short periods of wandering but with no signs of excitement, and one would expect HR to decrease at night with an increase in parasympathetic activity. But when the study design was altered to eliminate feeding at 7 p.m., the HR at night was 51 bpm, being comparable to the daylight value. These findings indicate a dependency of the dark phase increase in HR on feeding. This phenomenon may be due to autonomic nervous fibers located in the wall of the digestive tract that react to the filling of the gastrointestinal tract. The pig is known to digest slowly, taking at least 24 h to empty the digestive tract after

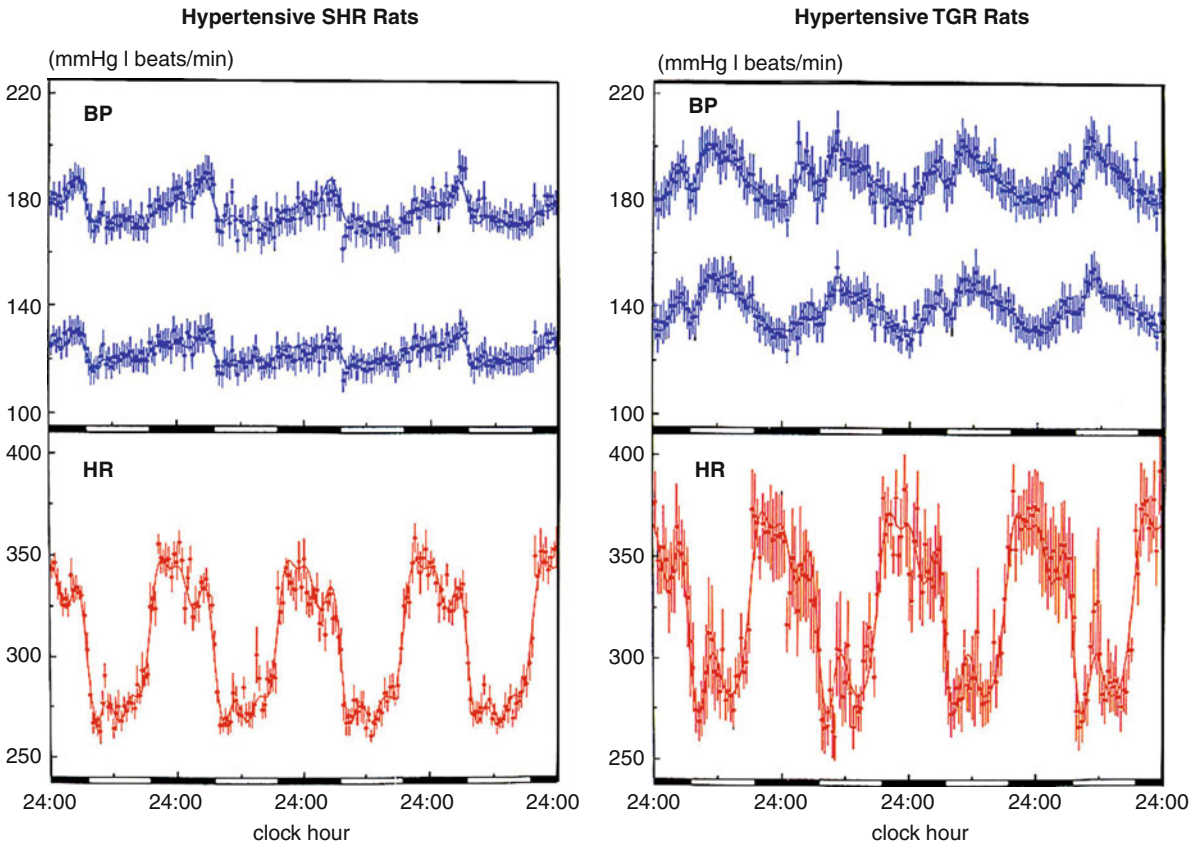

Fig. 21.26 Circadian rhythms in blood pressure (BP) and heart rate (HR) in two hypertensive strains of rats (Acc. Lemmer et al. (1993a))

a meal (Hossain et al. 1990). The point to consider is that the effect of feeding on HR can extend well beyond over 3 h and that this must be taken into account when designing studies involving Göttingen minipigs.

Systolic and diastolic BP over 24 h was remarkably stable with or without feeding (Fig. 21.10). Earlier reported values for arterial BP from minipigs ranged from 135 ± 12 to 160 ± 11 for systolic and 88 ± 4 to 96 ± 14 for diastolic (Beglinger et al. 1975b; Cimini and Zambraski 1985). However, nobody prior to Markert's group (Stubhan et al. 2008; Markert et al. 2009; Markert 2011) reported arterial BP data from freely moving minipigs (Markert et al. 2009). The slightly higher pressures reported previously may be attributable to the restraint used when taking the measurements. That restraint increases in BP and HR has recently nicely demonstrated – in accordance with the data in minipigs – in rats in which BP and HR were monitored both by telemetry as well as by the tail-cuff method (Grundt et al. 2009).

Markert's group (Stubhan et al. 2008; Markert et al. 2009; Markert 2011) also described fluctuation of the body temperature of the minipigs between daytime and nighttime (Fig. 21.11). During daytime (before feeding), body temperature was 37.0°C, whereas during the night, it increased to 37.5°C (Fig. 21.12). The body temperature cited in literature for miniature pigs ranged from 37–38°C (Bollen and Ellegaard 1997), 37.7 ± 0.2°C (Georgiev et al. 1972), to 38.2–39.9°C (Zambraski and Fuchs 1980). The increased HR during the night phase may contribute to the increase in body temperature. Nevertheless, temperature also increased in the experiment without feeding in which HR is not affected, indicating that factors other than elevated HR could be responsible. At least in rats and man, it is well

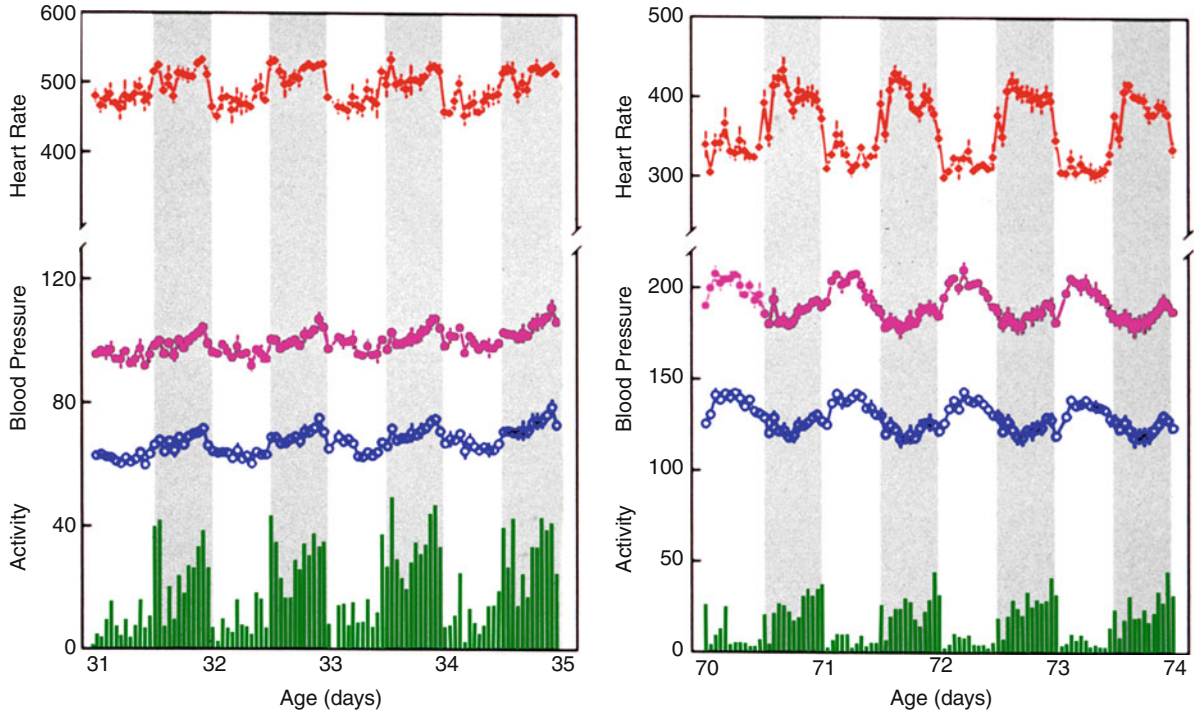

Fig. 21.27 Development of circadian rhythms with age in blood pressure, heart rate, and motility in transgenic hypertensive rats (TGR) (Acc. Witte and Lemmer (1999), Lemmer et al. (2003))

known that the body temperature is mainly governed by the internal clock, i.e., is really circadian (see below). However, such experiments have not been performed in minipigs.

ECG intervals reported from freely moving minipigs (Table 21.1) also range from earlier published data from nontelemetric studies. QT strongly correlated to the RR interval. The spontaneous changing of the T-wave polarity, which is not body-position-dependent, is thought to be due to the vegetative influences and may affect measurement of the QT interval. Markert's recommendation to use an individual correction of the QT interval for HR changes in swine is supported by observation from Harlan Laboratories (Jackson and Pohl 2010) and LAB Research (Authier et al. 2011), although the later two sources conclude that Fridericia correction (Fridericia 1920) can also be used.

However, in conclusion, it is important to note that the "circadian" (endogenous) character of rhythms in minipigs has not been investigated since no free-run data are available.

21.3.2 Small Animals

21.3.2.1 Rats and Rabbits

Limitations of the use of small animals in cardiovascular safety pharmacology studies are lack of I_{kr} channels in rat (no effects of ventricular repolarization can be studied) and nocturnal behavioral pattern of rats (see details in Sect. 4.1.3) and rabbits. With the advent of radiotelemetry (Brockway et al. 1991; Brockway and Brockway 1996; Mattes and Lemmer 1991; Lemmer et al. 1993a, see below), the circadian rhythms of hemodynamic data of rats and (later on) rabbits were investigated by numerous authors (Lemmer et al. 1993a; Akita et al. 2002; Eijzenbach et al. 1986; Hashimoto et al. 1999; Sato et al. 1995; Schnell and Wood 1993; Teelink and Clozel 1993; Kramer et al. 2000; Anigbogu et al. 2011; Kagohashi et al. 2008). Sato et al. (1995) collected by telemetry and compared BP and HR in conscious unrestrained rats and rabbits; unfortunately, a mixed set of both male and female animals was used. BP and HR in rabbits were lower than those in rats, but the circadian

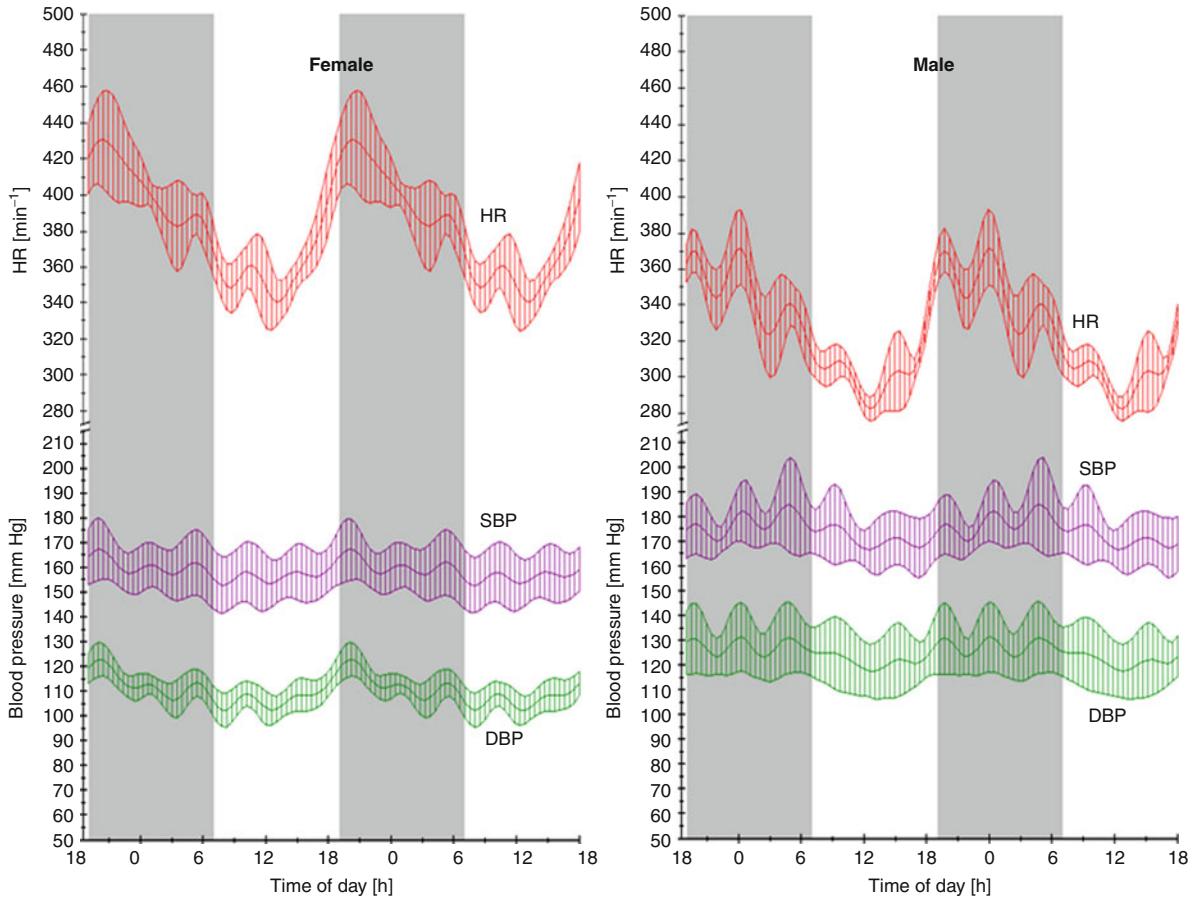

Fig. 21.28 Circadian rhythms in blood pressure and heart rate in female and male SHR rats (Acc. Grundt et al. (2006))

rhythms in rabbits showed nocturnal pattern similar to rats. In contrast, short-term variability in BP in rabbits was considerably larger than in rats. The hemodynamic data for 1-h intervals averaged over 4 days are shown in Fig. 21.12.

Data for any hour is the average of 120 points (30 points × 4 days). In each species, systolic BP, diastolic BP, and HR in the dark phase (08:00–20:00) tended to be higher than those in the light phase, showing nocturnal patterns. In rabbits, the line composed of hemodynamic data was almost flat in both the light and dark phases. The slope in each parameter during the transition period from the light phase to the dark phase (19:00–20:00) was gentle and obscure. In rats, values in the light phase were almost even, but those in the dark phase showed one peak in each parameter. The peaks appeared at 8 p.m. for systolic and diastolic BP and at 8–11 p.m. for HR. The slopes in each parameter during the switching period (19:00–20:00) were steep and obvious because of the presence of the peak in the dark phase, in contrast with the changes in rabbits.

Original recordings of mean BP and HR plotted every 2 min for 7 consecutive days in both species are presented in Fig. 21.13.

It clearly demonstrates not only the circadian rhythm but also short-term variability. It should be noted that the circadian rhythms of mean BP and HR in the rabbit showed nocturnal patterns, as already shown earlier for the rat (Brockway et al. 1991; Brockway and Brockway 1996; Mattes and Lemmer 1991; Lemmer et al. 1993a), and that the short-term variability in rabbits was appreciably larger than that in rats. The cause of short-term variability can be roughly

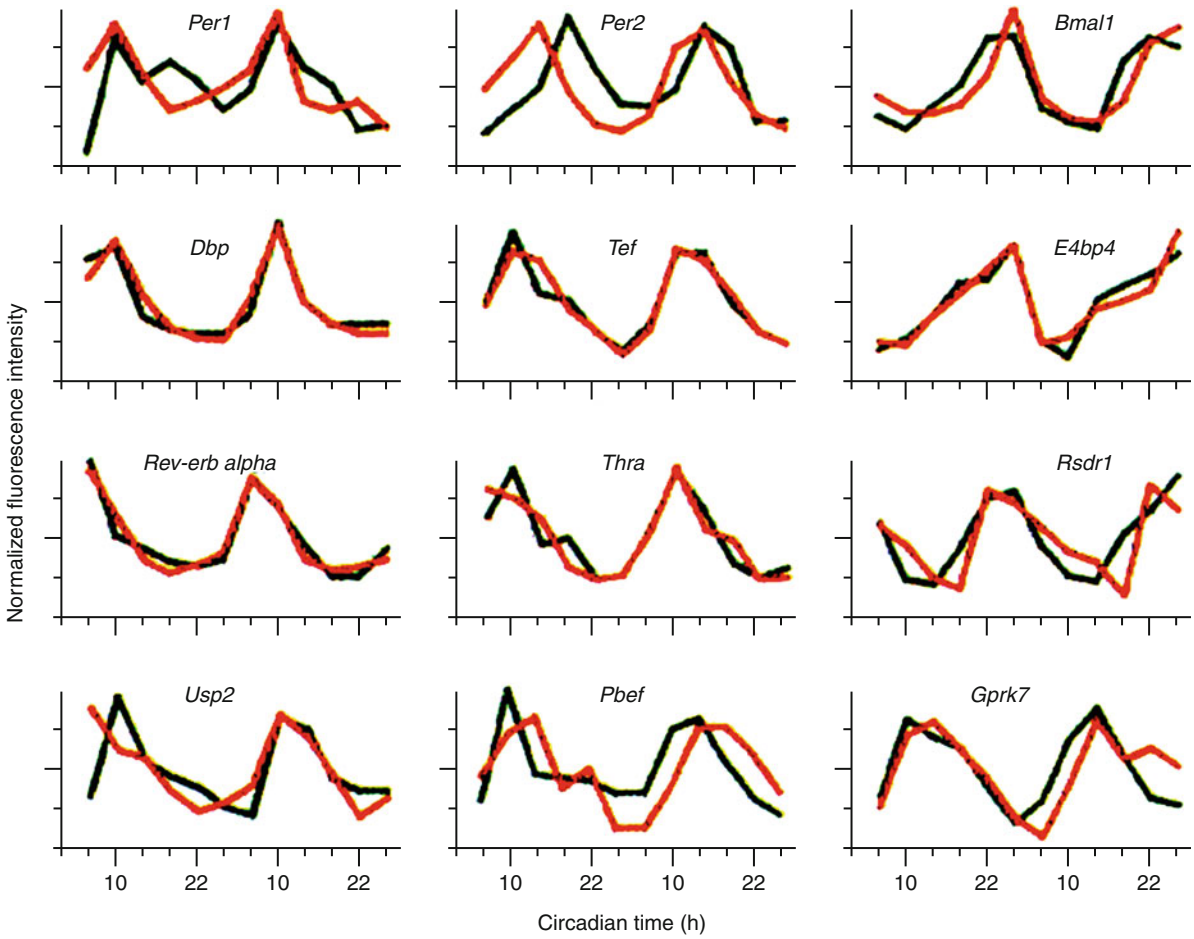

Fig. 21.29 Circadian gene expression profiles in mouse liver and heart; data for liver in black, those for heart in red color (Acc. Storch et al. (2002))

classified into two types: one is due to the routine behavioral changes including eating, drinking, and ambulating and the other is emotional or neural changes independent of body movement, which is considered to be more complex (Janssen et al. 2000). The effects of environmental conditions such as auditory stimuli also have to be taken into consideration. The analysis of the circadian rhythm of BP in rabbits is difficult to conduct, although it has been described that other physiologic functions in rabbits, including locomotor activity, food and water intake, urine and feces excretion, and body temperature, show clear nocturnal patterns in their circadian (Akita et al. 2002; Eijzenbach et al. 1986; Eisermann 1988; Varosi et al. 1990). The reason for the difficulty is considered to be primarily due to wide fluctuations in BP over short periods in rabbits. Another important condition for detecting the circadian rhythm in rabbits was considered to be reduction of stress. Since the swivel-tether system is stressful for the animals, some early studies failed to find the nocturnal pattern in rabbits even while evaluating HR (Hayashi 1981), which showed a clearer rhythm than BP in this study.

The data published by Sato et al. (1995) clearly indicate the complexity of biologic systems, their dependency on the internal clock and receptiveness to numerous signals. Knowledge of the test system's specific biological rhythms, awareness of animal models' responsiveness to internal and external

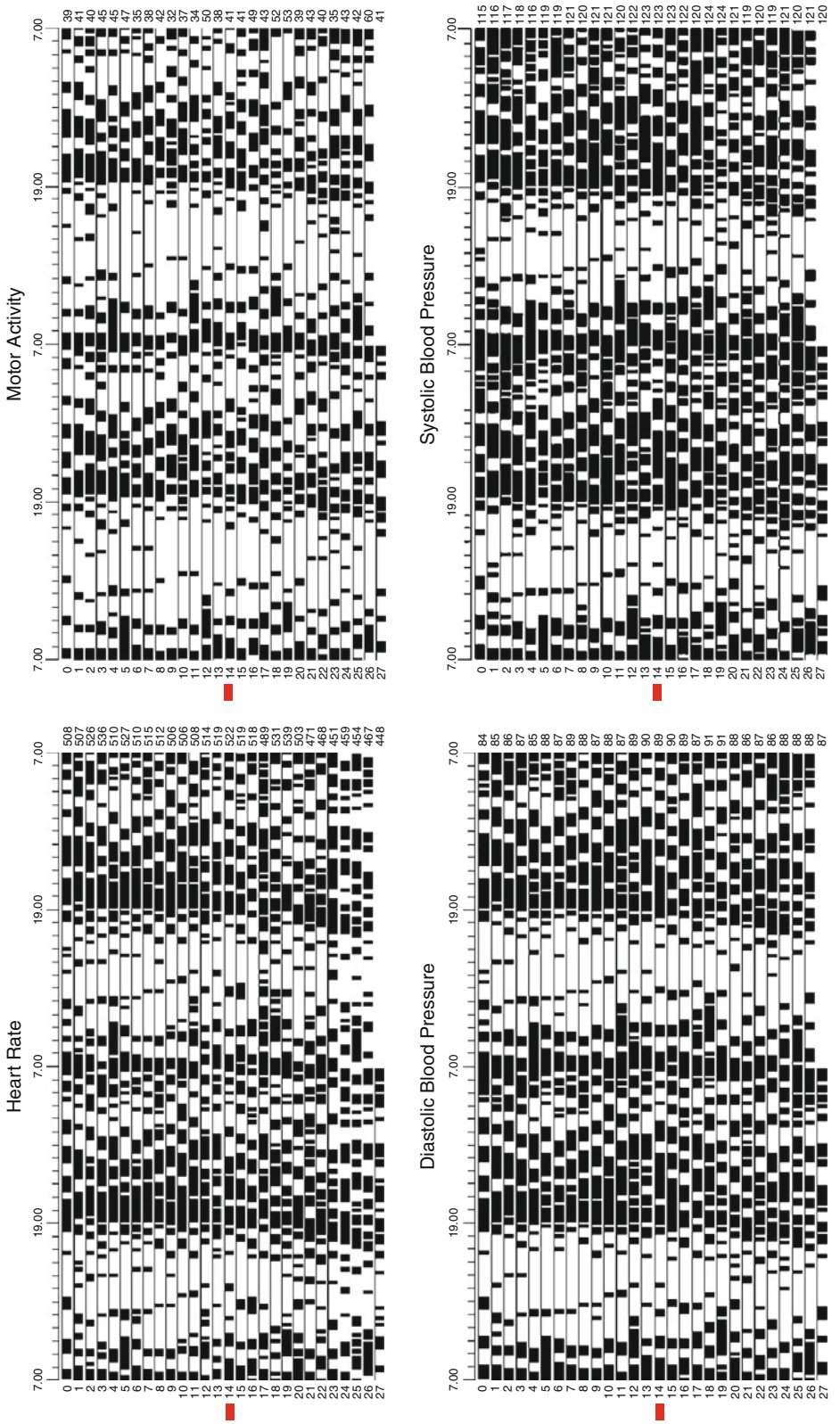

Fig. 21.30 Circadian rhythms in heart rate, blood pressure, and motility under synchronized conditions of LD 12:12 h light–dark and after free run in total darkness DD (Acc. Arraj and Lemmer (2007))

stimuli, and the extreme sensitivity of modern implantable telemetry should be taken into account for data analysis and interpretation.

21.4 Biological Clock and Chronopharmacology in Animal Models

21.4.1 Small Animals: Rats and Mice

Rodents such as mice and rats are widely used in testing drug toxicity and drug effects. In both species several strains were bred to study the influence of genetics on drug effects. Within the last century, several models of transgenic rats gained a lot of interest; however, rats are not suitable for developing knock-out animals. This can be much more efficiently done in mice; thus, new knock-out mice appear even daily on the market. In order to gain stable knock-outs, one needs to breed them for several generations, which, however, has not always been done.

21.4.1.1 Drug Toxicity

Already in the 1950s, toxicity of drugs was studied in rats and mice in relation to time-of-day. In 1951, Halberg et al. (1951) reported on daily variations in the blood eosinophil levels in mice. In rats, these authors showed, in addition, daily variations in tissue mitosis and rectal temperature (Halberg et al. 1954).

It is not possible to mention in detail all the data obtained on the circadian rhythm in drug toxicity in mice and rats; the interested reader will find such data in reviews (e.g., (Lemmer 1989; Reinberg and Smolensky 1983; Haus and Halberg 1959; Reinberg and Halberg 1971; Redfern and Lemmer 1997; Koukkari and Sothern 2006; Dunlop et al. 2004)).

Some examples may demonstrate circadian-phase-dependency in toxicity.

In 1976, Heinz von Mayersbach (1976) clearly demonstrated that the mortality of several compounds in mice can vary between 0% and 100% depending on the time of day of drug application (Fig. 21.14), a finding which has been a shock to many researchers. That LD_{50} values without looking at the time of day of drug application may be without any value of information is also seen in data on LD_{50} of procainamide (Fig. 21.15) and diazepam (Fig. 21.16) in mice (Ross et al. 1981; Bruguerolle 1984).

21.4.1.2 Behavioral Aspects

Activity is clearly circadian-phase-dependent in rodents which are night-active animals. Many studies have shown that both in rats and mice, motor activity predominates in the activity period during darkness. This is documented in telemetric studies (Figs. 21.17 and 21.18) and should be taken into account when testing drugs, which might influence motor activity.

In rats and mice, feeding and drinking behavior (Fig. 21.19) occur mainly during the activity period (Witte and Lemmer 1999). A rhythm in water intake and urine excretion could also be demonstrated in spontaneously hypertensive rats (SHR) using metabolic cages (Abu-Taha and Lemmer 2006). It is self-understanding that these rhythms must have implications for drug application. As a result, drugs given in either the drinking water or in the food pellets will *not* results neither in constant drug supply nor constant drug levels in these animals! This has been shown, e.g., after oral application of imipramine in the drinking water in rats (Fig. 21.20) (Lemmer and Holle 1991).

Food intake is rhythmic and persists also under free-run conditions in LL (light-light) (Rosenwasser et al. 1981), indicating that it is run by the internal clock.

Similarly, gastric emptying (Armstrong et al. 1978) (Fig. 21.21) and cytological liver organization (Müller 1971) are also not independent from circadian time. This indicates that even feeding the animals with drugs by a stomach tube will not result in constant drug absorption over time.

21.4.1.3 Cardiovascular System

It is generally accepted that multiple endogenous systems/mediators (sympathetic nervous system and catecholamines, renin-angiotensin-aldosterone system, endothelin, nitric oxide, vasoactive peptides, the hypothalamic-pituitary-adrenal axis, etc.) contribute to the regulation of BP. However, the regulation of the rhythmicity of BP and its disease-induced disturbances are not fully understood (Lemmer et al. 2005a, 2003).

There is some debate on whether and to what degree human rhythms in BP and HR are endogenous in nature (Kerkhof et al. 1998; Krauchi et al. 2002; Lemmer 1996). Therefore, animal models of primary and secondary hypertension can contribute to a better understanding of the mechanisms involved. We used different strains of normotensive, hypertensives, and

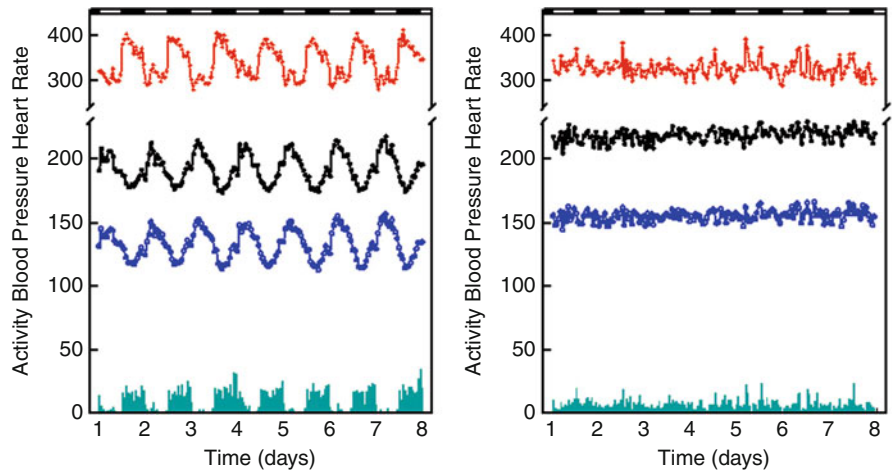

Fig. 21.31 Effect of SCN lesion (SCN-x) in transgenic-hypertensive rats (TGR) on blood pressure, heart rate, and activity (Acc. Witte et al. (1998c))

transgenic rats as well as wild-type and knock-out mice to address this question with the help of radiotelemetry.

21.4.1.4 Radiotelemetry

The availability of implantable radiotelemetric devices made it possible to study respiratory (Brockway and Brockway 1996) and various cardiovascular functions in freely moving, unrestrained animals (Brockway and Brockway 1996; Lemmer et al. 1993a; Kramer et al. 2000; Lange et al. 1991). There is a clear-cut advantage of radiotelemetry over the conventional tail-cuff method since radiotelemetry does not induce stress reactions in experimental animals, and furthermore, they can be monitored over several months. This was shown recently when comparing in the same animal radiotelemetry with tail cuff (Fig. 21.22). The procedure of tail cuff significantly increased BP and HR due to stress (Grundt et al. 2009).

We have shown that handling the animals and supplying food and water or cage change can increase BP and HR (Schnecko et al. 1996) (Fig. 21.23) as well as increase plasma concentrations of norepinephrine (NE) and angiotensin II (Schiffer et al. 2000, 2001). We have used these devices (Dataquest IV system, Data Sciences Inc., St. Paul, MN) in rats and mice either to measure simultaneously systolic and diastolic BP, HR, and activity by implanting the BP transmitter into the abdominal aorta (rats) (Lemmer et al. 1993a) or into the carotid artery (mice) (Lemmer et al. 2004a; Meier et al. 2004; Arraj et al. 2004), or an ECG-transmitter was used (Arraj and Lemmer 2006) (Fig. 21.24) to allow continuous monitoring of the HR as well as temperature and activity in mice.

Moreover, radiotelemetry can be applied to chronopharmacological studies in rodents, having the advantage that each single animal can be its own control, and dose–response studies can be performed at any circadian time after adequate wash-out periods (Meier et al. 2004; Lemmer et al. 1992; 1994; Schnecko et al. 1995; Witte et al. 1998a, 2001; Lemmer 2000; Pummer and Lemmer 2000; Janssen et al. 1998, 1993), these studies will not be further discussed here.

For data analysis, the Chronos-Fit program (Zuther and Lemmer 2004b) was used which fits partial Fourier series to the data (algorithm presented in (Zuther et al. 1996)) and allows presentation of a rhythmic pattern for single and grouped animals as well as transforming data into actograms (data marked black above the 24-h mean) to better demonstrate its rhythmicity.

21.4.1.5 Circadian Rhythm in Blood Pressure and Heart Rate in Rodents

Aside from normotensive control rats (models of human normotension: Sprague–Dawley: SPD, Wistar-Kyoto: WKY), spontaneously hypertensive rats (SHR) (Lemmer et al. 1993a) were used as a model of human primary hypertension, and transgenic TGR(mRen2)27 rats (TGR), as a model for human secondary hypertension (Lemmer et al. 1993a, b, 2003, 2005a) (Figs. 21.25 and 21.26). Experiments

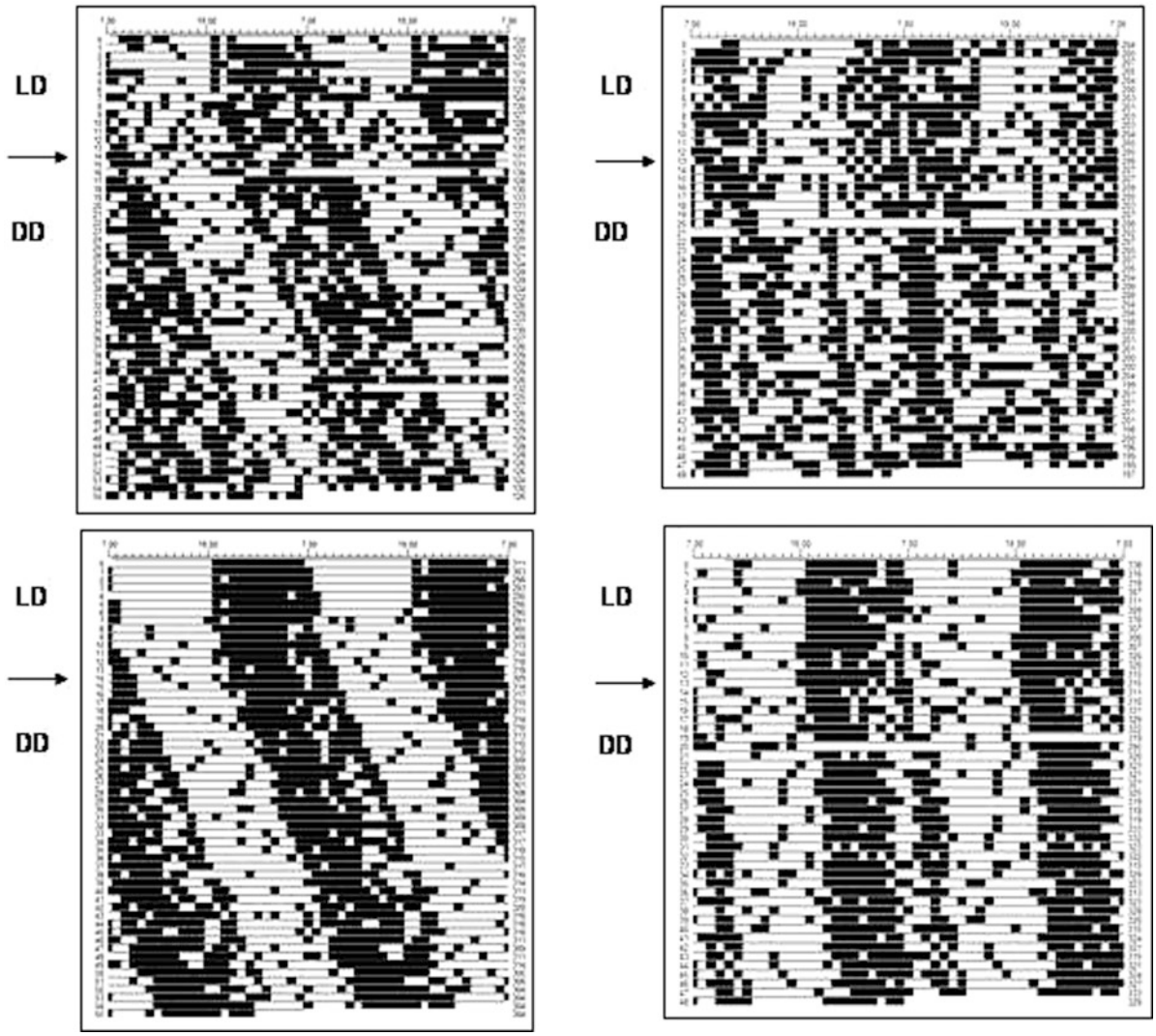

Fig. 21.32 Effects of LD (12:12 h) or DD (total darkness) on circadian rhythms in systolic blood pressure (SBP) and heart rate (HR) in normotensive and hypertensive TGR rats (Acc. Lemmer (2006))

were also performed in wild-type (C57) mice (Lemmer et al. 2004b, 2005b); several strains of transgenic (Witte et al. 2004) or knock-out mice are under investigation.

TGR(mREN2)27 rats develop a severe hypertension through the introduction of an additional mouse renin gene into the rat genome (Mullins et al. 1990). This is accompanied by an increase in the plasma concentrations of angiotensin ll (Schiffer et al. 2000, 2001; Tokita et al. 1994), renin, aldosterone, and corticosterone (Lemmer et al. 2000a), all, with the exception of renin, exhibiting pronounced circadian rhythms.

In telemetric studies, not only was the severe hypertension confirmed but surprisingly an inverse circadian BP pattern with peak values in the resting phase of the rats, i.e., during the light phase, was found in this rat strain, whereas the rhythm in HR and activity were not disturbed with peak values in the dark phase (Lemmer et al. 1993a, b) (Figs. 21.27 and 21.28). Thus, there was an internal desynchronization between BP and HR in TGR, obviously induced by the transgene.

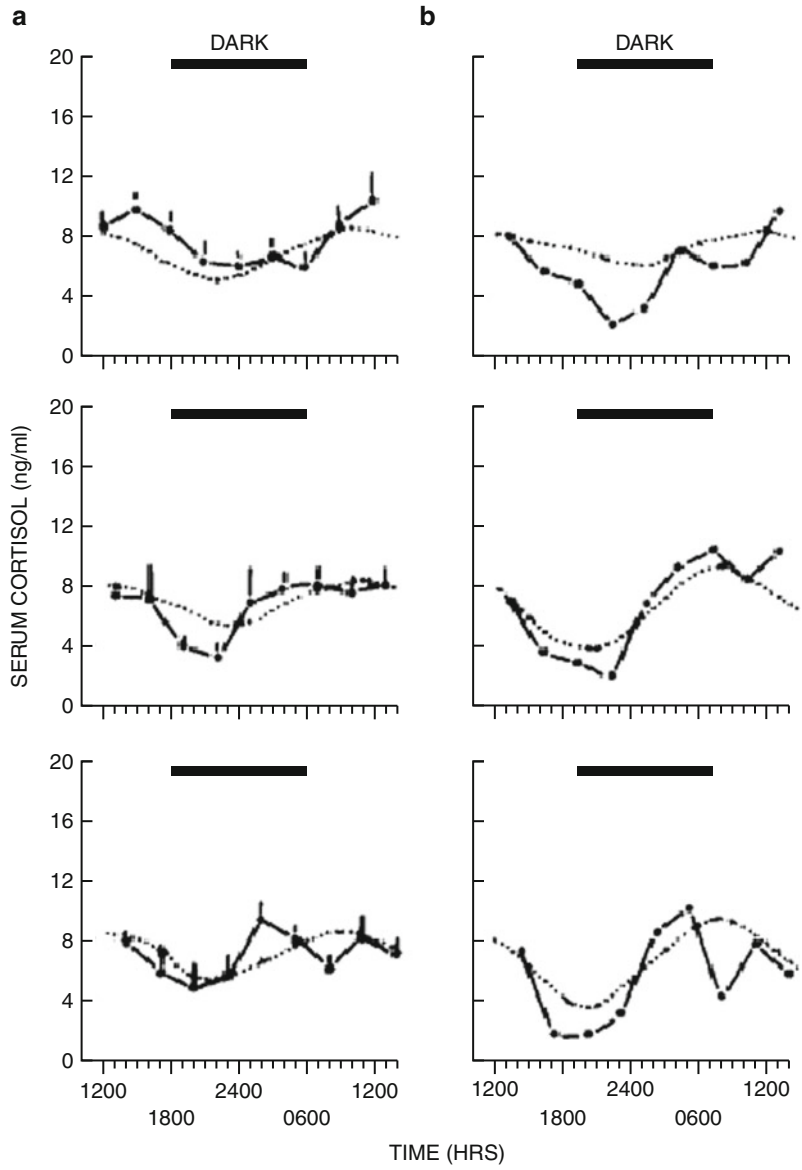

Fig. 21.33 Serum cortisol in female beagle dogs (Acc. Palazzolo and Quadri (1987))

Due to the nondipping behavior of BP during the rest phase, we proposed TGR as an animal model of human secondary hypertension (Lemmer et al. 1993a, b). Transgenic rats are normotensive up to about 7 weeks after birth and also display a normal circadian profile in BP, HR, and activity with peak values in the activity period during darkness (Witte and Lemmer 1999; Lemmer et al. 2003, 2005a; Witte et al. 1998b). Thereafter, TGR develop hypertension simultaneously with an about 12-h shift of the peak in BP from the dark into the light/rest phase (Witte and Lemmer 1999; Lemmer et al. 2003) (Fig. 21.29). This seems due to an ontogenic regulation of the mouse renin gene (Zhao et al. 1993). These data underline the need to monitor *before* drug testing whether a circadian rhythm in the parameter investigated is normal or possibly disturbed in relation to the age of the animal.

The circadian rhythms in BP and HR of WKY, SDR, and SHR – but not of TGR(mRen-2)27 – mirrored their

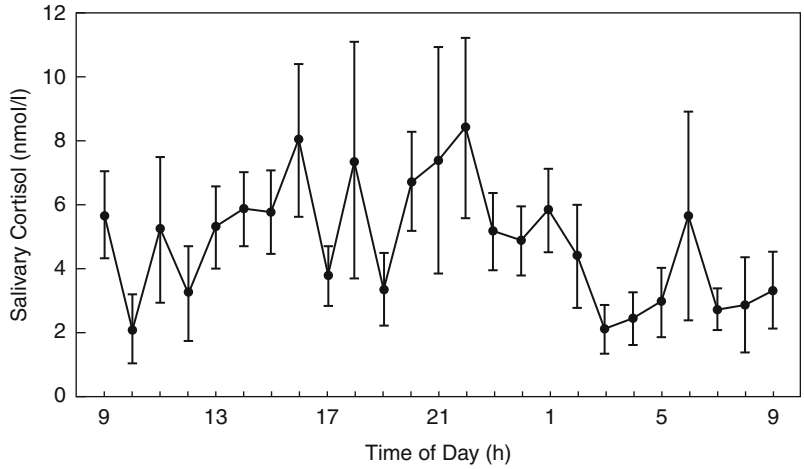

Fig. 21.34 Salivary serum cortisol in dogs (Acc. Koyama et al. (2003))

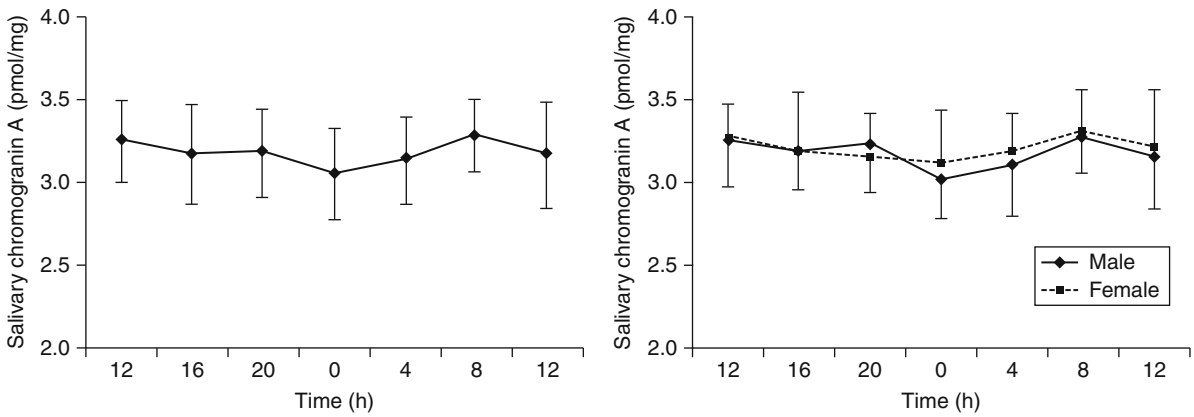

Fig. 21.35 Daily variations in salivary chromogranin in dogs (Acc. Kanai et al. (2008))

activity patterns in that peak values were observed in the rats' activity phase (Lemmer et al. 1993a). The circadian patterns in BP and HR of WKY, SDR, and SHR are similar to patterns observed in normotensive and primary hypertensive humans with the BP dipping in the rats' resting phase.

Radiotelemetric studies in male and female SHR also gave evidence for a gender-dependent rhythm in BP and HR, BP being lower and HR being higher in female than male rats (Fig. 21.28) (Grundt et al. 2006).

Interestingly, hypertensive TGR have a normal circadian pattern in kidney function, including renal plasma flow (Pons et al. 1996), and in the cerebral blood flow (Wauschkuhn et al. 2005), both peaking in the dark span. Thus, in TGR, renal and cerebral blood flow are internally desynchronized from the systemic flood flow, the underlying mechanisms are under investigation.

These data clearly show that studies on cardiovascular active drugs in rodents cannot be performed without taking the circadian rhythms and/or their internal desynchronization in cardiovascular functions into account.

Although BP is significantly elevated in TGR, we, however, found that plasma catecholamine

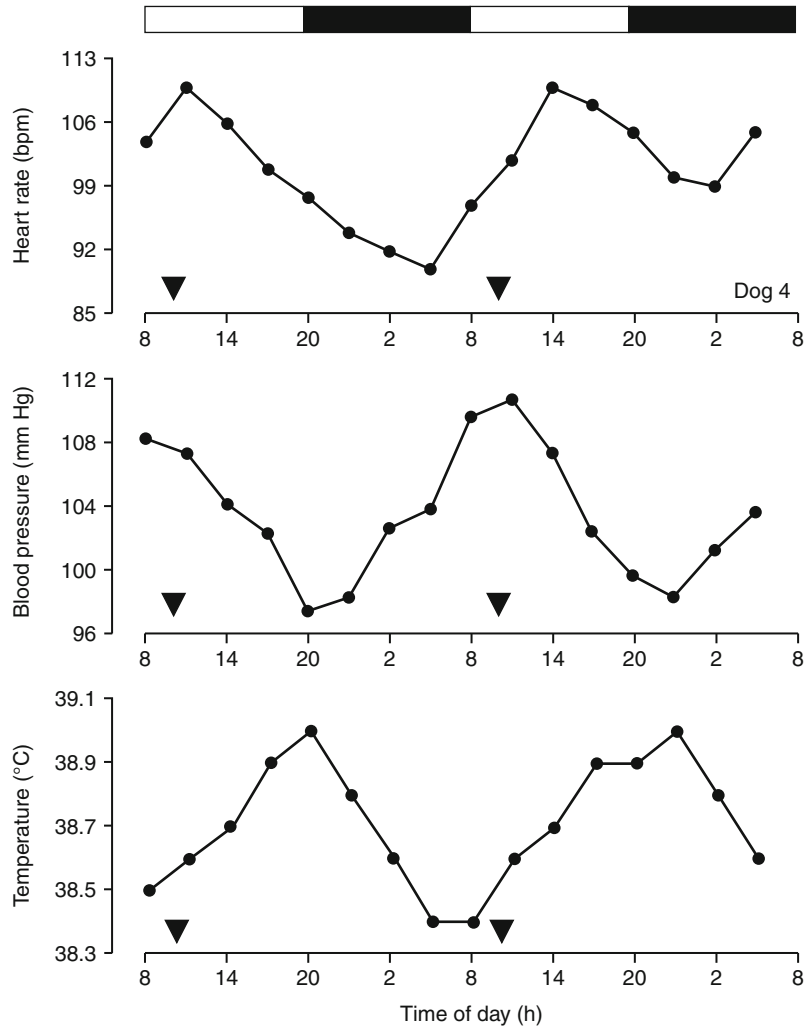

Fig. 21.36 Cardiovascular rhythms in fed and fasted dogs; there is an effect of feeding (triangle) (Acc. Piccione et al. (2005))

concentrations were reduced rather than increased in TGR; however, the rhythmic pattern and nocturnal peak were unaltered (Schiffer et al. 2000, 2001). Thus, the reduction in peripheral NE could be the result of different pathophysiological changes, such as an increased turnover of catecholamines, which is not detectable by measurement of plasma catecholamines, or an exhausted adrenergic system due to chronic stimulation by the renin-angiotensin-system, possibly with influence on the synthesizing enzyme tyrosine hydroxylase.

In a recent study, we were able to demonstrate by RT-PCR/Western blot in both nonhypertensive (4 weeks of age) and hypertensive (10 weeks) TGR lower NE concentrations and a reduced expression of the tyrosine-hydroxylase in cardiac tissue and adrenal glands. In the hypothalamus, NE concentrations were not different between the strains; however, tyrosine hydroxylase mRNA was significantly higher in TGR than SPD (Lemmer et al. 2003, 2005a). The turnover of NE was also reduced in hypertensive TGR in heart tissue and increased in the hypothalamus, both in the light and the dark phase. In adrenal glands of TGR, the mRNA of NE reuptake$_1$ transporter was also reduced, which could not be detected in hypothalamus (Lemmer et al. 2005a). These data indicate that the transgene in TGR leads to an increased central stimulation of the sympathetic nervous system and a consequent downregulation in the peripheral organs.

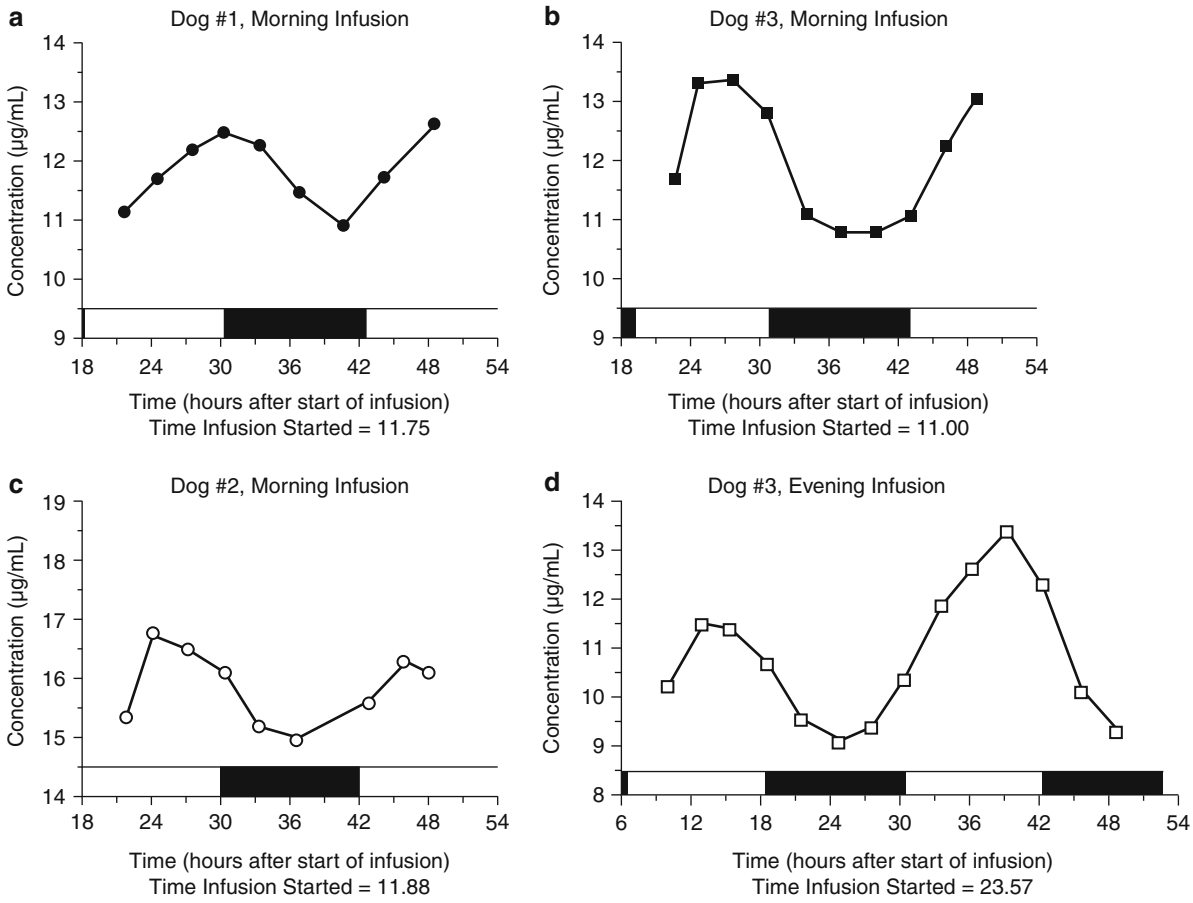

Fig. 21.37 Rhythm in serum theophylline concentration after constant infusion for 48 h in dogs (Acc. Rackley et al. (1988))

The data give evidence that the circadian pattern in cardiovascular functions can also be traced down to the molecular level. This observation also implies that studies on the genetics of, e.g., hypertension must be time-specified. Already in 2002, Storch could give evidence that the gene expression in mouse liver and heart follows a reproducible circadian rhythm (Fig. 21.29) (Storch et al. 2002).

In wild-type mice, a circadian rhythm in HR and BP similar to that found in rats was found by telemetry (Lemmer et al. 2004c, 2005b; Witte et al. 2004) with evidence for being endogenous since the rhythms persisted under total darkness under free-run conditions in DD (Fig. 21.30). This observation was confirmed by radiotelemetric ECG registration in mice (Lemmer et al. 2005b) (Fig. 21.24).

21.4.1.6 Cardiovascular Rhythms Driven by a Biological Clock?

Most behavioral and physiological parameters in mammals display at least some evidence of a 24-h temporal structure, reflecting an innate temporal program provided by biological clocks. The suprachiasmatic nucleus (SCN) of the hypothalamus serves as the main zeitgeber for such circadian rhythms. Light induction of clock genes might be a general factor through which the body clock is brought into synchronization with the external environment (Albrecht et al. 1997; Shigeyoshi et al. 1997; Spoelstra et al. 2004; Albrecht 2004). In rodents, light-induced phase shifts of behavioral rhythms are known to be positively correlated with the induction of the transcription factor Fos in the SCN, and it has been suggested that Fos itself mediates these light-induced

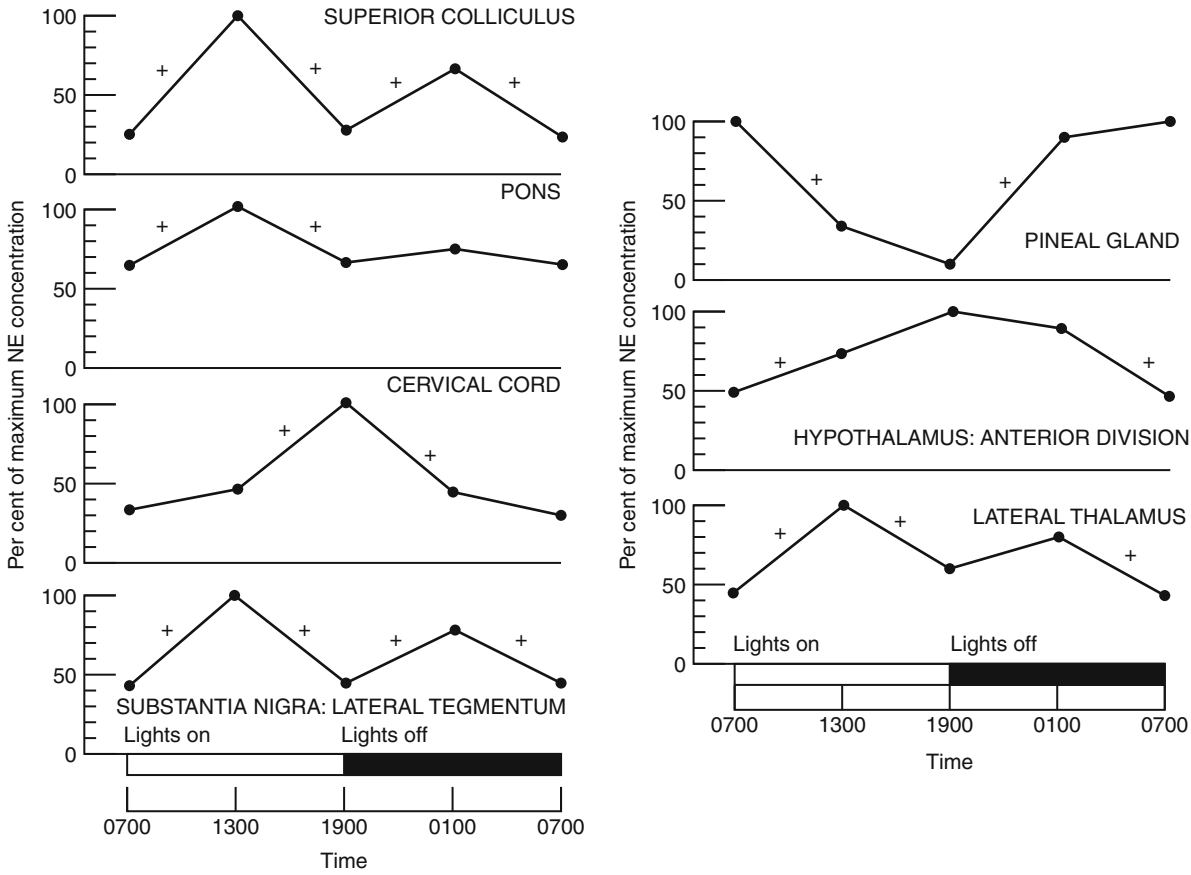

Fig. 21.38 Circadian rhythm in norepinephrine concentration in cat brain (Reis et al. 1968)

phase shifts (Kornhauser et al. 1996; Shimomura et al. 1998; Rusak et al. 1992; Sutin and Kilduff 1992; Lemmer et al. 2000b). As described above, alterations of endogenous rhythms, including severe changes in the rhythmic pattern of BP have been detected in TGR (mRen2)27 rats (Lemmer et al. 1993a, 2003, 2005a). Both in normotensive rats and in TGR, ablation of the suprachiasmatic nucleus eliminates 24-h BP variability (Janssen et al. 1994) and abolishes the rhythm in activity as well as in HR and BP (Witte et al. 1998c) (Fig. 21.31). This observation gives evidence that – at least in the rat – cardiovascular rhythms must also be under the control of the central clock(s) located in the SCN.

Another important feature of circadian rhythms is that they free run under constant environmental conditions, i.e., during constant darkness, indicating that they are really governed by an internal clock. Both in rats (Witte and Lemmer 1995) and mice (Lemmer et al. 2005b), we were able to demonstrate that the rhythms in cardiovascular functions (BP, HR) persisted under free run with a period deviating from 24 h. In normotensive rats, an increase (Figs. 21.18 and 21.31), and in normotensive mice, a shortening (Fig. 21.30) of the periods was found. Interestingly, BP and HR in TGR did not free run under DD (Fig. 21.32) (Lemmer 2006), indicating that light perception must be disturbed in this transgenic rat strain (Witte and Lemmer 1995).

In conclusion, the data obtained in various strains of rodents can help to better understand the rhythmic regulation of BP and HR and the underlying mechanisms involved. These data convincingly demonstrate that preclinical drug testing on cardiovascular functions and studies on safety pharmacology in rats and mice cannot be performed without taking the circadian rhythmic organization of these animals into account. Of additional importance are the observations

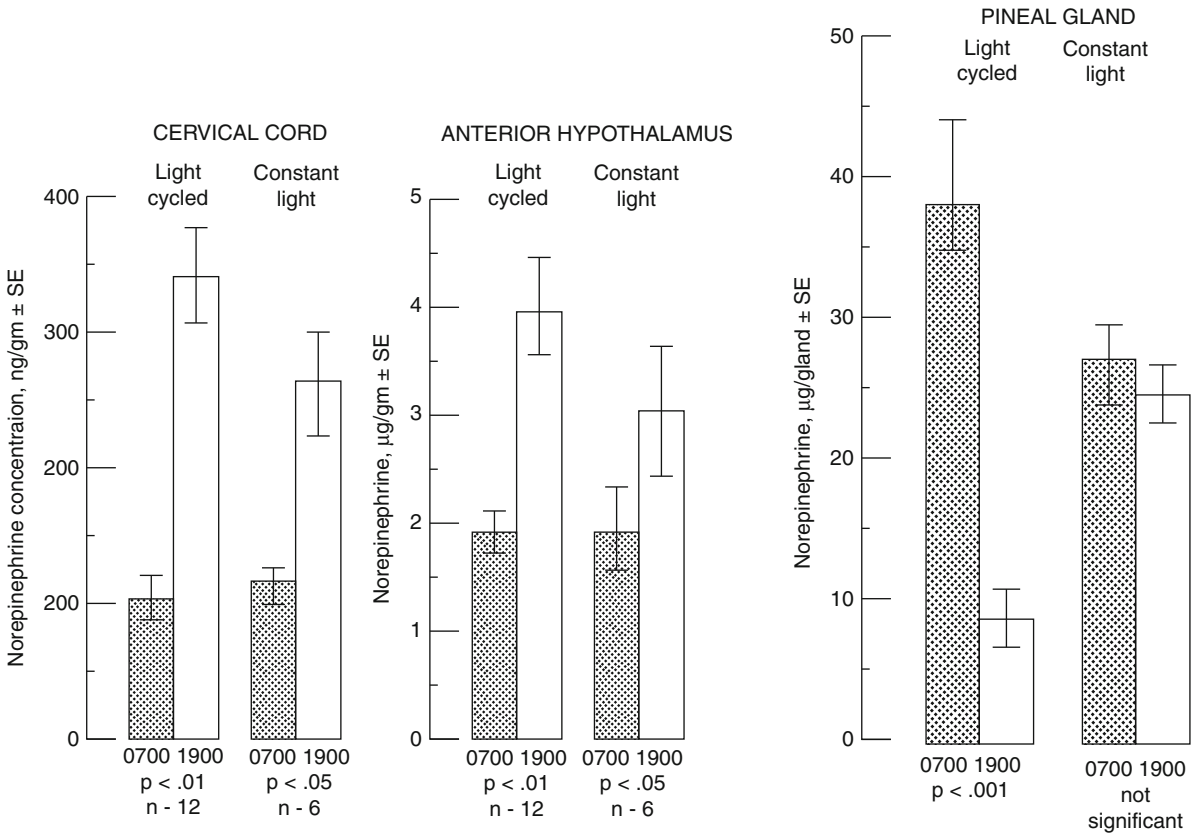

Fig. 21.39 Day-night differences in norepinephrine concentration in different areas of cat brain, either during a light–dark cycle or during continuous light (Acc. Reis et al. (1968))

that strain, gender, and age-dependent differences can occur in these rhythmic patterns. Finally, it is evident that radiotelemetry is the gold standard to study cardiovascular function in unrestrained, freely moving rodents. It should be mentioned that tissue pieces taken from brain, kidney, or liver even exhibit circadian rhythms during in vitro incubation (Reppert and Weaver 2002), clearly indicating the wonderful circadian organization of living material.

21.4.2 Other Experimental Animals

As already mentioned above, chronobiological investigations were rarely performed in other animal species used in preclinical studies and in safety pharmacology. Some examples are outlined below. With the exception in monkeys, experiments were generally not performed under free-run conditions to test for the endogenous component of a rhythmic pattern.

21.4.2.1 Dogs

In female beagle dogs, the rhythm in serum cortisol (radioimmunoassay) was studied at three different ages (Palazzolo and Quadri 1987). Blood samples were sampled every 3 h for three 24-h periods. In adult animals (3.3 years), a circadian rhythm could be detected, but not in puppies (8.4 weeks) and in older dogs (12.1 years). The authors concluded that the circadian rhythm was disrupted in old animals while not yet developed in puppies (Palazzolo and Quadri 1987) (Fig. 21.33).

More or less in line with the findings mentioned above are data on salivary cortisol (enzyme-linked immunosorbent assay) in adult beagle dogs in which no significant circadian rhythm was detected (Koyama et al. 2003) (Fig. 21.34).

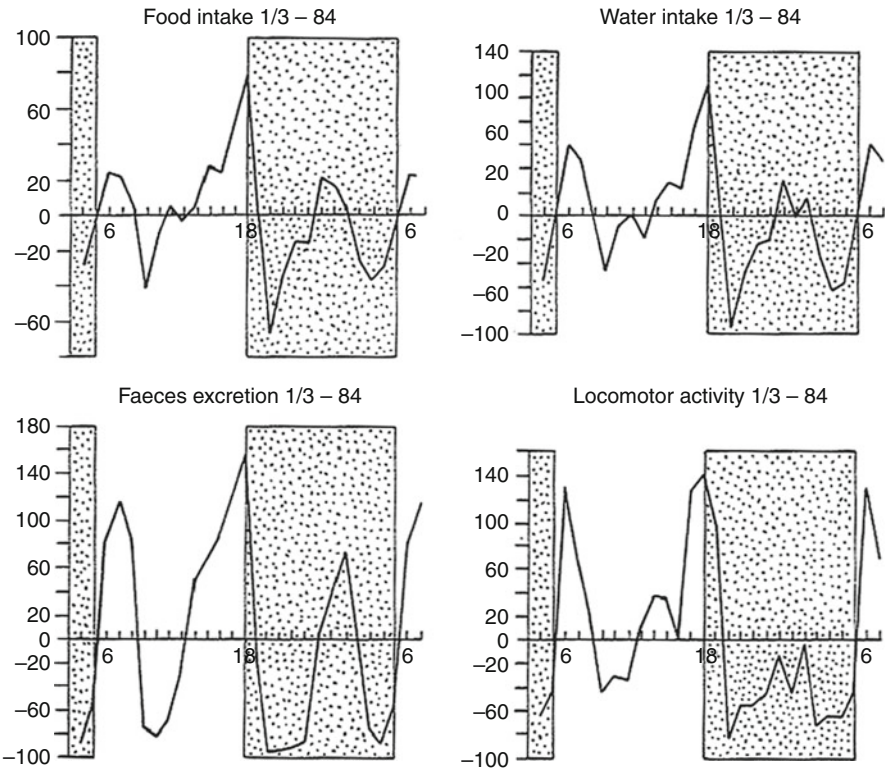

Fig. 21.40 Behavioral rhythms in guinea pigs (Acc. Jilge (1985))

There was also no circadian rhythm in salivary chromogranin in 16 beagle dogs (Kanai et al. 2008) (Fig. 21.35). Chromogranin exists in chromaffin granules of the adrenal medulla and is co-released with epinephrine or NE.

As discussed already earlier, with the use of telemetry, body temperature in female beagle dogs, in which the rectal temperature was determined by an electronic thermometer for 7 days, however, seems to be really circadian rhythmic (Refinetti and Piccione 2003). The authors speculate that the small robustness and a not-very-stable environmental condition may be responsible that, in the past, a significant rhythm was not detected.

The same group also reported on significant circadian rhythms on HR, BP, and temperature in 1-year-old beagle dogs which were not (??) altered by feeding (Piccione et al. 2005) (Fig. 21.36); this cannot be definitely concluded since no data are available under free-run conditions.

In eight unrestrained mongrel dogs, telemetry also gave evidence for circadian rhythms in the 24-h patterns of circulation as shown for HR, BP, peripheral resistance, and cardiac workload (Ashkar 1979).

In three mongrel dogs, the disposition of a theophylline infusion at a constant rate over 48 h was studied (Rackley et al. 1988). A characteristic 24-h rhythm was observed which was attributed to theophylline disposition with a draught around the night and a peak around the end of the light phase (Fig. 21.37).

In conclusion, the chronobiological studies in dogs give evidence for a 24-h rhythm in the cardiovascular system, which, however, was not verified under free-run conditions. It is possible that the daily routine and the feeding pattern of dogs may act as a prominent zeitgeber for these rhythms. It is of interest that the cortisol rhythm was not constantly rhythmic as found under entrained as well as free-run conditions in rats, man, and monkeys.

21.4.2.2 Cats

In mongrel adult cats, daily variations in NE concentration were reported in different areas of the brain (Fig. 21.38) (Reis et al. 1968).

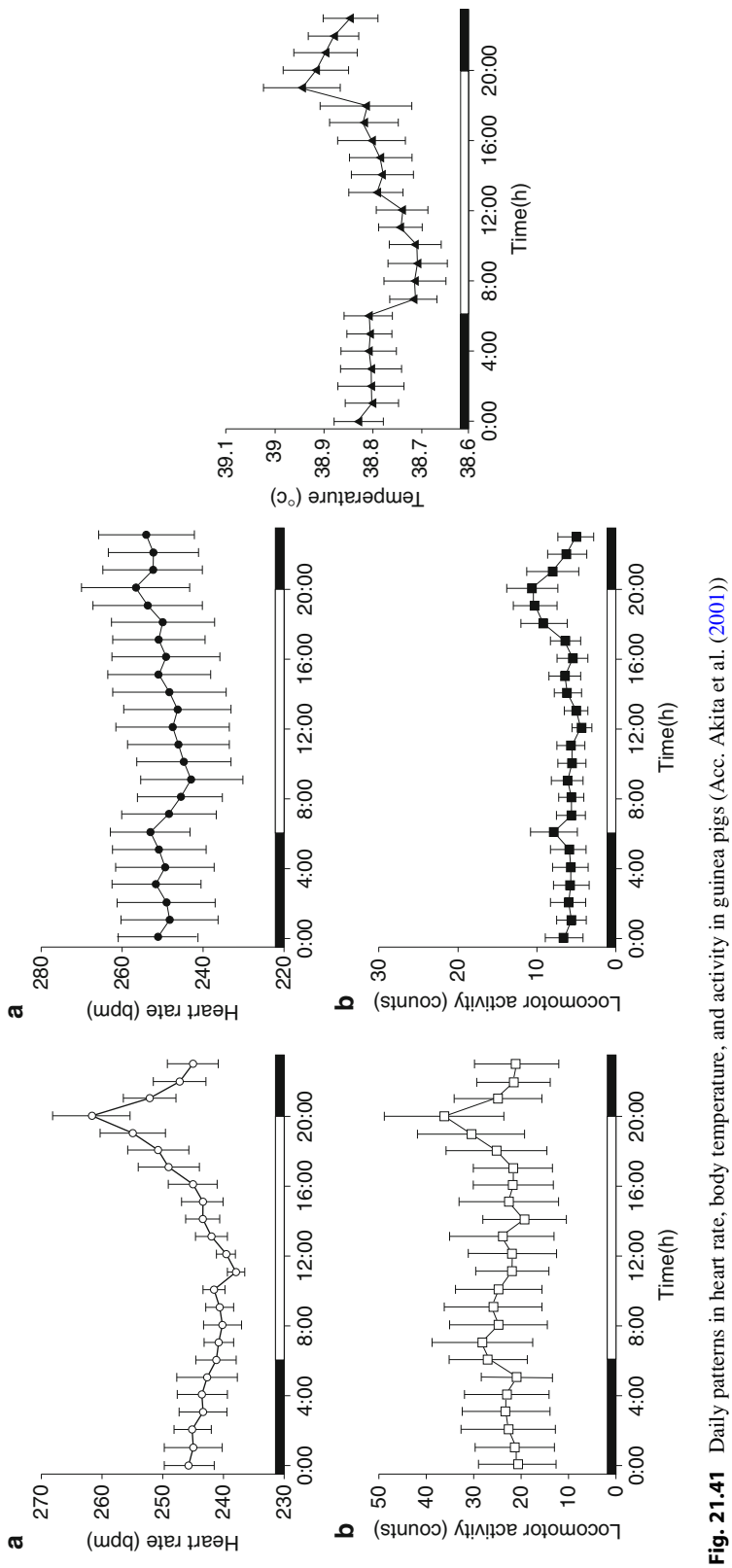

Fig. 21.41 Daily patterns in heart rate, body temperature, and activity in guinea pigs (Acc. Akita et al. (2001))

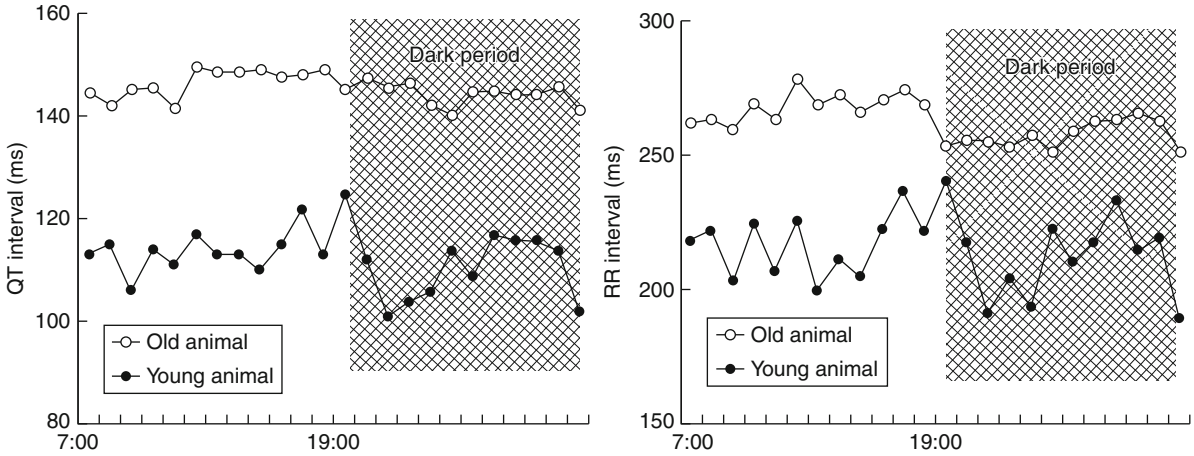

Fig. 21.42 Rhythm- and age-related changes in guinea pigs (6 weeks; 23 months) (Acc. Shiotani et al. (2008))

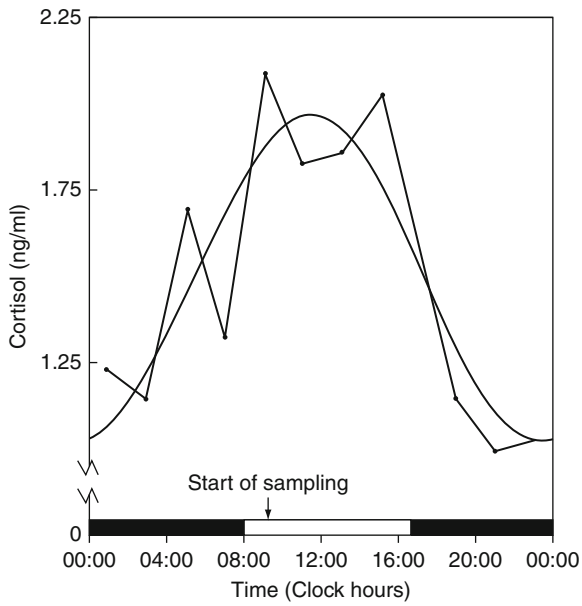

Fig. 21.43 Circadian rhythm in salivary cortisol in pigs (Acc. Ekkel et al. (1996))

Whereas in the pineal gland this rhythm did not persist under constant light, constant light did not abolish the rhythm in the cervical cord and anterior hypothalamus, giving evidence for the endogenous nature of this rhythm (Reis et al. 1968) (Fig. 21.39).

In conclusion, there are too few chronobiological data in cats. However, some 24-h rhythms were described and have to be taken into account in experiments when performed in cats.

21.4.2.3 Guinea Pigs

In guinea pigs, only very few data were reported. Jilge showed (Jilge 1985) that behavioral aspects such as food intake, water intake, feces secretion, and locomotor activity displayed 24-h rhythms (Fig. 21.40). However, the rhythm in locomotor activity as well as in HR and body temperature were only observed in some animals (Akita et al. 2001) by radiotelemetry (Fig. 21.41) which is the best method available. The authors underline that guinea pigs should not be used in chronobiomedical research.

In addition, cardiovascular data on QT and RR intervals showed some rhythm in young guinea pigs, but not in elderly ones (Fig. 21.42) (Shiotani et al. 2008).

In conclusion, the documentation of chronobiological data in guinea pigs is small.

21.4.2.4 Pigs

Data on minipigs were presented above. Additional chronobiological data were published in other pig strains. Salivary cortisol displays a significant circadian rhythm with a peak at the onset of the light period (Ekkel et al. 1996) (Fig. 21.43).

Similar data were reported for peripheral levels of melatonin (Fig. 21.44) (Bubenik et al. 2000). Heat production seems to be also circadian-phase-dependent both under feeding and during starvation and refeeding (Fig. 21.45) (Chwalibog et al. 2004).

In domestic pigs, a circadian rhythm exists also in the rhythm of body temperature (Fig. 21.46) with an

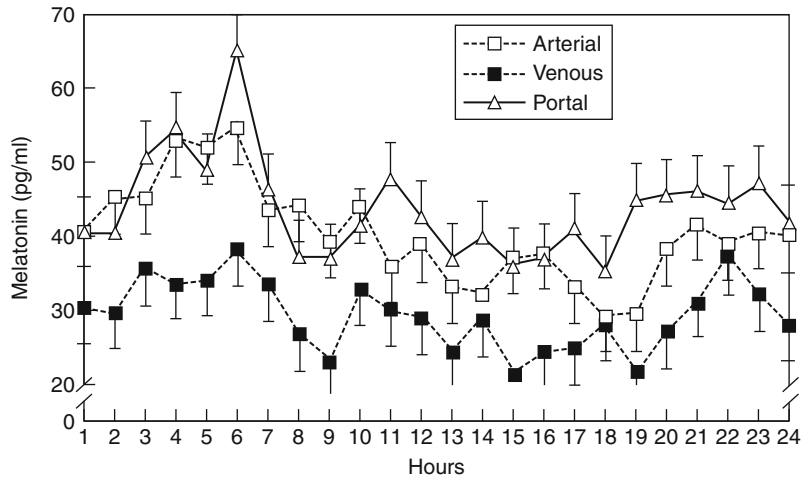

Fig. 21.44 Daily variation in peripheral melatonin in ten pigs (Acc. Bubenik et al. (2000))

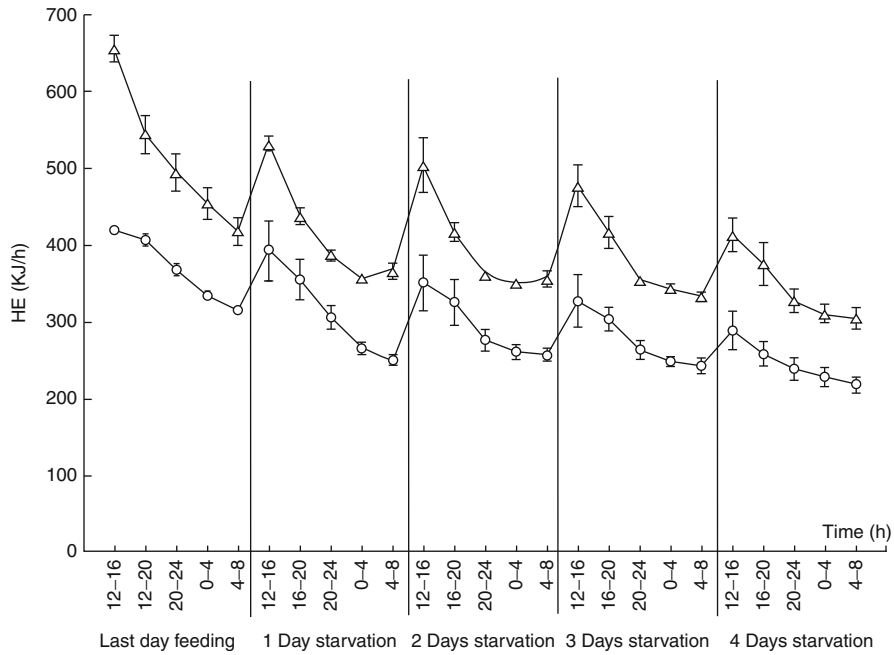

Fig. 21.45 Rhythm in heat production in pigs (Acc. Chwalibog et al. (2004))

acrophase at the end of the light phase (Hanneman et al. 2005).

21.4.2.5 Monkeys

Much more chronobiological data were obtained in monkeys. The temperature rhythm – which in man is really governed by the internal clock – displays pronounced circadian variations in cynomolgus monkeys with peak values in the activity period during the light phase as discussed earlier and shown in Fig. 21.47 (Almirall et al. 2001).

Circadian rhythms were also described for melatonin and ultradian rhythms in NE concentrations in rhesus (Fig. 21.48) and in Japanese monkeys (Levin et al. 1978; Nozaki et al. 1990).

Fig. 21.46 Circadian temperature rhythm in laboratory swine (Acc. Hanneman et al. (2005))

Circadian temperature rhythm parameters for growing–finishing domestic pigs

Subject	Amplitude(°)	Mesor(°)	Period(h)	Acrophase (military time)
1	0.18	38.15	24.4	17:50
2	0.14	38.55	24.5	21:18
3	0.13	38.77	23.1	17:22
4	0.08	40.33	24.1	22:06
5	0.22	38.55	25.9	21:18
6[a]	0.31[a]	37.83[a]	20.5[a]	22:25[a]
7	0.14	39.13	22.9	20:33
8	0.21	38.48	22.7	16:05
9	0.25	38.17	23.9	18:32
Mean (SE)	0.18(0.02)	38.66 (0.24)	23.6 (0.50)	19:44

[a]indicates the pig with noncircadian period.

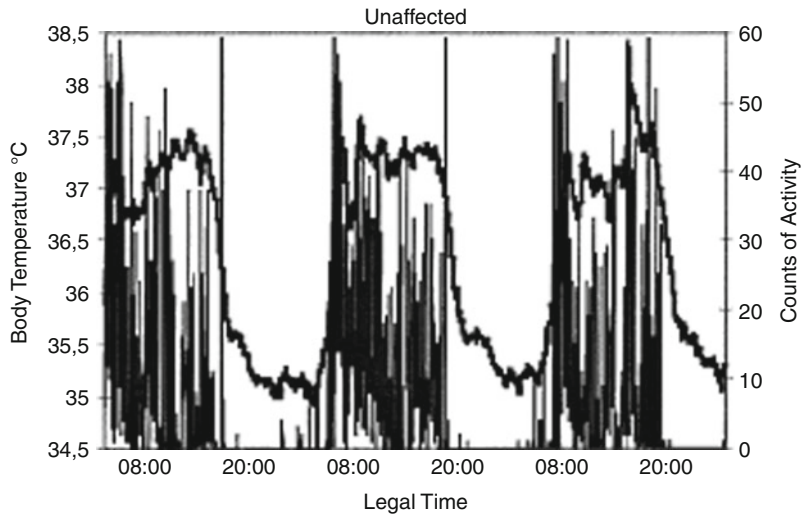

Fig. 21.47 Rhythms in body temperature and activity in cynomolgus monkeys (Acc. Almirall et al. (2001))

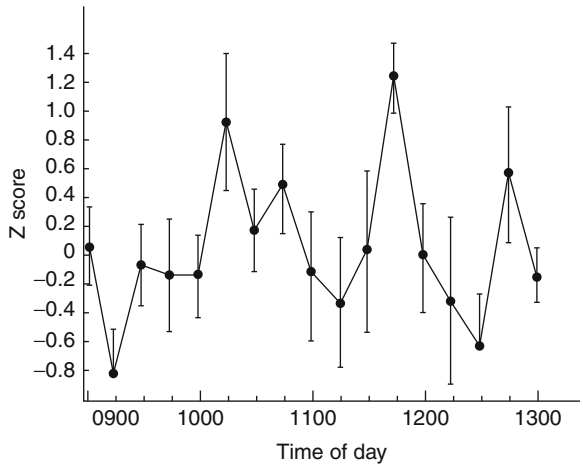

Fig. 21.48 Ultradian plasma norepinephrine concentration rhythm in rhesus monkey (Acc. Levin et al. (1978); Nozaki et al. (1990))

In capuchin and squirrel monkeys, circadian rhythms were documented, in activity, temperature, ACTH, cortisol (Torres-Farfan et al. 2008), and in temperature and activity (Boulos et al. 1996) (Fig. 21.49), as well as in pig-tailed macaques (Weed and Hienz 2006).

Most interestingly, the rhythm in temperature in squirrel monkeys persisted under free-run condition during total lightness (Fig. 21.50), clearly indicating the endogenous nature of this rhythm (Fuller and Edgar 1986).

Quite recently, it has been shown that the sleep rhythm in the primate Macaca mulatta persisted under constant dim light (CDL) – which again indicates the involvement of the biological clock in rhythm generation – and was reduced age-dependently (Zhdanova et al. 2011) (Fig. 21.51).

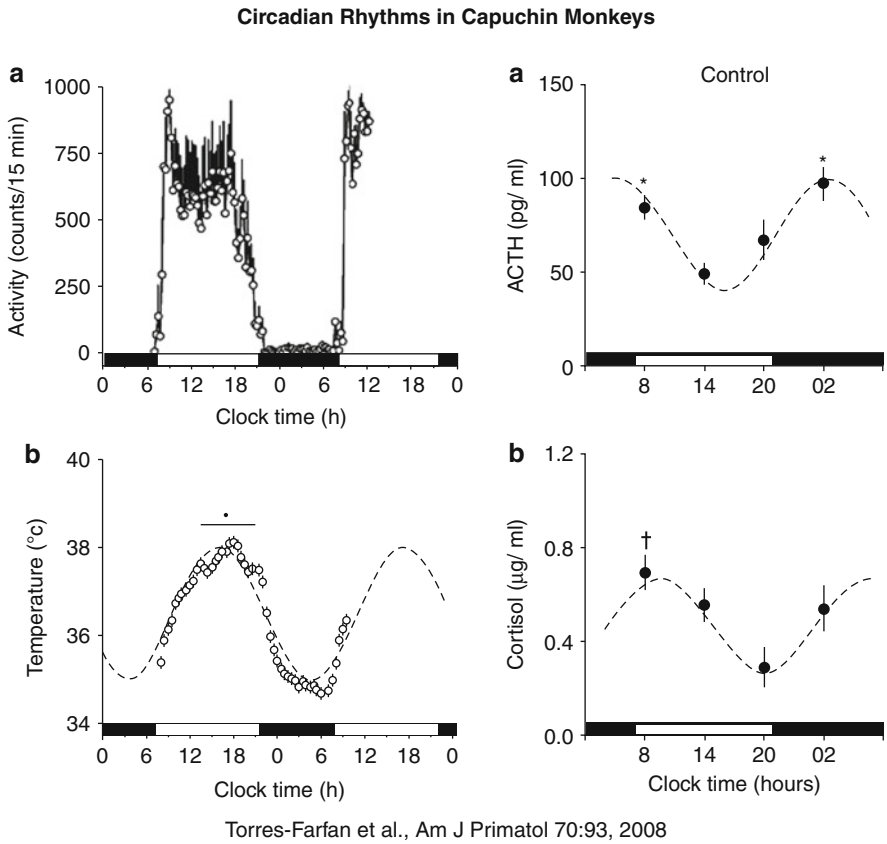

Fig. 21.49 Circadian rhythms in capuchin monkeys (Acc. Torres-Farfan et al. (2008))

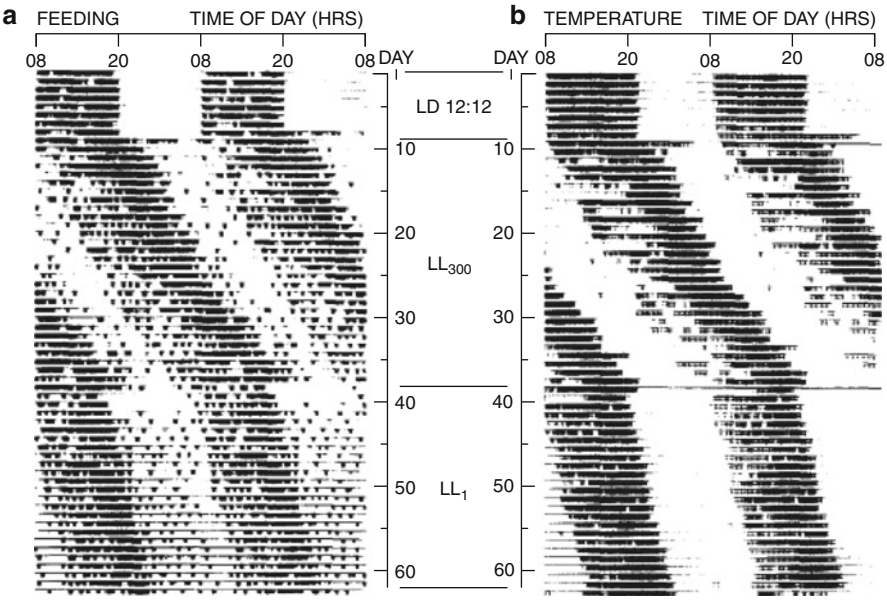

Fig. 21.50 Effect of light intensity on circadian temperature and feeding rhythms in squirrel monkey (Acc. Fuller and Edgar (1986))

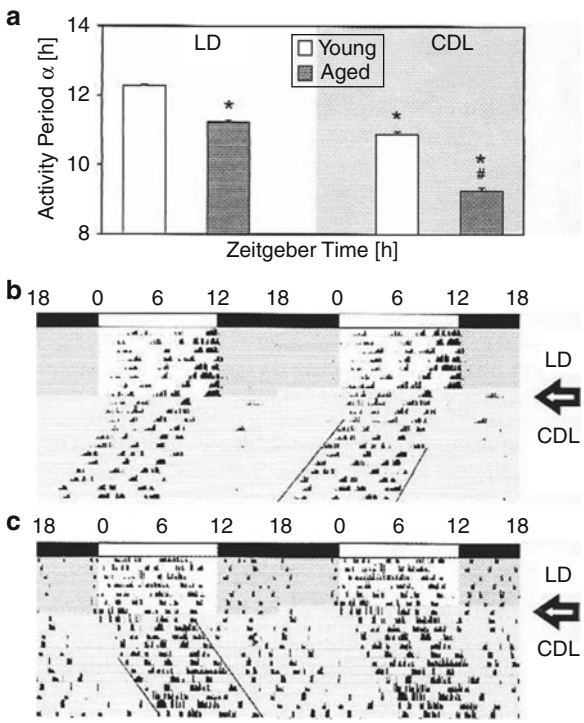

Fig. 21.51 Effect of aging on circadian rhythm in sleep in primate *Macaca mulatta*. LD = light–dark (**a**); CDL = continuous light, in young (**b**) and in aged (**c**) monkeys (Acc. Zhdanova et al. (2011))

21.5 Conclusion

In conclusion, monkeys convincingly display circadian rhythms in various body functions which must be considered in safety pharmacology studies. In this respect, monkeys seem to be very similar to humans in which many functions were nicely documented to be circadian-phase-dependent.

In this chapter, we have tried to document that circadian rhythms in bodily functions do exist in many animal species. There is no doubt that safety pharmacology experiments as well as other preclinical studies MUST take into account the rhythmic organization in planning and performing drug studies.

I would like to underline the importance of radiotelemetry for chronobiology, chronopharmacology, and safety pharmacology. When about 20 years ago, we received a cardiovascular telemetric device from Brian Brockway – the inventor and founder of DataSciences – and when our group (B.L.) and his were the first to publish telemetric data from rats ((Brockway et al. 1991; Brockway and Brockway 1996; Mattes and Lemmer 1991; Lemmer et al. 1993a)), we were already fully convinced that radiotelemetry in freely moving, unrestrained, unstressed animals would become the gold standard of cardiovascular research in the future. This has turned out to be true as we can demonstrate in this chapter. However, we have to admit that the concept of chronobiology/chronopharmacology should be more intensively incorporated in safety pharmacology as well as in other preclinical studies. The biological clock which is present in all living creatures must be looked at as a keystone in animal research in order to get a better understanding of bodily functions and in order to obtain a sound and reliable evaluation of a drug's kinetics and effects/side effects!

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Biomaterials in Their Role in Creating New Approaches for the Delivery of Drugs, Proteins, Nucleic Acids, and Mammalian Cells

22

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22.1 Introduction

The use of biomaterials to enable and enhance life-saving therapies in medicine has its documented roots in the 1600s. Historically, drugs were administered systemically for acute emergency situations. As injectable drug technologies developed, it was soon observed that drug administration would often be more effective if given in sustained-release or tailored-release profiles. However, the potential safety risks of drugs administered by these novel devices and biomaterials were also quickly realized. This chapter will examine the documented evolution of the delivery of such compounds, the challenges encountered, and the solutions developed to address the need for safe delivery of drugs, proteins, and cells for human use. Each biomaterial-based drug delivery system is unique to the drug, peptide, or protein employed in the system. Therefore, this chapter will focus on the safety considerations along with the therapeutic efficacy offered by this evolving and developing area of therapeutics.

22.1.1 Biomaterial Beginnings

The first documented scientific drug delivery studies may be traced to 1655, when Dr. Richard Lower in England successfully transfused blood. His initial transfusion experiments kept an exsanguinated dog alive by using a sharpened quill to connect a donor dog to the carotid artery of (Blundell 1829) the second animal (Lower 1932).

Lower's work was followed by attempts to conduct animal-to-human transfusions by various investigators

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in Europe in the late 1600s. These experiments quickly revealed the toxicity and the immunologic differences between animal and human blood. Consequently, the Paris Society of Physicians quickly outlawed animal-to-human transfusions in 1678 because of numerous adverse reactions, many resulting in death (Learoyd 2006). These observations represented some of the first documented potential toxicities of biologic compounds administered from one organism to another as early advances in biomaterials allowed transfusions to take place.

In 1817, Dr. John Leacock at Edinburgh Medical School described experimental transfusions between dogs and cats using an ox ureter connected with two crow's quills (Leacock 1817). In 1825, James Blundell, a British obstetrician, performed the first successful transfusion of human blood to a patient for the treatment of postpartum hemorrhage based on Dr. Leacock's studies (Schmidt and Leacock 2002), (Blundell 1829). He extracted a small amount of blood from a husband's arm, and he successfully transfused the man's wife. Between 1825 and 1830, he performed ten documented transfusions, five of which proved beneficial to his patients and five failures due to blood type incompatibility, and published these results (Blundell 1829). It was not until Karl Landsteiner developed the ABO blood-typing system in 1900 did safe human blood transfusions became feasible to avoid immunological differences among humans (Landsteiner 1900).

22.1.2 Development of the Hypodermic Needle

Dr. Francis Rynd described the invention of the hollow needle in 1844 in order to replace the quills derived from bird feathers (Rynd 1845). In 1853, Charles Pravaz is credited with the development of the first practical metal syringe made entirely from silver (Dippenaar 2007). The trocar and cannula were made of gold or platinum (Schorr 1966). Later in the same year, Alexander Wood experimented with the use of a hollow needle and syringe for the more effective subcutaneous administration of morphine in the treatment of neuralgia (Wood 1855). Injections of drugs such as morphine were initially targeted at treating local pain among individuals. It soon became apparent that there was systemic absorption of these drugs as ailments far removed from the site of injection also benefited. Ultimately, this observation would become the basis of all injection therapies. Virtually all therapeutic protein drugs are administered in aqueous solutions using needles and syringes.

22.1.3 Disinfection of Surgical and Drug Administration Devices

All of these instruments used for injection were of course subject to bacterial contamination. John Erichsen lectured through the 1860s that surgery was traditionally conducted without any attempts to avoid infection (Erichsen 1974). His student, Joseph Lister, experimented with the use of phenol to disinfect wounds and found a significant reduction of gangrene among surgical patients (Lister 1867).

22.2 Establishment of Standards and Regulatory Guidance for Biomaterials and Drug Combinations

These early attempts to develop syringes, needles, biomaterials, and drugs foretold the same basic challenges confronting the delivery of drugs today using advanced biomaterials:

(a) The drug–biomaterial stability and compatibility
(b) The immunologic consequences of drugs interacting with the recipient
(c) The need for sterile injectables (Siegel et al. 2007)

Each country's regulatory bodies have established the safety standards for the combination of biomaterials and drugs. The United States' Food and Drug Administration (FDA) and the European Commission set their standards independently. Each regulatory agency also takes guidance from the nongovernmental "International Organization for Standardization" (ISO) whose headquarters are in Geneva, Switzerland (Biological Evaluation of Medical Devices 2007). The biological evaluation of medical devices is outlined in ISO 10993.

The ISO guidelines apply to biomaterials that are either:

(a) Solid and nonbiodegradable
(b) Degradable and/or resorbable
(c) Nonsolid, such as porous materials, liquids, pastes, and particulates

There are 19 sections of the ISO standards covering tests for genotoxicity, carcinogenicity, and reproductive toxicity; tests for interactions with blood; tests for in vitro cytotoxicity; tests for local effects after implantation, identification, and quantification of potential degradation products; tests for irritation and skin sensitization; tests for systemic toxicity, toxicokinetic study design for degradation products and leachables; and principles and methods for immunotoxicology testing of medical devices (International Organization for Standardization 2010).

Drug and delivery device combinations for the FDA are defined in 21 CFR § 3.2(e) of the Code of Federal Regulations as (Code of Federal Regulations, Title 21 2005):

1. A product comprised of two or more regulated components, i.e., drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity
2. Two or more separate products packaged together in a single package or as a unit and comprised of drug and device products, device and biological products, or biological and drug products
3. A drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, e.g., to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose
4. Any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect

Additionally, the FDA has issued a draft Guidance for Industry and Staff entitled "Classification of Products as Drugs and Devices" with the goal of providing direction of the process to obtain a formal determination of whether a product is classified as a drug, device, biological product, or combination product (Office of Combination Products Food and Drug Administration 2011). Biomaterials that release low-molecular-weight drugs and proteins are considered combination products.

The complexity of the drug approval process can potentially result in products that meet the drug definition or both the drug and device definitions and that also meet the definition of biological product, might be classified as biological products, rather than as devices or drugs, and be subject to licensure under the PHS Act (Office of Combination Products Food and Drug Administration 2011).

22.3 Immunogenicity of Proteins and Biomaterials

The interaction of recombinant proteins and biomaterials (Meade and Silver 1990) can result in immunogenic reactions as a result of exposing the body to foreign compounds (Kessler et al. 2006; European Medicines Agency 2006) (Swanson). Any variables that may cause a change in protein structure, i.e., pH, temperature, interactions with packaging materials, storage conditions, etc., may potentially elicit the production of antibodies. Production of antibodies in preclinical studies can affect the regulatory path of the protein. Serious side effects may be observed in humans. For example, antierythropoietin antibody formation and pure red-cell aplasia has been observed in patients treated with recombinant human erythropoietin (Casadevall et al. 2002).

Therefore, antibody monitoring with appropriate assays is required to ascertain the safety and efficacy of therapeutic proteins. The combination of biomaterials and therapeutic proteins may accelerate protein unfolding and structure change. Preclinical immunogenicity assessment is important to determine toxicology and antibody neutralization potential. In general, initial responses to new compounds are IgM with low affinity and of low concentration. High-affinity mature IgG class antibodies are more likely to neutralize therapeutic proteins. These studies take at least 6–12 months to determine both binding and neutralizing antibodies to the protein or biomaterial. In patients, clinically relevant antibodies (Ab) include (a) the clearing Ab, (b) the sustaining Ab, (c) the neutralizing Ab, (d) allergic reactions, and (e) cross-reactions with endogenous proteins. Immunoassays are developed to detect antibodies using ELISA, RIA, surface plasmon resonance (BIAcore), or electrochemiluminescence.

22.4 Biotechnology Needs for Drug Delivery

The modern biotechnology revolution began in 1974 with the first expression of a foreign gene implanted in bacteria using recombinant DNA methods (Morrow et al. 1974; Berg et al. 1974). The need to administer most of these therapeutic protein compounds by injection is compounded by their short half-lives, and therefore, there is a need to inject these drugs frequently (Duckworth et al. 1998). There are over 70 self-administered injectable drugs approved for marketing in the United States with the goal of encouraging better therapy through improved patient compliance (Indepdence Blue Cross 2010). The vast majority of these compounds are therapeutic proteins. Therapeutic protein drugs such as insulin, interferon, and growth hormone are now dispensed from injector pens (Fischer et al. 2008). The therapeutic advantages, device safety, ease of use, accuracy of dose, and small needle size of injector pens are helping to increase patient compliance and positive outcome among those who self-administer therapeutic proteins. From a regulatory viewpoint, the drug stability in these prefilled self-contained delivery systems must be assessed to the same degree as a new chemical entity. Table 22.1 is a representative summary of biomaterials and drugs that have been combined to enable delivery of a variety of pharmaceuticals for therapeutic benefit to the patient.

22.5 Drug Delivery Technologies to Alter Drug Pharmacokinetics

The short pharmacodynamic half-lives of these biotechnology compounds have stimulated much research to deliver these drugs by the oral route, by the nasal route, by inhalation, or in a sustained-release fashion. Unfortunately, only a small subset of peptide-based therapeutics has been marketed through noninvasive routes (e.g., cyclosporine, insulin, calcitonin, desmopressin). This is primarily a function of their low molecular weights and small dosing requirements (Brown 2005).

22.5.1 Chemical Excipients to Alter Pharmacokinetics of Proteins

The most successful approaches for increasing the biological and pharmacodynamic half-lives of therapeutic proteins have been the use of chemical excipients. The use of ionic complex formation, covalent pegylation or glycosylation, and amino acid alterations of proteins has resulted in several successful FDA-approved products. Chemical excipient biomaterials that are used to enhance the half-life of therapeutic compounds include both naturally occurring and synthetic compounds.

22.5.1.1 Ion Complex Formation

Many significant advances in protein drug delivery systems have been accomplished using insulin as a model. This is a consequence of insulin being the first pure protein therapeutic molecule discovered and that diabetes affects an increasing percentage of the world's population. Therefore, the discovery of insulin by Frederick Banting and Charles Best in 1922 is naturally associated with the beginning of research aimed at finding optimal means to administer this exogenous protein to patients (Banting and Best 1922) and altering its delivery profile.

Insulin complexes came into use 15 years after its discovery. Hagedorn showed that insulin combined with the basic protein protamine derived from salmon sperm resulted in continuous insulin action for 3–12 h (Hagedorn et al. 1936). Protamines are small arginine-rich peptides that are positively charged. When combined with insulin, they shift the isoelectric point of insulin to physiologic pH. This lowers its solubility, forming a subcutaneous depot after injection and significantly extends its half-life. Hallas-Moller showed an unique interaction of proteins with metal ions in 1952 when he developed the Lente insulin form by adding zinc to insulin in an acetate buffer which also effectively decreased insulin's solubility (Hallas-Moller et al. 1952). The primary chemical and amino acid structure of insulin remains unaltered in both these examples of ion complex formation.

22.5.1.2 Pegylation

There are several FDA-approved therapeutic proteins whose pharmacokinetic properties improved and

Table 22.1 Biomaterial–drug combinations

Biomaterial/drug attachment	Drug	Purpose	Benefits/adverse reactions	Reference
PLGA/physical	Leuprolide, goserelin	Prostate cancer	Increased patient compliance/lower dose, injection site pain	Knobil (1990), Okada (1997)
PLGA/physical	Octreotide (Sandostatin)	Acromegaly	Increased patient compliance/injection site pain	Scarpignato and Pelonsini (2001), Anthony and Freda (2009)
PLGA/physical	Exenatide	Type II diabetes	Increased patient compliance/injection site pain, pancreatitis, gastrointestinal pain, hypoglycemia	Bunck et al. (2009), Bydureon
PLGA/physical	hGH	Pediatric growth hormone deficiency	Increased patient compliance/injection site pain, nodules, erythema, bruising, itching	Nutropin Depot Package Insert (1999)
Protamine/ionic	Insulin	Diabetes	Extended-release basal insulin/hypoglycemia	Hagedorn et al. (1936)
Zinc/ionic	Insulin	Diabetes	Extended-release basal insulin/hypoglycemia	Hallas-Moller et al. (1952)
Polyethylene glycol (PEG)/covalent	Enzyme adenosine deaminase	Severe combined immunodeficiency disease	Better efficacy, longer half-life/allergies, bleeding, dizziness	Adagen® Package Insert, Enzon Pharmaceuticals (2008)
Polyethylene glycol (PEG)/covalent	Interferon	Hepatitis C, cirrhosis, bridging fibrosis	Better efficacy, longer half-life/immune reaction	Avramis and Panosyan (2005)
Polyethylene glycol (PEG)/covalent	hGH	Acromegaly	Better efficacy, longer half-life/immune reaction	Schreiber et al. (2007)
Polyethylene glycol (PEG)/covalent	Filgrastim	Febrile neutropenia infection	Better efficacy, longer half-life/immune reaction, bone ache, bleeding, injection site redness	Neulasta® Package Insert, Amgen, Inc. (2002)
Glycosylation/covalent	Erythropoietin	Anemia	Better efficacy, longer half-life/immune reaction, cardiovascular problems	Macdougall et al. (2007)
Fatty acid/covalent	GLP-1 receptor agonist	Type II diabetes	Increased patient compliance/injection site pain, pancreatitis, gastrointestinal pain, hypoglycemia	Watson et al. (2010)
Fatty acid/covalent	Insulin	Diabetes	Sustained release/injection site redness, hypoglycemia, allergic reaction	(Levemir® Insulin Detemir (rDNA) Injection, Package Insert Novo Nordisk)
Amino acid substitutions/ covalent	Insulin	Diabetes	Faster onset or longer half-life/injection site redness, hypoglycemia, allergic reaction	DeFelippis et al. (1988), Home et al. (1999), Dailey et al. (2004), Gillies et al. (2000), Luzio et al. (2003)
Drug-eluting stents, stainless steel, cobalt-chromium-nickel-molybdenum alloy, tantalum, nitinol, EVAc, PBMA/physical	Paclitaxel, sirolimus (rapamycin), zotarolimus, everolimus	Prevention of restenosis following angioplasty	Longer interval between coronary blockage/bleeding, allergic reaction, myocardial infarction, stent thrombosis	Morice et al. (2007)
EVA copolymer, PVA/physical	Ganciclovir	Cytomegalovirus retinitis	Extended half-life of drug/visual acuity loss, hemorrhage, retinal detachment, cataracts	Vitrasert Package Insert, Bausch and Lomb (2003)
Silicone, PVA/physical	Fluocinolone acetonide	Posterior uveitis	Extended half-life of drug/increase intraocular pressure, glaucoma, optic nerve damage, visual acuity field defects, cataract formation, infection	Retisert Package Insert, Bausch and Lomb (2009)

extended by formation of covalent polyethylene glycol (PEG) conjugates with the drug (Alconcel et al. 2011; Mehvar 2000). The most common chemical approach has been to activate the PEG with functional groups suitable for reaction with lysine and N-terminal amino groups (Kozlowski and Harris 2001). Pegylation of a protein generally masks the protein's surface. This effectively increases the protein's molecular size, reduces renal ultrafiltration, inhibits antibodies or antigen processing cells, and reduces degradation by proteolytic enzymes. In vivo circulation lifetimes are increased, but the specific activity of the compounds may be compromised. Thus, the protein's distribution is significantly altered. From a chemistry viewpoint, pegylating a protein can result in variations in the number of PEG chains bound to the protein. The protein may then have increased polydispersity with regard to the molecular weight of the newly formed molecule. There may also be difficulties in determining the exact sites of conjugation in polypeptides (Veronese 2001; Overbay and Manley 2002).

There are many approved pegylated proteins currently in clinical use (Brown 2005). The first FDA-approved pegylated product was Enzon's Adagen®. It is a PEG-modified version of the bovine enzyme adenosine deaminase (ADA). It is used to treat ADA, commonly known as the "bubble boy disease." Another Enzon product called Oncaspar® is a PEG-modified version of the enzyme L-asparaginase used as a chemotherapeutic agent for acute lymphoblastic leukemia (Avramis and Panosyan 2005; Mehvar 2000).

Clinical studies examined the efficacy and safety of Genentech's Pegasys® peginterferon-**α**2a in patients with hepatitis C-related cirrhosis or bridging fibrosis. The studies showed that 180 μg of peginterferon-**α**2a administered once weekly was significantly more effective than 3 million units of standard interferon-**α**2a administered three times weekly (Heathcote et al. 2000; Heathcote et al. 2000). Merck's PEG-Intron®, peginterferon-**α**2b, is also indicated for the treatment of chronic hepatitis C. Pfizer's pegvisomant (Somavert®) is a pegylated hGH receptor antagonist used in the treatment of acromegaly (Schreiber et al. 2007). Amgen's pegfilgrastim (Neulasta®) is used to decrease the incidence of infection, manifested by febrile neutropenia.

22.5.1.3 Glycosylation

Darbepoetin-α (Aranesp®) is an erythropoiesis-stimulating protein similar to recombinant human erythropoietin. It is for the treatment of chronic renal disease-induced anemia or in patients with anemia caused by chemotherapy (Overbay and Manley 2002). It is produced in Chinese hamster ovary cells by recombinant DNA technology. It differs from human erythropoietin by the addition of two N-linked oligosaccharide chains. These two additional sites result from amino acid substitutions in the peptide backbone, which do not interfere with receptor binding. Darbepoetin-α has also has 22 sialic acid residues compared to 14 sialic acid residues in human erythropoietin. The carbohydrate chains increase the molecular weight of this glycoprotein from approximately 30,000 Da for human erythropoietin to 37,000 Da for darbepoetin-α. It has a threefold longer terminal half-life in humans than erythropoietin, leading to a decrease in frequency of administration and greater patient compliance (Macdougall et al. 2007).

22.5.1.4 Covalent Attachment of Long-Chain Fatty Acids

Liraglutide is an analogue of human GLP-1 that acts as a GLP-1 receptor agonist to stimulate insulin release from pancreatic beta cells. As a result, it is injected once daily for the treatment of type II diabetes (Watson et al. 2010). Clinical studies have confirmed reductions in HbA1c, low risk of hypoglycemia, and reductions in body weight and systolic blood pressure. Liraglutide differs from native GLP-1 by replacing arginine_{34} with a lysine residue. In addition, a C-16 fatty acid (palmitic acid) is covalently attached with a glutamic acid spacer on the lysine residue at position 26 of the peptide. This structural alteration increases the drug's half-life from 1.5 min to 13 h resulting in a once-daily injectable formulation for this 3,751-Da peptide.

Insulin detemir is a long-acting analogue of human insulin. This formulation has a covalently attached fatty acid to the molecule in order to increase its biological half-life. Insulin detemir (Levemir®) differs from human insulin in that the B-chain amino acid threonine in position B30 has been removed and C14-myristic fatty acid chain has been attached to the lysine at amino acid position B29 (Levemir® Insulin Detemir (rDNA) Injection, Package Insert Novo Nordisk). The 24-h prolonged action of this insulin

analogue is mediated by the slow systemic absorption of insulin detemir molecules from the injection site. The slow absorption is due to strong self-association of the drug molecules and albumin binding. Insulin detemir is distributed more slowly to peripheral target tissues since insulin detemir in the bloodstream is highly bound to albumin.

22.5.1.5 Amino Acid Substitutions

Several novel insulin formulations have been developed that dramatically affect insulin's pharmacokinetic properties. This has been accomplished by substituting amino acids in the primary structure of the protein in a manner that does not change the biological activity of the molecule. These amino acid substitutions can increase or decrease insulin pharmacokinetics.

22.5.1.6 Rapid-Onset Insulins

Insulin is composed of two polypeptide chains: A and B. Chain A consists of 21 amino acids and chain B consists of 30 amino acids. In human insulin, amino acids 28 and 29 on the B-chain are proline_{28} and lysine_{29}, respectively. Eli Lilly's Lyspro Humalog® insulin decreases the onset of action of insulin by a factor of 2 compared with insulin's native structure. In this insulin formulation, two amino acids have been reversed from native human insulin so that lysine appears in position 28 and proline is found at position 29 (DeFelippis et al. 1988; Home et al. 1999). This form of insulin favors the more soluble monomeric structure, and hence, it diffuses from the injection site more rapidly to control postprandial glucose compared with regular human insulin. Novo Nordisk's Novolog insulin-aspart analogue accomplishes the same rapid onset-of-action effect as the proline_{28} lysine_{29}-insulin by simply substituting the proline_{28} with aspartic acid in the same position of the B-chain (Home et al. 1999). Sanofi-Aventis' Apidra insulin glulisine is another rapid-acting human insulin analogue produced by recombinant DNA technology utilizing a nonpathogenic laboratory strain of *Escherichia coli* (K12) (Dailey et al. 2004). Insulin glulisine differs from human insulin in that the B-chain amino acids asparagine_3 is replaced by lysine and the lysine_{29} on position B29 is replaced by glutamic acid.

Each of these rapid-acting insulins is designed to benefit diabetic patient care by helping to maintain postprandial blood glucose close to the normal.

22.5.1.7 Slow-Release Insulin

Other insulin primary structure substitutions are designed to increase the biological half-life of insulin. Sanofi-Aventis' Lantus insulin glargine is a 24-h, long-acting recombinant insulin analogue. Structurally, the A-chain asparagine_{21} amino acid is replaced with a glycine and two arginine amino acids are added to the C-terminus of the B-chain (Gillies et al. 2000). This insulin analogue is injected in aqueous solution at pH 4. On subcutaneous injection, the insulin glargine precipitates at physiological pH and forms a slow-dissolving depot of hexameric insulin. Studies have shown a relatively constant 24-h concentration profile, with no pronounced insulin peak (Luzio et al. 2003). This profile effectively mimics physiological basal insulin release.

Thus, chemical alterations of existing proteins have resulted in dosage forms with significantly altered pharmacokinetics compared with their native molecules. These methods are not panaceas, for pegylation and amino acid substitutions can alter the biological activity of the molecules, their toxicities, or their bioavailabilities.

22.6 Biodegradable Sustained-Release Delivery Systems

Poly-lactide-glycolide (PLGA) is a biodegradable copolymer that has been used by most companies and investigators as a matrix for the sustained release of drugs. The rationale for its safe use is based on decades of extensive experience using the polymer in bioerodible sutures and studies showing its biocompatibility (Bourges et al. 2006). PLGA hydrolyzes to degrade into lactic acid and glycolic acid. Lactic acid is then oxidized to pyruvic acid and is fully metabolized by the tricarboxylic acid cycle. Lactate can also be converted to glucose in the liver. Glycolic acid is metabolized by glycolic acid dehydrogenase in the liver to oxalic acid (Fry and Richardson 1979). Thus, the degradation products of this polymer are readily metabolized as natural products and eliminated by the organism.

A key consideration for all polymer matrix-based delivery systems is the selection of compounds with wide therapeutic indices. This is a direct result of the difficulty in producing commercially viable, injectable

delivery systems that do not result in an initial "burst" of drug release within the first few hours after administration (Brown et al. 1986). Therefore, drugs must be effective at very low doses and with minimal side effects at very high doses.

The maintenance of the peptide or protein drug's chemical stability while releasing at physiological temperature and pH is a major concern in order to bring stable, reliable, and reproducible products to market.

22.6.1 LHRH Sustained-Release Matrices

The first marketed sustained-release products incorporated with luteinizing hormone-releasing hormone (LHRH) agonists into PLGA implants or microspheres were for the treatment of prostate cancer. LHRH is also known as gonadotropin-releasing hormone (GnRH). LHRH is a relatively low-molecular-weight 10-amino-acid-length peptide of approximately 1,100 Da. LHRH is normally secreted in pulses; however, sustained release of LHRH has been shown to inhibit the secretion of steroid hormones (Knobil 1990). Prostate cancer is a steroid-dependent tumor; therefore, blocking the release of testosterone results in shrinkage of the prostate tumor. Initially, 1 mg daily injections were used to deliver this peptide to shrink prostate tumors. The first PLGA product was a relatively large 1.5-mm subcutaneous implant containing an LHRH analogue called goserelin (Zoladex®) which released the drug for 1 month. The drug–device combination is delivered subcutaneously through a 14-gauge or 16-gauge needle. Pain occurring at the injection site has been associated with this relatively large needle used to inject the implant (R&D Directorate Report No. 54 1996). PLGA microspheres that were injectable through 20-gauge or smaller-bore needles were developed containing the leuprolide analogue of LHRH (Lupron Depot®) (Okada 1997). From a safety viewpoint, it is important to note that sustained release of LHRH agonists is a classic example of how total drug administered may be markedly reduced compared to daily injection. As described previously, 1 mg was originally administered in the daily dosage form. Sustained-release dosage forms of this peptide reduced the leuprolide dose four- to eightfold. Thus, the patient is exposed to significantly less systemic drug by virtue of the depot form compared to the daily injectable form. LHRH and its analogues have a very wide therapeutic index. They are effective in inhibiting steroid release at relatively small doses, and a toxic upper dose limit has never been identified. Thus, these LHRH compounds are ideally suited to sustained-release dosing from a safety and therapeutic perspective.

22.6.2 Octreotide

Another PLGA peptide sustained-release delivery system is Sandostatin LAR (long-acting release) (Novartis Pharmaceuticals) for the treatment of acromegaly. This delivery system releases octreotide acetate from a PLGA microsphere depot. Octreotide acetate is a long-acting peptide with pharmacological actions mimicking those of the somatostatin. Octreotide LAR is administered by injection into the gluteal muscle every 28 days. Local side effects of octreotide include pain, stinging, or burning at the injection site and occur in approximately 28% patients treated with octreotide LAR (Lancranjan et al. 1996).

These small therapeutic peptides all lack the three-dimensional structure of larger proteins. Therefore, these peptides are not very susceptible to denaturation and chemical degradation and are therefore less likely to induce an immunogenic response. These peptides also have fairly wide therapeutic indices (Anthony and Freda 2009). They have low toxicity potential even during the initial burst of drug release after drug administration (Scarpignato and Pelonsini 2001; Warner et al. 1983). These safety considerations plus their therapeutic benefit in treating conditions such as prostate cancer support their widespread use as a sustained-release biomaterial–drug combination.

22.6.3 Exenatide

Exenatide is a 39-amino-acid peptide possessing glucoregulatory activity. Presently, it is injected twice daily for the treatment of type II diabetes (Bunck et al. 2009). This 4,187-Da synthetic hormone was discovered in the saliva of the Gila monster. The incretin GLP-1 hormone stimulates insulin secretion from the beta cells of the islets of Langerhans in the pancreas. A PLGA-based microsphere has been developed and has recently been granted marketing authorization by the European Commission under the name

Bydureon (exenatide once weekly) for the treatment of type II diabetes. FDA approval in the United States had been delayed due to undisclosed cardiac safety, but was approved in 2012.

Therefore, from an extended-release drug delivery point of view, we see that the polymeric-based sustained-release technology has generally been limited to low-molecular-weight peptides rather than large therapeutic protein molecules.

22.6.4 Human Growth Hormone

A notable exception to the restriction of PLGA sustained-release delivery systems for small peptides was the Nutropin Depot® product developed by Alkermes and Genentech. This PLGA-based microsphere product provided sustained release of human growth hormone over a 2- or 4-week period from a single injection (Nutropin Depot®, Genentech, Inc. 2002). Human growth hormone (hGH) is a 191-amino-acid protein with a molecular weight of 22,125 Da. The depot product was approved in 1999 by the US FDA as a treatment for growth hormone deficiency in pediatric patients. In June 2004, Genentech and Alkermes announced their decision to discontinue commercialization of Nutropin Depot. The companies stated that their decision was based on the significant resources required by both companies to continue manufacturing and commercializing the product. The adverse event profile of the Nutropin Depot product showed several potential side effects when administering the PLGA dosage form to children. In studies involving 138 pediatric patients treated with Nutropin Depot, the most frequent adverse reactions were injection-site reactions, which occurred in nearly all patients (Nutropin Depot®, Genentech, Inc. 2002). On average, two to three injection-site adverse reactions were reported per injection. These site of injection reactions included nodules (61% of injections), erythema (53%), pain postinjection (47%), pain during injection (43%), bruising (20%), itching (13%), lipoatrophy (13%), and swelling or puffiness (8%). The intensity of these reactions was generally rated mild to moderate, with pain during injection occasionally rated as severe (7%). The discontinuation of this novel product is further evidence of the complexity of developing viable sustained-release delivery systems for high-molecular-weight protein molecules.

The Nutropin Depot was also plagued with a dosage form that used a comparatively large 21-gauge needle for injecting pediatric patients and had a volume of injection that could be as large as 1.2 mL. This competed with a relatively pain-free daily dosage form that uses a very small 30-gauge needle. Thus, PLGA-based systems for protein-based therapeutics still remain limited and suboptimal. A single sustained-release delivery platform for all proteins remains elusive despite many attempts.

22.7 Site-Directed Local Delivery of Drugs from Medical Devices

All the previous examples describe systemic distribution of a drug from chemically altered compounds, PLGA implants, and microspheres. There are also conditions where the maintenance of local high concentrations of drug is necessary. In these cases, the goal is to obtain the appropriate local therapeutic affect without exposing the patient to high concentrations of drugs systemically. This section reviews three examples of local delivery: drug-eluting coronary stents, intravitreal delivery of ophthalmic medications, and the cell-targeting challenges of nucleic acids.

22.7.1 Drug-Eluting Stents

Drug-eluting stents represent one of the most intensely studied biomaterial drug delivery system. Coronary balloon angioplasty often results in occlusion and restenosis of the artery. Stainless steel bare metal stents were then inserted to act as an intravascular mechanical support to prevent occlusion of the coronary artery following balloon angioplasty (Sigwart et al. 1987). Several trials in the 1990s showed the superiority of stent placement over balloon angioplasty. Initially, restenosis was reduced because the stent acted as a scaffold to hold open the dilated segment of artery. Unfortunately, over time, coronary stents remained vulnerable to restenosis. Restenosis was usually caused by neointimal tissue growth. Investigators attempted to inject drugs at the time of the stent insertion. Unfortunately, the local concentration of these drugs could not be maintained within the coronary artery and restenosis occurred. Hwang et al. showed in 2001 that the continuous release of drugs from

a coated stent could achieve local high concentrations (Hwang et al. 2001). Drugs like rapamycin were shown to inhibit neointimal growth within the artery. As a result, rapamycin was coated onto stainless steel stents in an ethylene vinyl acetate copolymer and poly *n*-butyl methacrylate matrix. Drugs such as paclitaxel were also introduced onto coronary stents for clinical use. These drug-eluting stents were shown to be superior to bare metal stents 5 years after implantation (Morice et al. 2007). There are now new concerns that after extended time periods within the artery, fully released stents again put patients at risk for the formation of a clot within the stent. Therefore, new drug-eluting biodegradable stents fabricated from polylactic acid are currently under development to address stent thrombosis (Tsuji et al. 2003).

The evolving complexity of drug-eluting stents requires numerous tests to account for assessment of the composition, dimensions, and functional attributes of the stents. A series of tests are recommended to characterize the drug delivery properties of the stents (Center for Devices and Radiological Health 2010). The FDA has therefore issued guidance describing the nonclinical engineering tests and recommended labeling for intravascular stents and associated delivery systems (Center for Devices and Radiological Health 2010).

22.7.2 Intravitreal Drug Delivery Devices

Topical eyedrops are the most common mode of ophthalmic drug delivery. However, this route severely limits the dose, the class of drug, and the molecular weight of the drug that can be administered to treat posterior eye disease such as age-related macular degeneration (AMD). New protein-based drugs such as Lucentis (ranibizumab) and EYLEA (aflibercept ophthalmic solution) require intravitreal injection (Fung et al. 2007). These drugs target and bind vascular endothelial growth factor (VEGF) that is involved in stimulating abnormal blood vessel growth in those affected by AMD. These drugs must be injected monthly or bimonthly, respectively. There are practical limits to the number of intravitreal injections that can be administered through the eye. In general, a 27–30-gauge needle is used for intravitreal injections. Therefore, there are numerous efforts underway to develop sustained-release systems such as implants or microspheres to extend the half-life of for these ophthalmic drugs.

Biocompatibility of either nonerodible or degradable systems require that all components be chemically inert, noncarcinogenic, hypoallergenic, and mechanically stable at the implantation site (Choonara et al. 2010). Several intravitreal sustained-release implants have been approved for the delivery of drugs such as fluocinolone acetonide over a 2.5-year period to treat uveitis (Retisert Package Insert, Bausch and Lomb 2009) and ganciclovir (Vitrasert Package Insert, Bausch and Lomb 2003) for the treatment of cytomegalovirus retinitis. These products require surgical implantation into the eye. There are several untoward side effects associated with these implants including retinal detachment, glaucoma, and the need for cataract surgery (Bourges et al. 2006; Choonara et al. 2010). This has limited their use to ophthalmic applications with no alternative therapies.

22.7.3 Nucleic Acids

The discovery of small interfering RNA (siRNA) opened the possibility of specifically downregulating excess protein expression involved in various diseases (Fire et al. 1998). The delivery of nucleic acid therapeutics presents several unique technological and safety hurdles for the pharmaceutical industry. Diseases such as hypercholesterolemia, liver cancer, respiratory syncytial virus infection, kidney injury, age-related macular degeneration, and Duchenne muscular dystrophy have been targeted by numerous companies and research groups. The challenge for delivering nucleic acids is that these molecules must be delivered into the cell rather than interacting with receptors on the cell surface. It is chemically and physically challenging to target specific cells and deliver 13,300-Da-sized double-stranded nucleotides into a cell (Zabner et al. 1995).

Cationic lipids have been used to form ionic complexes with siRNA and antisense oligonucleotide therapeutics in order to facilitate delivery across cell walls (Lv et al. 2006). There is little to no effect on organ function or tissue architecture at low doses. However, acute inflammation and significant tissue damage often occurs at higher doses. Intravenous

administration tends to cause more severe adverse effects and can be lethal at higher doses of the complex (Yew and Scheule 2005). Therefore, at present, most leading candidate siRNA drugs are focused on targets such as kidney and liver diseases where systemic clearance naturally concentrates the drug in those organs.

22.7.4 Tissue Engineering of Cell-Based Structures

The combinations of natural and synthetic scaffolding to allow the growth of new tissue for body structure and functional implants are an important and exciting new field of combination biomaterials. An early experimental example of tissue engineering demonstrated the growth of chondrocyte cells on a human ear-shaped PLGA polymer template (Yilin et al. 1997). This implant was successfully implanted on the dorsa of athymic mice. It is important to note that in these tissue engineering procedures, autologous cells are harvested from the subject. Then the patient's own cells are seeded and grown on a sterile template in vitro. Then after several days time such that enough cell proliferation has occurred on the scaffold, the tissue-engineered implant is implanted into the patient.

Elle-Behnke has demonstrated the ability to restore sight in an animal model using a novel peptide-based nanofiber hydrogel scaffold (Ellis-Behnke et al. 2006). The scaffold consisted of a self-assembling beta-sheet nanofibril approximately 10 nm in diameter that contains 99% water (Zhang 2003). Elle-Behnke showed that the treatment with synthetic biological materials solution enabled reconnection of brain tissue after acute injury in hamsters.

Early laboratory successes with this technology have prompted several notable clinical applications. Myelomeningocele is a birth defect in which the backbone and spinal canal do not close before birth. High pressure or poorly compliant bladders are a common complication of this birth defect. A study in 2006 showed that bladder repair and reconstruction was possible by conducting a biopsy to collect urothelial and muscle cells from these patients. These cells were then grown in tissue culture and seeded onto a collagen and PLGA bladder-shaped scaffold. These autologous cell-based artificial bladders were then implanted into the patients. Four-year postsurgery follow-up showed improved bladder function in all patients who received these tissue-engineered bladder tissues (Atala et al. 2006).

Recently, a report from the Karolinska University Hospital in Sweden described the implantation of a synthetic trachea into a patient stricken with tracheal cancer (Naik 2011). A scaffold formed from the biodegradable PLGA polymer was impregnated with the patient's own stem cells over a 2-day period. The synthetic trachea was surgically implanted into the patient with the stem cells directed to differentiate as normal trachea cells. About 48 h after the transplant, imaging and other studies showed cells in the process of populating the artificial windpipe, which had begun to function like a natural windpipe. There was no rejection by the patient's immune system, because the cells used to seed the artificial windpipe came from the patient's own body. The patient no longer has cancer and is expected to have a normal life expectancy. Previously, this laboratory at the Karolinska University hospital had conducted this procedure using cadaver tracheas.

22.8 Conclusions

This chapter has presented the interface of biomaterials with the delivery of biotechnology-based drugs to man. This is an ever-evolving field that remains very dependent on the role of the original needle and syringe first developed by investigators and physicians in the 1800s. Today, injection needles and devices are designed to be minimally invasive, sterile, and disposable. Thus, they are insuring the safe pharmacological delivery of these new drugs to man. The use of biodegradable polymers such as PLGA for delivery of proteins and peptides is very limited by the inherent biological properties of individual drugs and the nature of the diseases that they are treating. Therefore, these important developments cannot yet be termed "platform technologies" applicable to every drug, peptide, protein, or nucleic acid. Each compound must be developed and tested on its own as if the drug–biomaterial combination was itself a new chemical entity. The recent advances in tissue engineering are among the most exciting developments in the field of biomaterials. However, at present, it is clear that each tissue engineering procedure will be highly individualized.

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Nanotechnology and Safety Pharmacology 23

A. Michael Bloh

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23.1 Introduction

The advent of nanotechnology is considered to be the biggest engineering innovation since the Industrial Revolution. Proponents of this new technology promise to reengineer the man-made world, molecule by molecule, sparking a wave of novel revolutionary commercial products from machines to medicine This "industrial revolution" in molecular manufacturing will alter the relationship of materials so profoundly that this change may produce both positive and negative effects on health and the environment. The worldwide market for products produced using nanotechnology is estimated to reach US $1 trillion by 2015 (Gwinn and Vallyathan 2006a).

Study in nanotechnology began in the 1900s, expanded slowly, then reached extensive growth in the later twentieth and early twenty-first centuries. Nanotechnology has expanded into many fields including, but not limited to, physics, chemistry, energy, agriculture, electronics, and medicine (Wikipedia 2011a). Figure 23.1 shows some products already impacted by nanotechnology (Whiteley and Hawxhurst 2006). A list of the numerous consumer products impacted by nanotechnology is maintained by The Project on Emerging Nanotechnologies (www.nanotechproject.org/inventories/consumer/). However, this list should not be considered complete because of the rapid growth of the industry and the evolving nature of the commercial definition of "nano."

Because of the many facets of nanotechnology, there are several definitions that are application dependent. A common definition is the deliberate manipulation of matter in the structural size range of 1–100 nm. A nanometer is one-billionth of a meter or 1/100,000th of a millimeter (National Health Laboratory Services 2011; National Nanotechnology Initiative 2011; US Centers for Disease control (CDC) 2009a). Figures 23.2 and 23.3 provide some comparative perspective on nanomaterials (McNeil 2005; Yokel and MacPhail 2011).

A full review of the complex and growing topic of nanotechnology and its implications is beyond the scope of this chapter. This discussion will be limited to an overview of engineered (deliberately created) nanotechnology used in medical devices and diagnostics and medications intended for human use, and their implications for drug safety, pharmacovigilance, and risk management. The discussion would not be complete without some comment on the environmental impacts of these products and an overview of their regulation. Because of the rapid growth of nanotechnology, it is strongly suggested that readers thoroughly research their specific areas of interest using the information in this chapter as a starting point (see Sect. 23.12 listed at the end of the chapter).

Fig. 23.1 Illustration of some products impacted by nanotechnology (Whiteley and Hawxhurst 2006)

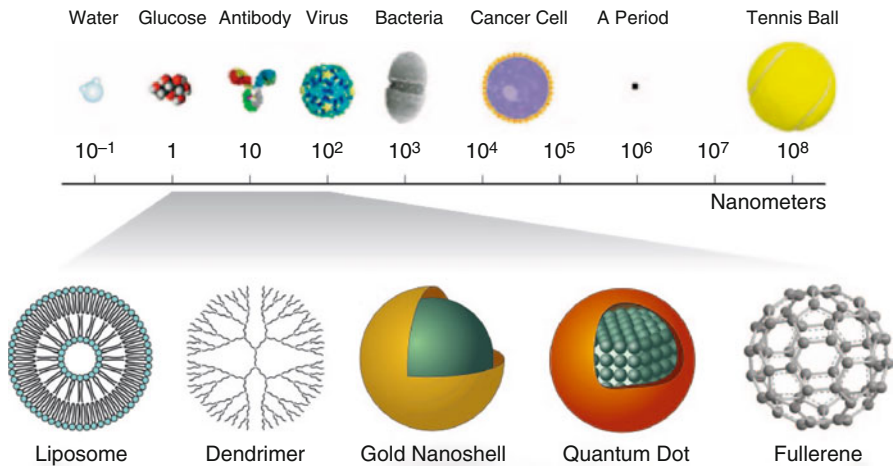


Fig. 23.2 Relative size of nanoparticles compared with familiar items (McNeil 2005)

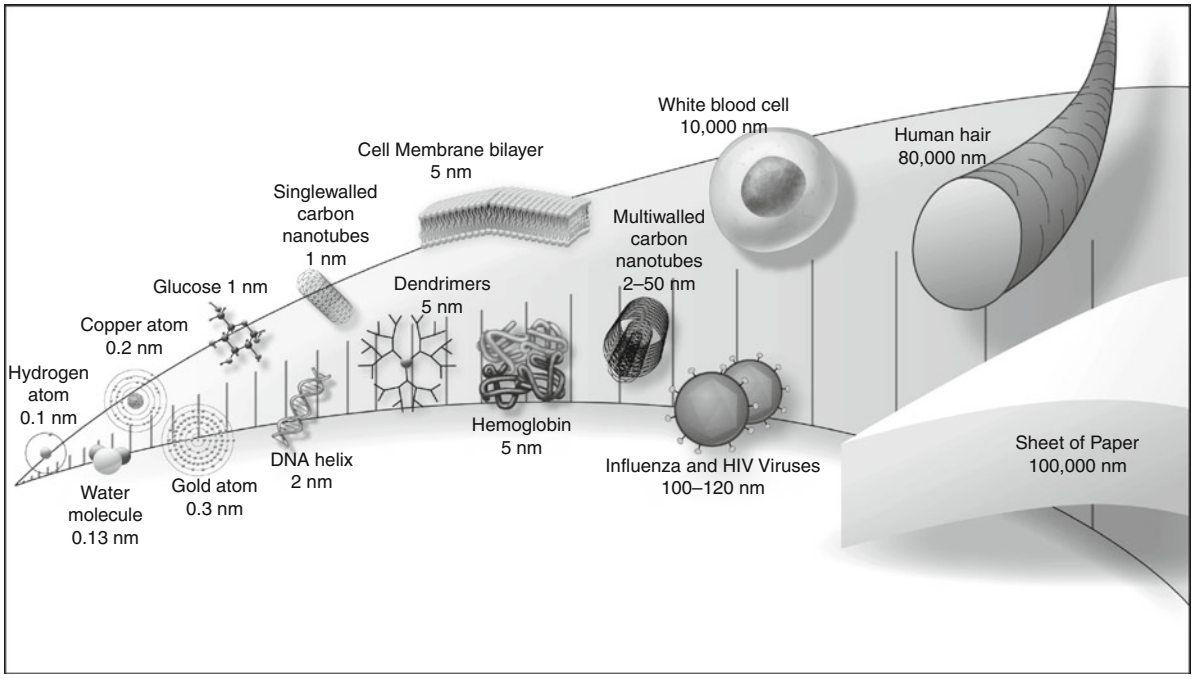


Fig. 23.3 The sizes and shapes of some nanomaterials as compared to more familiar materials. Shown for comparison are materials that are below, within, and above the nanoscale range, to put nanomaterial size in perspective (Yokel and MacPhail 2011)

23.2 Unique Properties of Nanomaterials

Because of their diminutive size, nanomaterials have an increased surface area; their physical, chemical, and biologic properties can unpredictably change and be different from their bulk counterparts. There are no product class distinctions with nanomaterials as of this writing. A 10 nm particle may have properties different from a 20 nm particle of the same material (Pautler and Brenner 2010a). For example, nanogold is being studied for cancer, antibiotic use, and its other unique nanoproperties (Fig. 23.4). Above 60 nm in particle size, gold retains its known properties. However, below this size

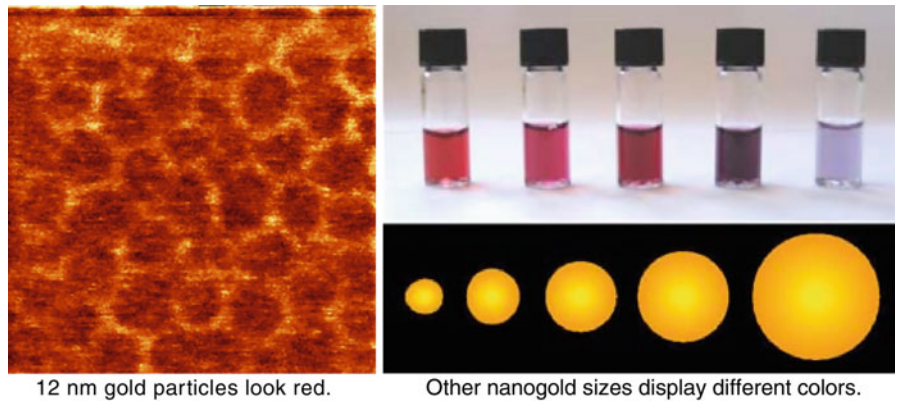


Fig. 23.4 Illustration of physical characteristics of nanogold (SRI International 2005)

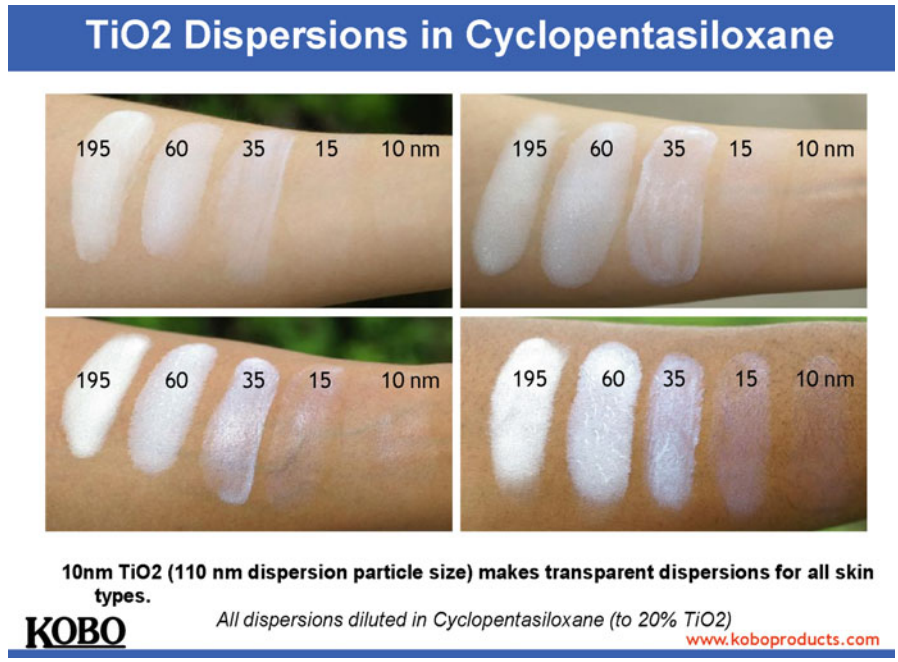


Fig. 23.5 Topical appearance of nano-titanium dioxide dispersions on different skin types (Schlossman et al. 2011)

nanogold's color changes from gold to red; its melting point and its reactive properties also change (Alanazi et al. 2010). As particle size decreases, optical properties of nanomaterials change. Titanium dioxide, a common ingredient in sun protection products, is opaque (white) in its macro form, becomes more transparent as particle size decreases, and ultimately becomes clear in appearance. Figures 23.5 and 23.6 illustrate some of these properties (Tzeng 2011; SRI International 2005; Schlossman et al. 2011). For drug delivery, engineered nanoparticles may be used as carriers; however, the drug itself may be formulated on a nanoscale and then function as its own carrier (DeJong and Borm 2008a).

23.3 Lack of Standardization for Toxicology

As of this writing, there are several dilemmas that confound the safety evaluations of nanomaterials, in addition to the differences in chemical identity and behavior among varying sizes of a specific nanomaterial mentioned above. A nanomaterial may have a variety of uses: the nanomaterial itself may be used as a medical treatment, as a carrier for a pharmacologic, biologic or diagnostic agent, or as a carrier for genetic material, in humans or in other species. Additionally, the same nanomaterial may be used in the manufacture of tires,

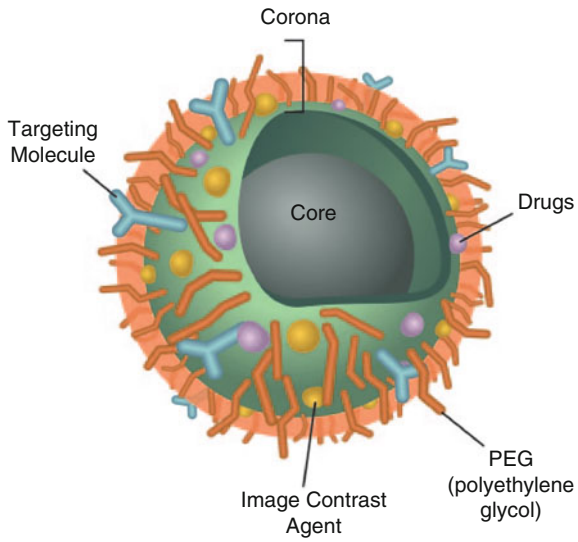


Fig. 23.6 Multifunctional nanoparticle. A nanoparticle's "corona" can be functionalized with hydrophilic polymers, targeting molecules, therapeutic drugs, and image contrast agents. The interior core can be solid (e.g., quantum dots) or liquid (e.g., liposomes). Molecules are not shown to scale. *PEG* polyethylene glycol (Harper et al. 2011)

paint, or automobile wax. It may have other applications in agriculture, electronics, or sporting equipment. Additionally, nanomaterial may not be used as an entity unto itself; it may be combined with other agents, for example, surface-active agents, that facilitate dispersal in solution. Targeting molecules may be added to the nanomaterial to facilitate biologic or chemical activity (Fig. 23.6). Figure 23.7 highlights some physical and chemical factors that can influence the biological effects of nanomaterials.

To compound the above, a lack of standardization of nanomaterials creates ambiguity in their safety assessment. Table 23.1 lists some of the issues that surround the standardization of nanotoxicology reported by Broverhof and David in 2007 (Broverhof and David 2010). In a review of nanomaterial standardization (or the lack thereof) as related to informatics, Thomas DG, et al. list the needs for standardization (Table 23.2) and report on the activities of several organizations devoted to the topic (Thomas et al. 2011). In an effort to demonstrate the importance of standardization in the bioevaluation of

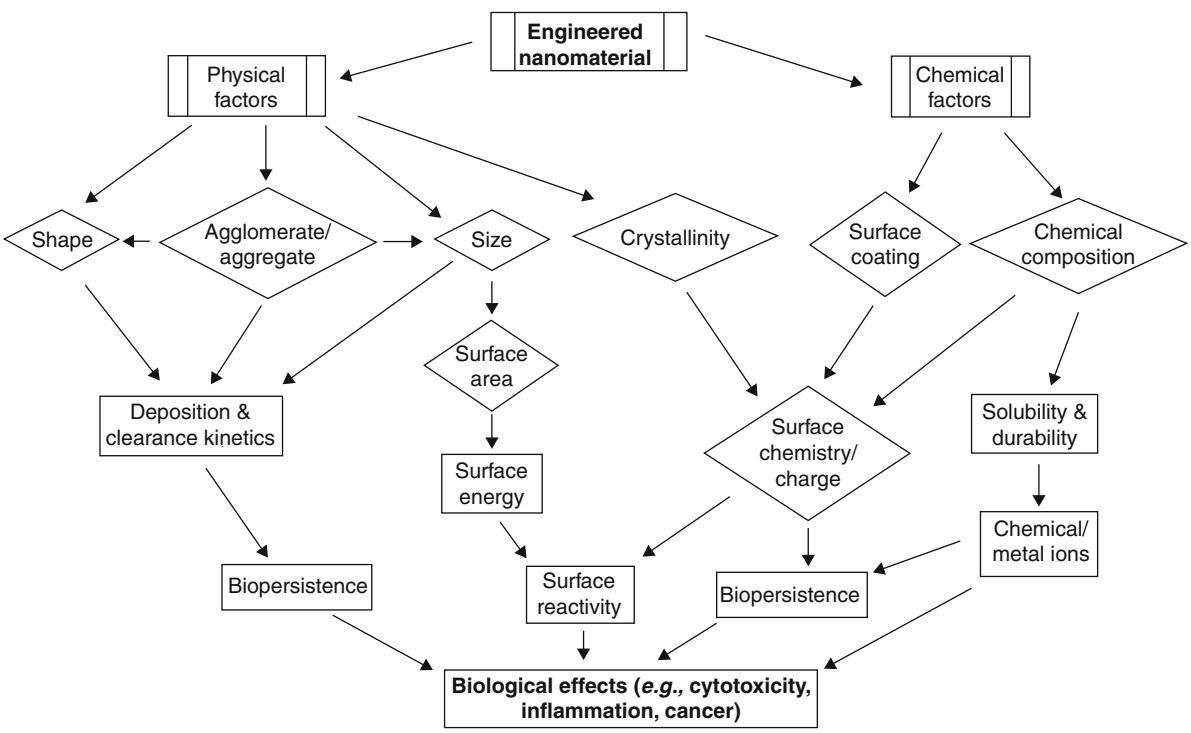


Fig. 23.7 Some physical and chemical factors that can influence the biological effects of nanomaterials (Lai 2011)

Table 23.1 Issues in nanotechnology characterization (Broverhof and David 2010; Lai 2011)

Engineered nanomaterials have unique characteristics than their macro counterparts
May or may not be water soluble
May behave differently (e.g., agglomeration) in a biologic medium (e.g., ionic buffers, protein, serum)
Existing testing strategies for chemicals do not always apply to nanomaterials
Nanoparticles consist of more than one molecule
Dissolution in aqueous medium may be poor
Crystalline structure, water insolubility, and slow dissolution can impact biologic systems differently
Composition, shape, and dimensions may play a role in toxicity assessments
May require new specific toxicology assessments to be developed
Traditional methods (e.g., sonication) may alter the nanomaterial structure and affect subsequent assessments
Dose (e.g., mg/kg) may not be the most appropriate metric for assessment
Dose assessments may be based on surface area, particle number, or other measure (e.g., hemolytic potential)
Laboratory to laboratory variation in culture medium preparation
Lack of characterization may lead to conflicting results
Surface coatings of nanomaterials may influence assessments
Nanomaterials may vary in their impact on different cell lines
Varying structure-activity relationships due to lack of characterization
Broad characterization may be impracticable, even overwhelming
Paucity of nanoparticle reference standards
Minimal knowledge of how known reference standards influence toxicologic techniques

Table 23.2 Rationale for standardization (Thomas et al. 2011)

Enable collaboration
Data sharing
Unambiguous representation and interpretation of data
Semantic (meaningful) search
Integration of data
Ensuring quality data
Reliability
Reproducibility
Nonstandardized data does not lend itself to computerized analysis or data mining
Nonstandardized information may confound protocol development

nanomaterials, Harper et al. exposed zebra fish embryos to nanogold particles of different sizes and varying charges. While uncharged nanogold particles appeared to have no detrimental effects, positively charged particles caused mortality and negatively charged nanogold induced malformations. Rigorous controls demonstrated that only the change in charge produced deleterious effects. Large differences in biologic response can occur as a result of small changes in nanomaterial properties (Harper et al. 2011). These concepts were underscored in a 2010 presentation by Geertsma who stated that because of the lack of standardization, nanomaterials must be evaluated on a case-by-case basis (Geertsma 2010). Toxicologists must be aware of all of the nuances of nanotechnology evaluations to deliver meaningful results.

In an effort to address much of the above, Lai proposed a paradigm in toxicologic testing of nanomaterials for moving forward into the future. He states that use of a "one size fits all" approach to testing nanoparticles ignores the complexity of the toxicity and mechanism of nanoscale materials. The proposed paradigm for testing and evaluating the health hazards of various classes/subclasses of nanomaterials uses reference materials, short-term in vivo studies in conjunction with high-throughput screenings, and class-specific low-throughput in vitro assays (Fig. 23.8).

23.4 Pharmacokinetics

Figure 23.9 illustrates various exposure types and biosystems that may be affected by nanoparticles and provides an excellent starting point for the pharmacokinetic discussion. Pharmacokinetic study is complicated by the issues that face the toxicology outlined above and the complexities of combining a therapeutic agent with a nanoproduct. Pharmacokinetic study in humans should include the therapeutic agent and the nanoproduct used and consider the issues raised by dose, metabolism, populations, ethnicity, other therapies, diet, disease states, and adverse events. Protein binding and tissue accumulations are other important pharmacokinetic factors. However, nano-pharmacokinetics is a discipline that is in its infancy. This section provides a starting point for researchers interested in the pharmacokinetics of nanomaterials. The pharmacokinetic

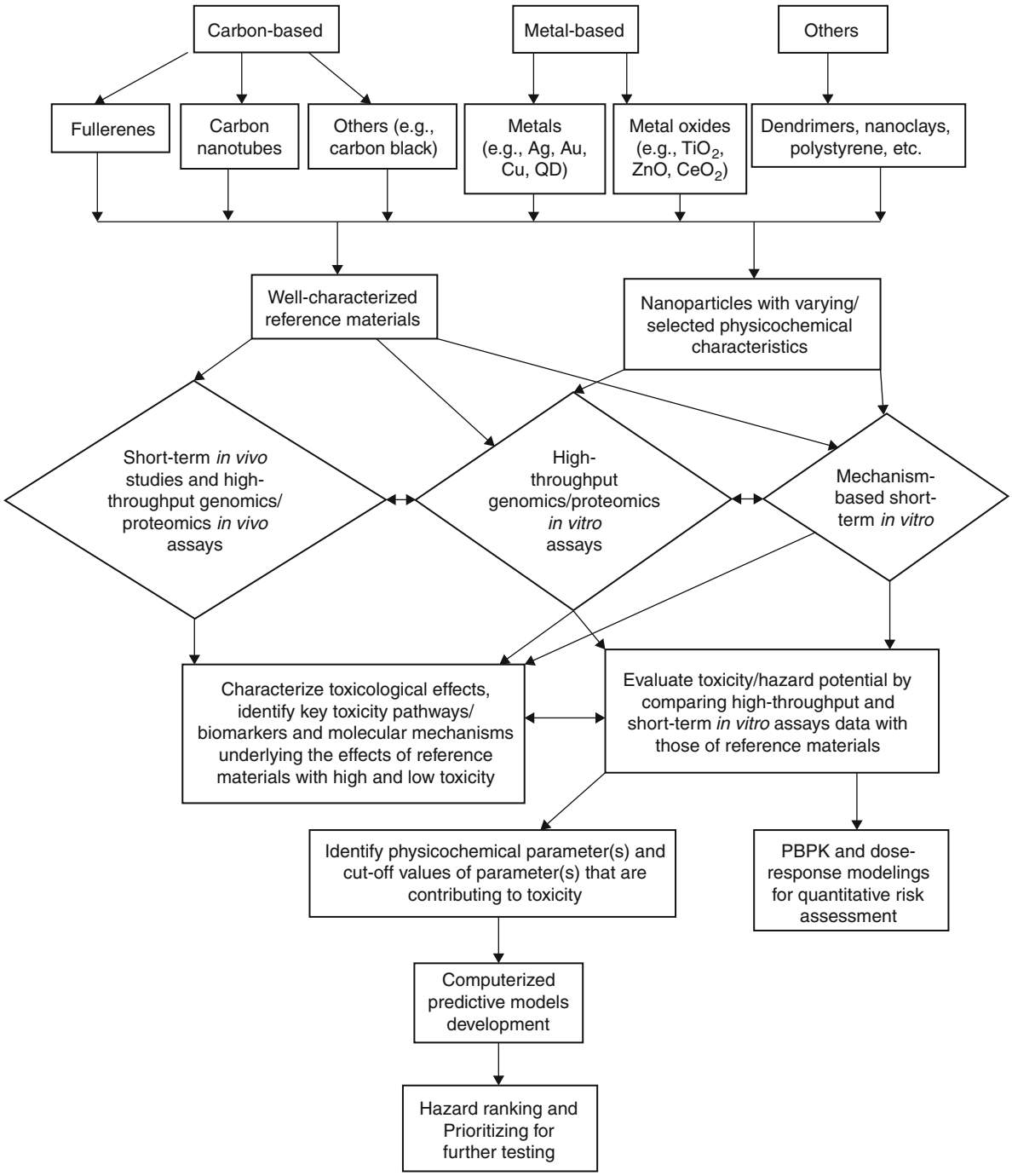


Fig. 23.8 Proposed paradigm for the toxicity testing of nanomaterials (Lai 2011)

principles of adsorption, distribution, metabolism, and elimination (ADME) are illustrated in Fig. 23.10. Riviere reported the pharmacokinetics of carbon nanotubes, fullerenes, and quantum dots following parenteral administration in rats. He reported that the pharmacokinetics of nanomaterials studied differ from those of traditional drug molecules. Decay in blood concentration may be related to the (nano)compound movement into tissues where further excretion does not occur (e.g., trapped in the

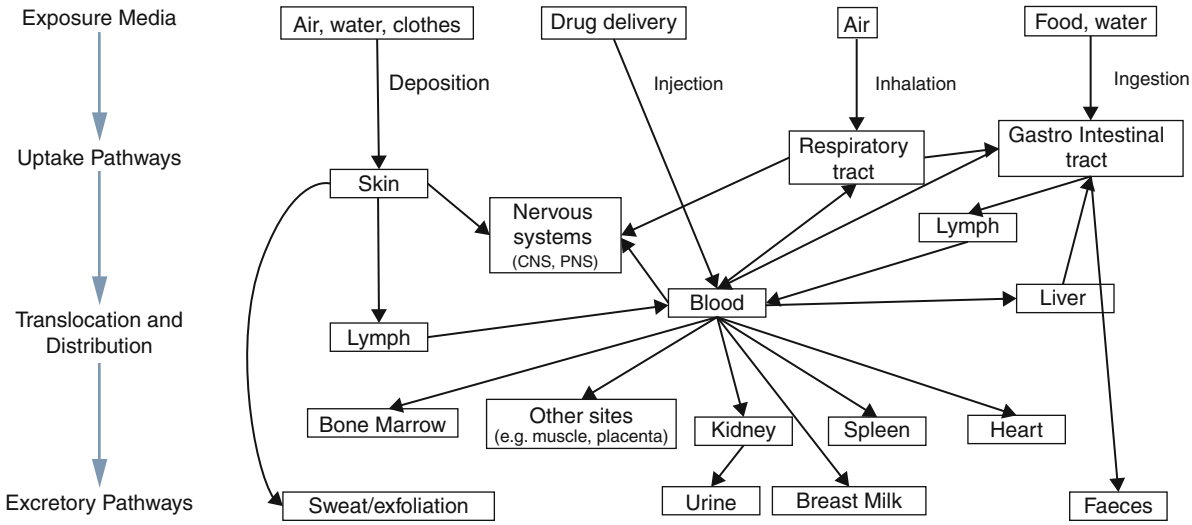


Fig. 23.9 Nanoparticle routes of exposure and affected bio systems (Rouhiainen et al. 2010)

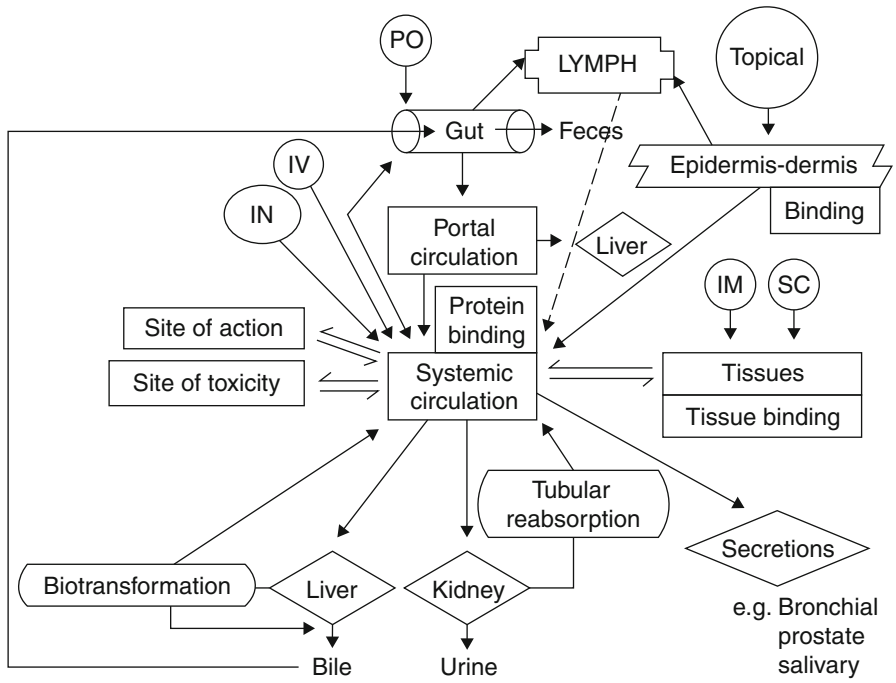


Fig. 23.10 Pharmacokinetic stages involved in adsorption, distribution, and elimination (ADME) (Riviere 2009)

reticuloendothelial (RE) system, bound to tissue proteins, post distributional aggregation). In these instances, blood half-life may be relatively short despite prolonged body persistence. "Nanomaterials may also be transported in the body via the lymphatic system, a phenomenon which complicates their pharmacokinetic analysis based on blood sampling and also exposes lymphoid tissue to higher concentrations than would be seen secondary to distribution from blood. These idiosyncrasies of nanomaterials compared to most drugs or small molecule xenobiotics require caution in using classic interpretations of the meaning of basic ADME parameters." From his literature review, he reported that pharmacokinetic analysis of fullerenes can be complicated by the lack of nanomaterial controls, attached ligands (e.g.,

antiviral, anticancer drugs), different vehicles, and tracking labels. Carbon nanotube studies were complicated by the lack of sensitive radiolabel analysis, small numbers of study animals, and lack of control in carbon nanotube length. Despite these limitations, carbon nanotube studies showed a pattern of distribution of larger material to tissues of the RE system, liver, spleen, lymph nodes, and bone marrow. The pharmacokinetics of quantum dots was influenced by coating with polyethylene glycol (to escape detection by the RE tissues, so-called stealth mode), size, charge, and periodicity. Renal excretion of particles 5–6 nm in size was reported; however, larger particles with specific surface characteristics (e.g., negative charge) may interact with the RE system or become protein bound and may not be eliminated from the body. Biodegradable nanomaterials found in marketed products can be described by standard pharmacokinetic approaches. Manufactured nanomaterials are not biodegradable and may accumulate in certain tissues. The unique surface chemistry of these manufactured materials may further alter their disposition of bioactivity. The preferential uptake of some nanomaterials by the lymphatic system coupled with the tendency of some nanomaterials to react with the RE system was also discussed. Nanomaterial clearance from the blood does not necessarily correlate to clearance from the body (Riviere 2009).

Li and Huang reviewed pharmacokinetic parameters focusing on chemotherapy and their pharmacokinetic nuances in the delivery of anticancer agents. In addition to many of the concepts mentioned above, they reviewed concepts at enhancing cargo drug delivery including targeting tissue-specific microvasculature, caveolae-mediated transcytosis and tumor micro-vessel clotting (Li and Huang 2008).

Longmire reported the concepts of nanoparticle flexibility may be important in the application of dendrimeres in imaging. Imaging tissue uptake and subsequent renal clearance was enhanced by dendrimeres exhibiting enhanced flexibility. Additionally, dendrimeres with long PEGylated tails underwent rapid renal clearance as opposed to those with shorter PEG tails (Fig.23.11). They proposed that their nontraditional models may have use in other therapeutic applications (Longmire et al. 2011).

For a thorough review of how nanomaterials (1–100 nm and >100 nm) behave in the blood and other organs with correlation to pharmacokinetics, see Moghimi et al. summarized in Table 23.3. This group also addressed future issues in nanomaterial pharmacokinetics presented in Table 23.4 Moghimi et al. 2012).

In addition to an excellent discussion of nanomaterial ADME, Li et al., proposed use of physiologically based pharmacokinetic modeling (PBPK) as a method to provide consistent pharmacokinetic information. However, PBPK may be limited because it requires large amounts of information, consistency in study designs, and consistent cross-discipline teamwork. Another limitation to PBPK modeling is that "tissues need to be harvested from animals in cohorts at each time point of study, resulting in a large number of animals needed for each study. A fairly large number of tissues and organs from each animal are required for PBPK modeling, further amplifying the number of samples to analyze. These limitations result in experimental designs being very time and cost consuming. Noninvasive measurement of nanoparticle bio-distribution, such as imaging, could greatly reduce this difficulty" (Li et al. 2010).

Overt and subtle manipulations of nanomaterials can greatly affect their pharmacokinetics.

23.5 Safety Issues

Nanomaterials by their nature have safety and environmental issues involved in their manufacture and laboratory use. Nanomaterials that escape the laboratory or manufacturing site can enter the environment where they may deteriorate or are free to interact unpredictably with anything and potentially create unknown environmental hazards. Because of the limited knowledge about them, organ toxicity, tumor development, and immune responses are possible concerns with exposures through the routes described below (Hoet et al. 2009; Gwinn and Vallyathan 2006b).

23.5.1 Inhalation Route

Inhaled nanomaterials can aggregate in the alveoli where their increased surface area places a burden on mucociliary and macrophage clearance. While in the lung, nanomaterials may translocate to the systemic circulation. Inhaled nanoparticles can also gain access to the CNS via olfactory nerves.

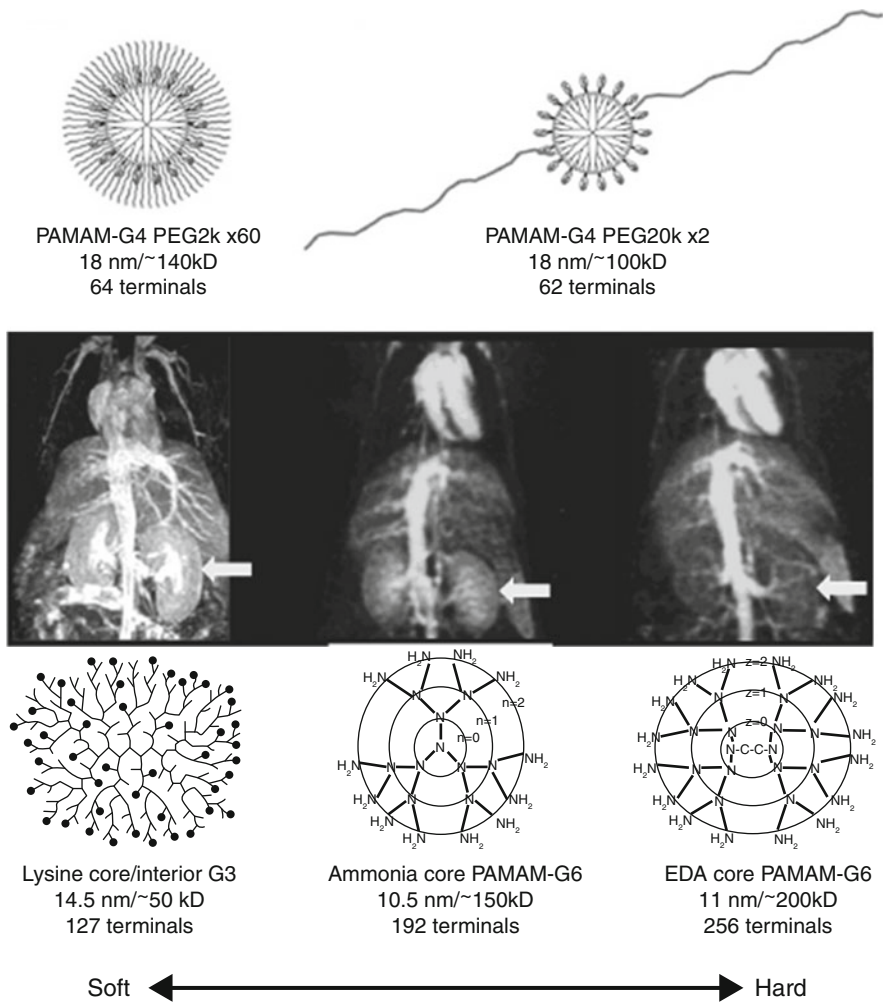


Fig. 23.11 PEGylated dendrimers and enhanced renal excretion of "softer" nanomaterials (Longmire et al. 2011)

Table 23.3 Summary points: factors controlling nanoparticle pharmacokinetics – an integrated analysis and perspective (Li et al. 2010)

1.	Intravenously injected nanoparticulate drug carriers provide a wide range of unique opportunities for site-specific targeting of therapeutic agents to many areas within the vasculature and beyond. Cancer nanomedicines have shown the success of this approach. This progress continues in other therapeutic areas such as inflammatory conditions
2.	PK and biodistribution of nanocarriers are controlled by a complex array of interrelated core and interfacial physicochemical and biological factors. Size, morphology, and surface characteristics of nanocarriers can be tuned to afford controlled PK and biological targeting according to the type, developmental stage, and location of the disease
3.	The plasma protein coat remains a key determinant in controlling nanocarrier PK and secondary responses, but not all deposited proteins play a role. The composition of the plasma protein coat is expected to differ considerably among nanoparticles in amount and heterogeneity, and in a time-dependent manner, on the basis of the physicochemical properties of the nanoparticles as well as the pathological state and the dosing regimen
4.	Geometric parameters play a significant role in nanoparticle PK – in flow properties (margination dynamics), vascular adhesion, cellular internalization, and escape routes from vasculature – but the bulk of current data is available for spherical nanoparticles
5.	As a result of altered PK, stealth systems with encapsulated therapeutic agents may induce new toxicity profiles

Table 23.4 Future issues: factors controlling nanoparticle pharmacokinetics – an integrated analysis and perspective (Li et al. 2010)

1. Breaching the biological barriers may induce diseases. Should we breach biological barriers with nanoparticles? Alternatively, should we concentrate on exploiting biological opportunities offered by the disease states for rational nanocarrier engineering and targeted drug delivery?
2. Sophistication in design and nanoengineering techniques may result in the generation of more heterogeneous populations of nanoparticles. These developments may cause more difficulties in interpretation of biological events following their administration. Can we develop technologies that yield more homogeneous populations of complex nanocarriers in terms of surface chemistry, architecture, and morphology?
3. Can we generate definitive maps that establish the interdependency of size, shape, and surface characteristics of nanoparticles in relation to biodistribution, controlled drug release, excretion, and adverse effects?
4. To what extent is the composition/conformational state of the outer surface proteins regulated by the type and the mode of deposition of the primary protein scaffold? Can appropriate methodologies be developed to control these factors?
5. Can carbon nanotubes, quantum dots, and many other nonbiodegradable nanoparticles/nanomedicines reach their clinical targets? Or should future efforts concentrate on the refinement of proven biodegradable nanocarriers?
6. How important are the physical and mechanical properties of nanoparticles and nanoassemblies with respect to target binding and therapeutic performance?
7. A detailed understanding of dynamic behavior and interactive forces between therapeutic agents (and particularly macromolecular cargo) and nanocarriers is still required and remains central for optimization strategies

23.5.2 Dermal Exposure

Nanosized particles may penetrate more deeply into the skin than their larger counterparts. It has been hypothesized that nanoparticles of titanium dioxide (5–20 nm) can penetrate the skin and enter the immune system or the systemic circulation. Quantum dots have been shown to penetrate porcine skin within 8 h of application.

23.5.3 Oral Route

Uptake of nanoparticles after oral exposure depends on particle size and surface chemistry (certain nanoparticles are combined with other materials). In rats, 50 nm to 3 μm particles were detected in the liver, spleen, blood, and bone marrow after oral exposure. Particles >100 nm did not reach the bone marrow and particles >300 nm did not reach the blood, suggesting that nanoparticles less than these sizes have been detected in bone marrow and blood. Nanomaterials can be used to enhance the GI absorption of a pharmacologically active compound.

23.5.4 Genotoxicity and Carcinogenicity

Nanomaterials can enter the cell and in some cases the nucleus where it is possible for them to interact with internuclear processes where DNA damage may occur. While useful in concept to cancer treatments, the genotoxic effects on normal cells must be considered. Carbon nanotubes (CNTs) administered to mice demonstrated genotoxic results that were similar to those of asbestos.

23.5.5 Development

Nanomaterials have been shown to cross the placenta in rats suggesting a risk to the developing fetus. Carbon nanotubes (CNTs) have shown to induce changes, in vitro, in cell proliferation and other cellular activities suggesting in vivo consequences in development.

23.5.6 Immunological Responses

Nanomaterials have demonstrated both positive and negative effects on the immune system that differ for inorganic and organic nanotypes. These effects may be desirable or undesirable. Therefore nanomedicine testing should exclude undesirable immunological responses. In general, positively charged (cationic) particles are more likely to induce acute inflammatory reactions (innate reaction) than negatively charged (anionic) particles. Two parameters – size and surface charge – play a central role in these responses. The phagocytic activity of macrophages in the lung has also been linked to particle size; although micrometer-sized particles stimulate phagocytosis, smaller, nanometer-sized materials often do not, or even reduce the capacity of the macrophages (Hoet et al. 2009).

Table 23.5 Classes of nanomaterials for use in medicine (Surendiran et al. 2009)

Type of nanomaterial/basic description	Potential medical use	Disease state
Liposomes Spherical nanoparticle, lipid bilayer membrane, hollow interior	Drug delivery system	Cancer
Nanopores 20 nM pores	Permits nutrients but limits immune penetrations	Transplanted tissue, genetics
Fullerenes, "soccer ball" framework	Encapsulates radioactive material; transports antibiotic, antiviral, and anticancer products	Imaging procedures, antibiotic with light stimulation, infection, HIV, cancer
Nanotubes 1–25 nm tubes with and without "caps"	Drug delivery, Amphoteracin B, DNA transport, increasing immune response	Can penetrate the cell wall, fungal, cancer therapy, vaccines
Quantum dots 2–10 nm nanocrystal	Drug delivery by conjugation	Diagnosis and treatments, prostate cancer, melanoma, breast cancer
Nanoshells Silica core and thin metallic shell	Immunologic manipulation	Cancer, immunoglobulin determinations
Nanobubbles Nanoscaled bubble	Drug transport, combined with heat or ultrasound	Cancer, increased uptake by target cells. Vascular clearing
Paramagnetic nanoparticles (e.?g., iron)	Diagnostics, imaging, rapid cell uptake	MRI imaging, cancer
Nanosomes Silica coated iron oxide nanoparticles with targeted antibody and contrast elements	Targeted diagnosis and treatment, combined with laser	Brain cancer
Dendrimers Nanomolecule with branching structures	Drug transportation within the branch "cavities"	Gene therapy, anti-retroviral, type 1 diabetes, potential for intranuclear cancer applications
Respirocytes Nano device hypothetical RBC	Deliver up to 236x more oxygen than RBC	Cardiac arrest

With nanoparticles, the smaller they are, the greater their surface area to volume ratio and the higher their chemical reactivity and biological activity. Greater chemical reactivity of nanomaterials can result in increased production of reactive oxygen species (ROS), including free radicals. ROS production has been found in a diverse range of nanomaterials including carbon fullerenes, carbon nanotubes and nanoparticle metal oxides. ROS and free radical production is one of the primary mechanisms of nanoparticle toxicity; it may result in oxidative stress, inflammation, and consequent damage to proteins, membranes, and DNA. Size is not the only variable influencing the safety of a nanomaterial, chemical composition, shape, surface structure, surface charge, aggregation and solubility, and the presence or absence of functional groups of other chemicals can also contribute (Fig. 23.6) (Nel et al. 2006).

23.5.7 Products in Use Today

Nanotechnology and nanomedicines are in use today. Many cosmetics and sunscreens use nanosized ingredients (e.g., nanotitanium dioxide, nanozinc oxide) (Friends of the Earth (FOTH) 2006; Nano Patents and Innovations 2009). Currently, there are nano-enabled drugs that have been approved by the FDA for the treatment of cancer, such as Abraxane®, which is used to treat breast cancer, and Doxil® for ovarian cancer (Table 23.5) (Surendiran et al. 2009).

23.5.8 Safety Challenges

Laboratory and manufacturer's workers may be at risk for accidental exposures through dermal, nasal, ophthalmic, and oral routes. Topically applied sunscreens and cosmetics that contain nanomaterials when washed off enter water ecology with possible unknown repercussions. Nanomedications and nanomaterials may confound the safety evaluation of bio/pharma agents to which they are combined. Nanomedications and bio/pharma products combined with nanodelivery systems may present unique safety issues. The preliminary evaluation of many products

is performed in normal animals and humans. However, sick individuals can be prone to unforeseen toxicities. Studies need to focus on therapeutic effects as well as nanoparticle disposition (Gwinn and Vallyathan 2006b; DeJong and Borm 2008b).

Nanomedications need to be evaluated for their effects on clinical laboratory tests, drug interactions, dose, hepatic and renal impairment, special populations (e.g., pediatrics, elderly), interactions with other existing pathologies, and interactions with foods and over-the-counter products. To the chagrin of consumer advocacy groups, at this time the FDA, EMEA, and MHRA do not believe that additional regulations are required for managing the licensure of nanomaterials/medications (Hoet et al. 2009; Friends of the Earth (FOTH) 2006; Nano Patents and Innovations 2009; Pautler and Brenner 2010b; DeJong and Borm 2008b). While unrelated to the clinical safety evaluation of nanomedicine, insurance providers may consider expensive nano treatments as "experimental" or environmental hazards and outside the scope of coverage of certain health insurance policies (Fink 2010).

23.5.9 Safety Summary

The current safety track record for nanomedicines is without clinical problems so far; however, because of rapid expansion in the area, much remains to be elucidated. Nanomaterials' bio/pharma properties are different from their macro counterparts, and there are at present no class distinctions. The use of nanomaterials in the laboratory and manufacturing process may require "nanoproof" protective equipment to prevent inadvertent exposures. Environmental implications regarding nanomaterials need to be reviewed and updated, especially wastewater testing standards. The environmental impact of nanomaterials and metabolites is largely unknown.

The implications for drug safety, pharmacovigilance and risk management are as limitless as the possible applications of the technology. Nanomaterials and nanomedicines will require specific safety evaluation on a case-by-case basis for their use in humans. Clinical research will need to focus not only on therapeutic safety and effectiveness but also on nanomaterial impact and disposition. Pharmacokinetics for active moieties, nanomaterials, and metabolism of both will need to be studied. Present concepts of risk detection may not apply to nanomedications especially in long-term exposures and effects or use in individuals with impaired health (DeJong and Borm 2008b).

Current regulations may or may not be adequate in managing nanomaterials' licensing process for commercial use. Current US labeling requirements do not stipulate that nanomaterials are used in a product (Hoet et al. 2009; Pautler and Brenner 2010b; CDC 2009b; CDC 2009c).

However, health authorities should always be notified early in the development process when nanotechnology is involved in a medical treatment. Regulators do not want to see nanomaterials turn into the twenty-first century's "asbestos" (Gwinn and Vallyathan 2006b).

The USEPA is taking a new path forward on regulation of manufactured nanomaterials under the Toxic Substances Control Act ("TSCA"). Revised regulations are expected in the near future (US Environmental Protection Agency 2011).

Nanotechnology, nanomaterials, and nanomedicines currently under development represent a revolution in the visualization, diagnosis, and treatment of many disease states. While current medical applications of nanotechnology appear to be "safe," in this rapidly growing promising area, much remains to be revealed regarding their environmental and clinical safety profiles.

23.6 Nanotechnology and Medical Devices

Appendix 2 lists some of the medical devices that may utilize nanotechnology in some form. It is beyond the scope of this section to address all of these topics, some of which have already been addressed while others are not germane to the discussion. Because of the variation in nanotechnology vernacular, some authors list nanoparticles that may carry therapeutic or diagnostic payloads also as "nano devices." These types of compounds have been addressed in earlier sections. This section will highlight technology that would be considered "hardware," but on a nanoscale. In this realm, nanoscale may refer to beyond the 1–100 nm scale. Research in this area also lacks standardization and is performed on a case-by-case basis. Many of these devices and techniques are in the early stages of development. Information changes rapidly; therefore researchers are again encouraged to review their specific area of interest for the most current information relevant to their topic.

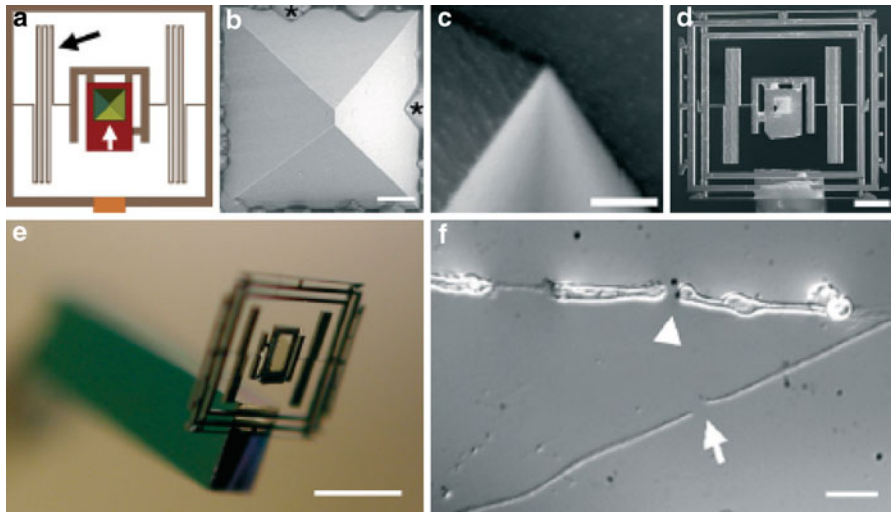


Fig. 23.12 Nanoknife (Chang et al. 2010). Nanoknife for microscale axon cutting. (**a**) Schematic planar view of microsuspension and cutting shell microassembled into a nanoknife. *Black arrow*, serpentine flexures acting as compliant microsuspension. *White arrow*, 1-μm thick silicon nitride cutting shell. Footprint of entire structure is 1 mm^2. (**b**) Scanning electron microscope (SEM) view of pyramidal-shaped cutting shell with apex serving as cutting edge (scale = 20 μm), (**c**) SEM showing cutting edge with an ~20 nm radius of curvature (scale = 100 nm), (**d**) Image of assembled nanoknife (scale = 200 μm). (**e**) Nanoknife mounted at an angle to a rod and held by a micromanipulator (not in view). For axon cutting in vitro, the nanoknife is positioned and angled, so that its planar footprint is parallel to the cell culture dish. The cutting stroke is executed as a downward movement delivered via the micromanipulator (scale = 500 μm). (**f**) Examples of cuts made by a nanoknife in an unmyelinated axon (*arrow*) and a myelinated axon (*arrowhead*) (scale = 25 μm)

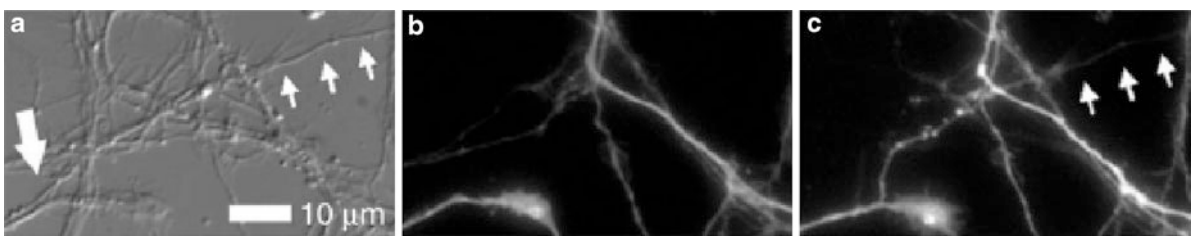


Fig. 23.13 Mouse axon electrofusion (Chang et al. 2010). Axon microelectrofusion demonstrated by the passage of soluble cytoplasmic green fluorescent protein (GFP) from one axon fusion partner into another. (**a**) Bright field image showing hippocampal axons in culture. *Single arrow* points to location where microelectrofusion was induced. (**b**) Perfusion image of the same field as in "a" showing axons containing soluble cytoplasmic fluorescent GFP. (**c**) After microelectrofusion, GFP passed from the axon at the *bottom left* into an axon fusion partner that did not originally have GFP, indicating successful fusion of the two axonal compartments

23.6.1 Nanoknife and Axon Refusion

Chang et al. discussed the nuances of neuronal damage and repair via a variety of techniques. They used a nanoknife with a cutting edge of 20 nm (Fig. 23.12) to sever mouse axons that were later rejoined by microelectrofusion (Fig. 23.13). While admittedly cumbersome and time consuming, the process demonstrated success, principally via the use of soluble cytoplasmic green fluorescent protein (GFP).

23.6.2 Nanoburrs

Nanoburrs (Fig. 23.14) are a relatively new development in nanotechnology and demonstrate its

multifunctionality. Nanoburrs reported were "60-nm core-shell hybrid NPs consisting of a polymeric core, a lipid interface, and a poly(ethylene glycol) (PEG) corona. For temporal control, we achieved the capacity for slow drug elution over 2 weeks using poly(lactic acid) (PLA) conjugates of paclitaxel as a model therapeutic agent, made by a modified drug-alkoxidering-opening strategy." Nanoburrs were reportedly successful in repairing vascular damage and as a vehicle for an anti-angiogenics used to inhibit cell division and prevent growth of scar tissue that can clog arteries. Paclitaxel was control released over a period of 10–12 days. Nanoburrs reportedly can be used in conjunction with or instead of traditional arterial stents. An advantage of nanoburrs is that they can be injected intravenously protecting patients from more invasive procedures (Fig. 23.15). This technology could have broad applications across other important diseases, including cancer and inflammatory diseases where vascular permeability or vascular damage is commonly observed (Langer 2010; Chan et al. 2010; Rajiv et al. 2011).

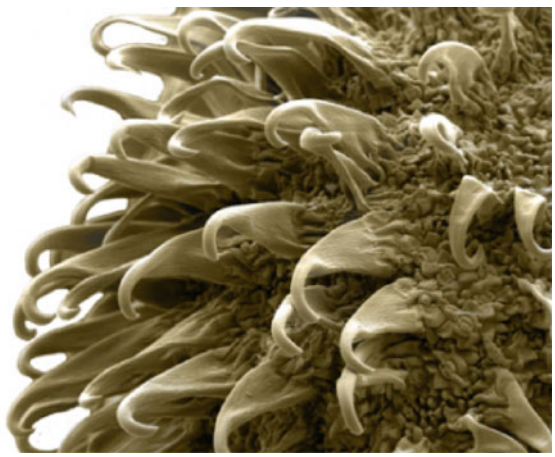


Fig. 23.14 Nanoburr (Langer 2010)

23.6.3 Nanoneedles

Demuth et al. reported the use of nanoneedles (Fig. 23.16) as an alternative delivery system for needle-based therapies (e.g., vaccines and drugs). Therapeutic materials are coated on nanoneedles on thin film arrays and administered through the stratum corneum (SC), promoting efficient and pain-free transcutaneous delivery. Their findings suggested the potential delivery of DNA vaccines, gene therapy as the administration of degradable polymer nanoparticles for controlled release of cargos in vivo (DeMuth et al. 2010).

23.6.4 Nanoscaffolds

In addition to tissue repair, nanotechnology holds promise in the area of tissue regeneration. This involves seeding tissue on a "scaffold" that can be constructed

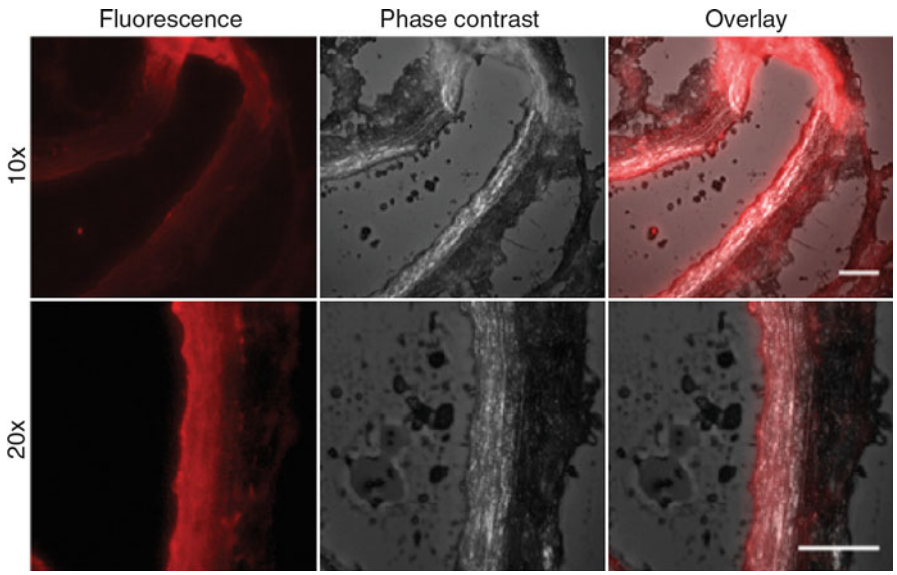


Fig. 23.15 Nanoburrs binding in vivo (Langer 2010). Representative images of nanoburr binding in vivo by i.v. delivery to angioplastied left common carotids. (*Left* to *right*) fluorescence, phase contrast and overlay images of angioplastied left common carotid arteries incubated with nanoburrs at 10× and 20× magnification (scale bar, 100 μm). Images were obtained with a DeltaVision deconvolution microscope using the 10× and 20× objective

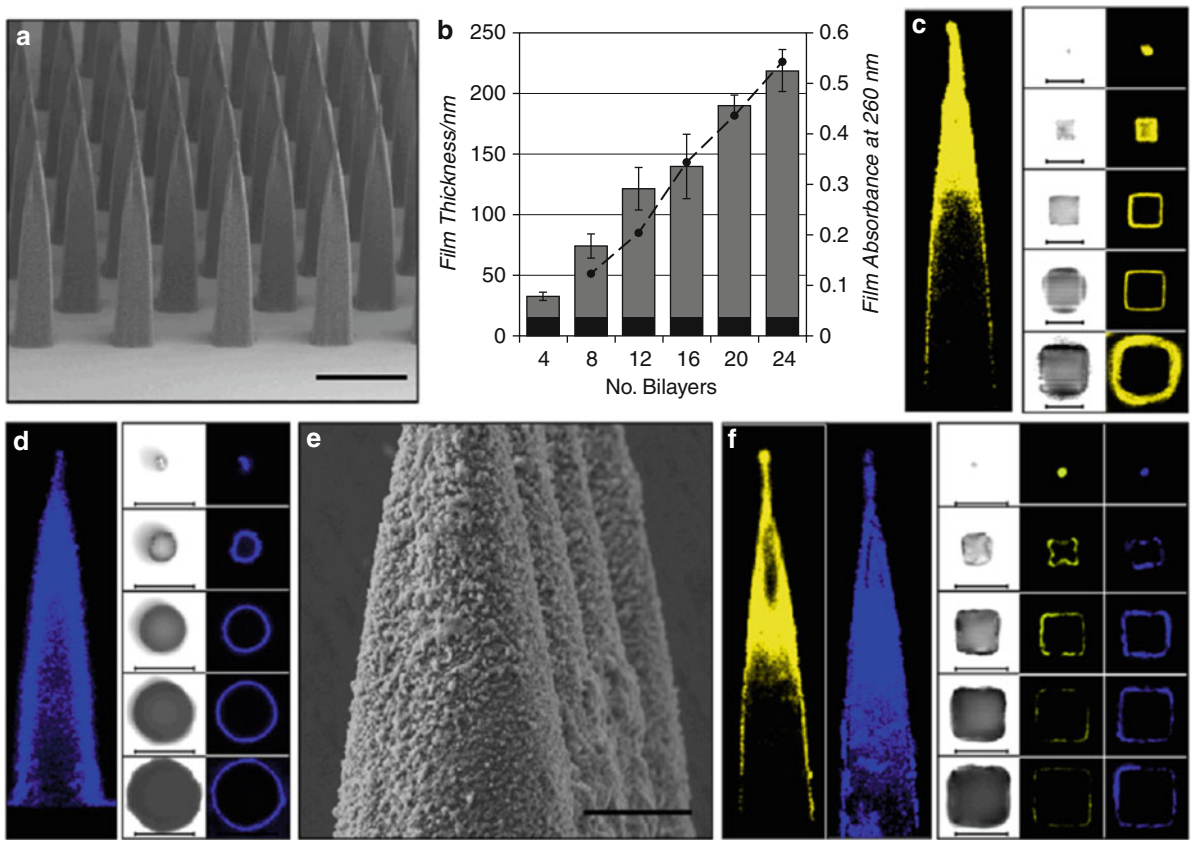


Fig. 23.16 Nanoneedles (DeMuth et al. 2010). (**a**) SEM micrograph of uncoated PLGA rnicroneedle arrays of pyramidal geometry (scale – 500 μm). (**b**) Film growth (*left* axis) and absorbance (*right* axis) for (Poly-1/pLLC)$_n$ multilayers assembled on silicon/quartz substrates bearing a (PS/SPS)$_{20}$ initiating layer (*black bar* – (PS/SPS)$_{20}$, *grey bar* – (Poly-l/pLUC)$_n$, *dashed line* – Ab-260 nm). (**c**, **d**) Representative confocal micrographs showing (C) (PS/SPS)$_{20}$-(Poly-1/Cy3-pLUC)$_{24}$-coated microneedle and (**d**) (PS/SPS)$_{20}$-(Poly-1/DiI-PLGA NP)$_4$-coated microneedle (*left* – transverse section, *right* – lateral sections, 200 μm intervals, scale – 200 μm). (**e**) SEM micrograph showing a (PS/SPS)$_{20}$-(Poly-l/PLGA NP)$_4$-coated microneedle array (scale – 50 μm). (**f**) Representative confocal micrographs showing a (PS/SPS)$_{20}$-(Poly-l/Cy3-pLUC)$_{24}$-(Poly-l/DiD-PLGA NP)$_4$ co-coated microneedle (transverse and lateral sections, *left* – Cy3-pLUC, *right* – DiD-PLGA NP, 200 μm intervals, scale – 200 μm)

from a variety of nanomaterials (Figs. 23.17, 23.18). Tissues under study include bone, cartilage, vascular, neural, and bladder tissues. Additional tissues under study include muscle, skin, kidney, liver pancreas, and immune system. Zhang and Webster's review provides an excellent starting point for those interested (Zhang and Webster 2009).

23.6.5 Nanobots (Nanorobots)

Nanobots remain an intriguing aspect of nanotechnology that has yet to be realized.

An excellent description of nanorobotics can be found at numerous websites including Wikipedia (Wikipedia 2011b). In brief, these are nano devices capable of performing any number of medical applications including cell repair, identification and destruction of cancer cells, diagnostics, and others. Such devices would require the ability to be navigated, non-replicating as well as being safe and effective. Figures 23.19–23.21 show some artistic renditions of futuristic nanobots. Mavroidis provides a comprehensive overview (Mavriodis 2011). Interested researchers should review the literature for updates in their specific field of interest.

A report published in *Nature* (Davis et al. 2010) received a lot of attention in the lay press as the first successful implementation of a "nanobot"

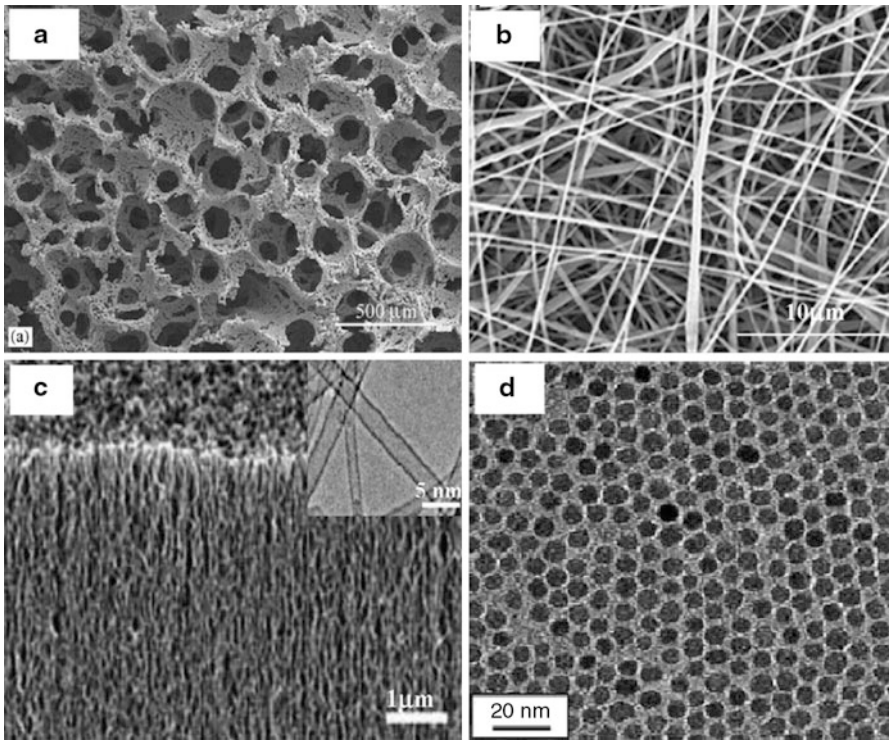


Fig. 23.17 Nanoscaffolds (Zhang and Webster 2009). (**a**) Scanning electron microscopy (SEM) image of poly(L-lactic acid) (PLLA) nanofibrous scaffold with interconnected spherical macropores created by a phase-separation technique. (**b**) Electrospun polycaprolactone/hydroxyapatite/gelatin (PCL/HA/gelatin, 1:1:2) nanofibers which significantly improved osteoblast functions for bone tissue engineering applications. (**c**) Densely aligned single wall carbon nanotube (SWCNT) forest grown with novel water-assisted chemical vapor deposition in 10 min. (**d**) Transmission electron microscopy (TEM) image of monodispersed magnetic Fe_3O_4 nanoparticles (6 nm) deposited from their hexane dispersion and dried at room temperature. Scale: A = 500 nm, B =10 nm, C = 1 µm (inset = 5 nm), D = 20 nm

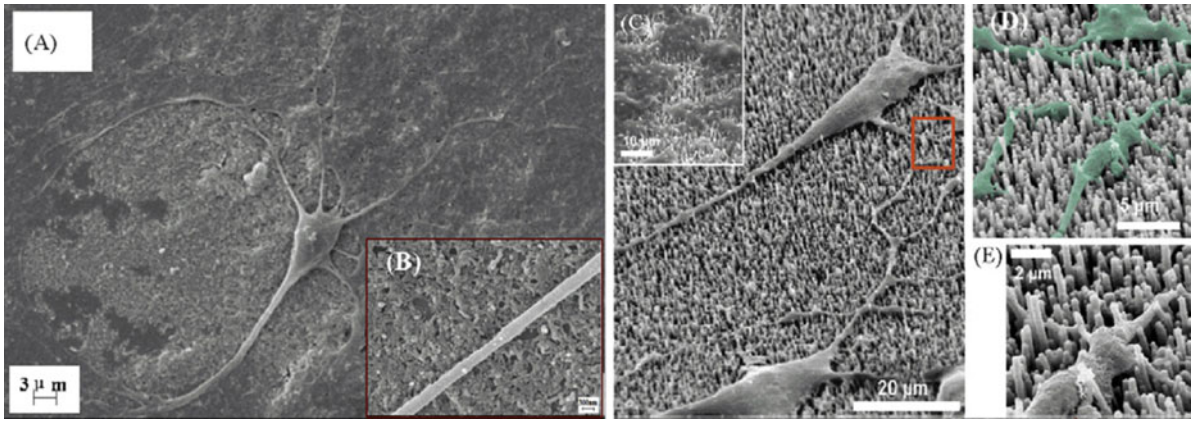


Fig. 23.18 Neural cells grown on nanoscaffolds (Zhang and Webster 2009). SEM images of neural cell adhesion on carbon nanotube/fiber substrates. (**a**) Neonatal hippocampal neurons adherent on purified MWCNT glass substrates with extended neurites after 8 days; *inset* image (**b**) shows a single neurite in close contact to CNTs (Images are adapted from). (**c–e**) PC12 neural cells grown freestanding on vertically aligned CNFs coated with polypyrrole at different magnifications. Scale: A = 3 µm, B = 300 nm, C = 20 µm (insert 10 nm), D = 5 µm, E = 2 µm

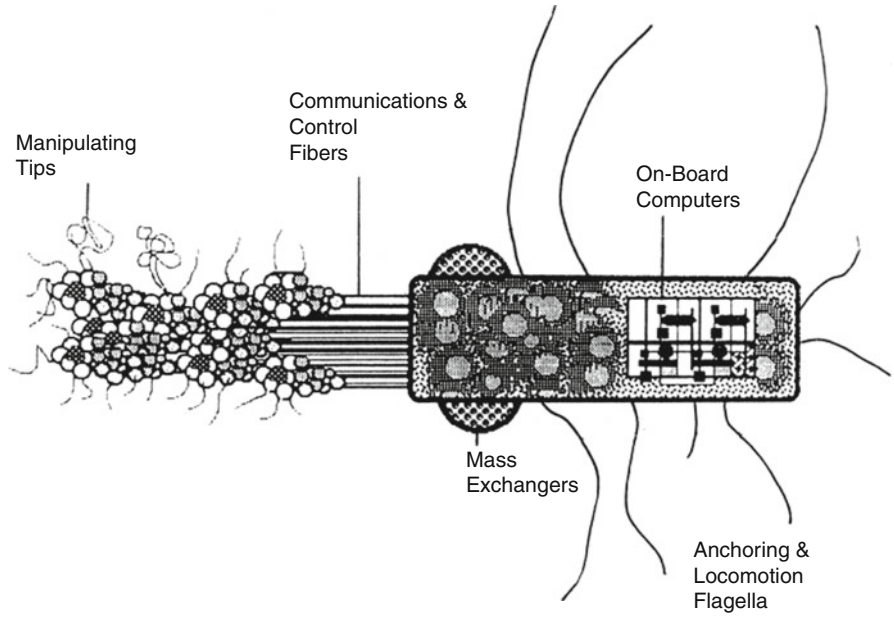


Fig. 23.19 Nanobot, fictionalized cell repair machine VI. http://www.foresight.org/Nanomedicine/Gallery/Captions/

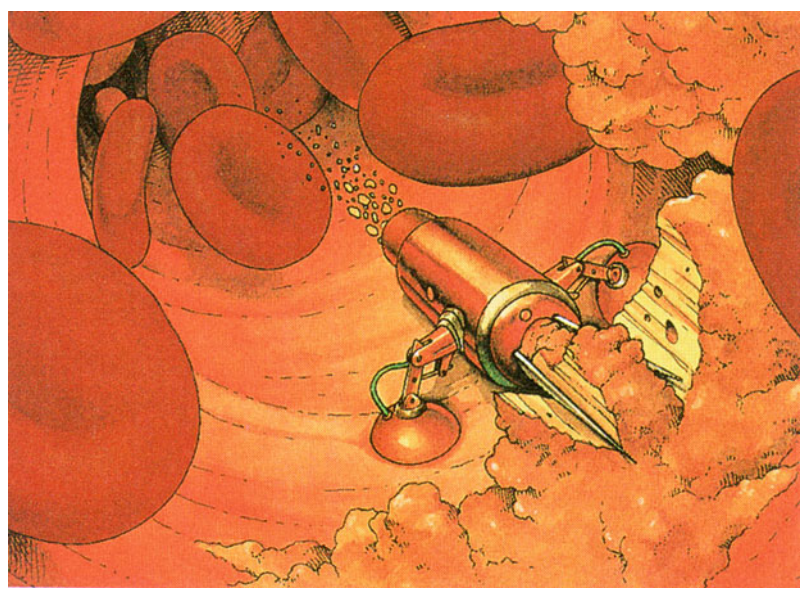


Fig. 23.20 Fictionalized nanobot manipulating cholesterol. http://www.foresight.org/Nanomedicine/Gallery/Captions/

(Figs. 23.22, 23.23). However, close inspection reveals that the group used a targeted nanoparticle administered intravenously and not a piece of "hardware" as described earlier in this section. It is a demonstration of the potential of nanotechnology that can deliver genetic therapy to targeted cancer cells. Results in humans of this clinical trial have yet to be reported. This study could also be used to demonstrate the ambiguity of nanotechnology vernacular.

Al-Fandi et al. (Al-Fandi et al. 2011) reported using flagellated *Escherichia coli* as potential nanobots, using biochemical gradients as a navigation system.

Another report (Muscat et al. 2011) described a programmable and autonomous "molecular robot"

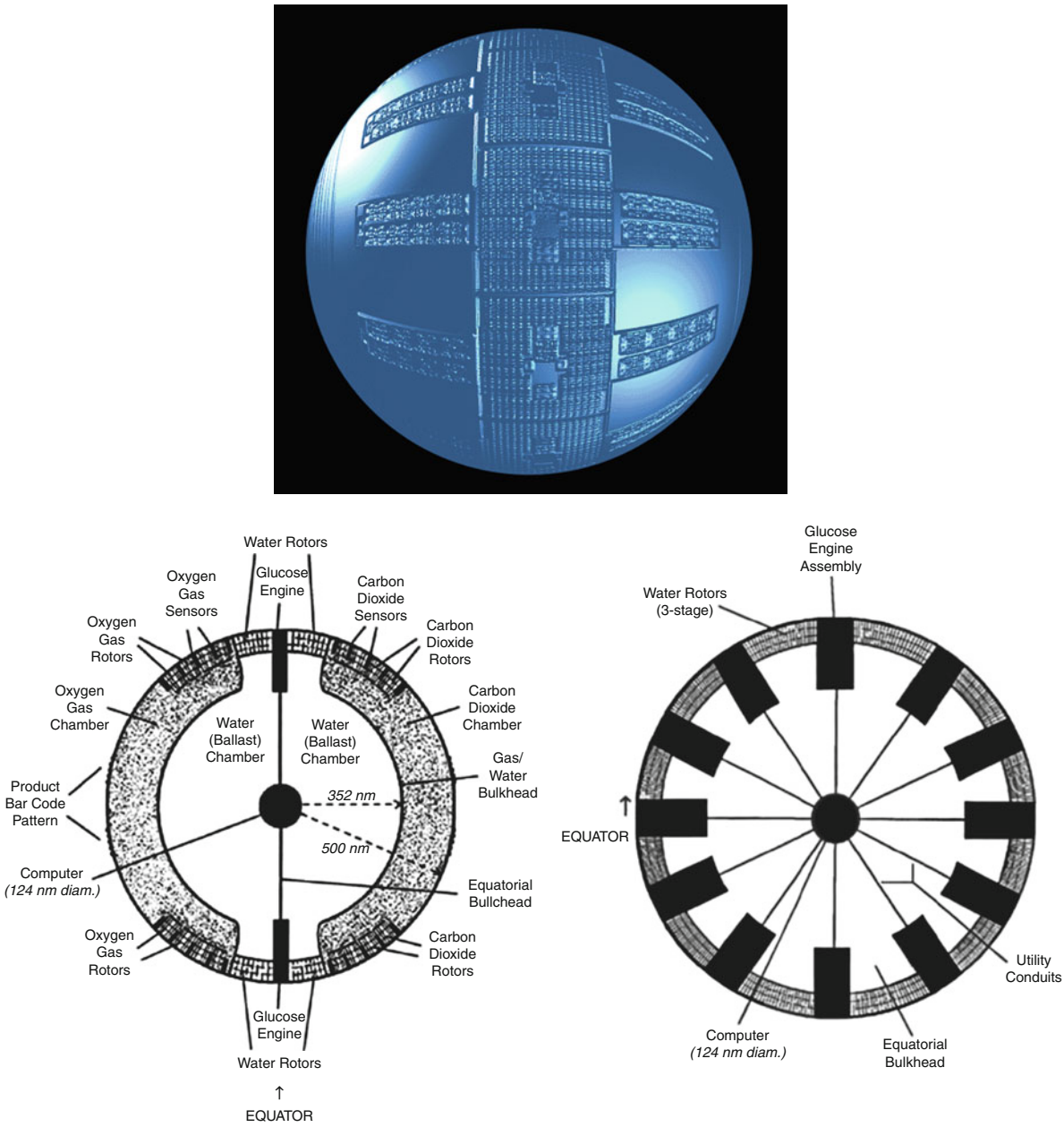


Fig. 23.21 Fictionalized respirocyte. http://www.foresight.org/Nanomedicine/Gallery/Captions/

whose motion was fueled by DNA hybridization and allowed precise control of genetic cargo delivery, not really a demonstration of a nanobot but another example of where research is headed.

Much maligned in science fiction (e.g., *Prey* – Michael Crichton 2002), nanobots promise significant impacts in medicine, surgery, drug delivery, and diagnostics in the future.

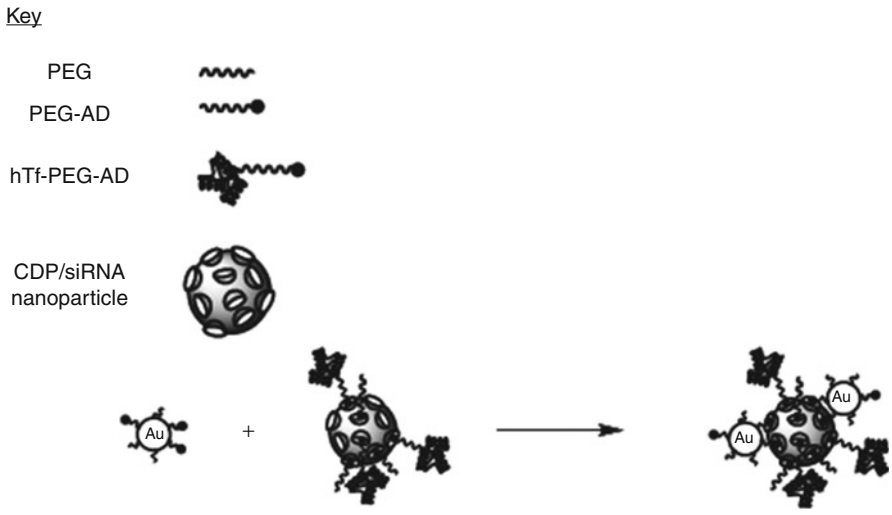
23.7 Nanodiagnostics

The capability of nanomaterials for specific targeting, payload transport, and elimination of payload and carrier makes them ideal for specific diagnostic applications. As in the previous sections, progress in this area has been in volume and this section is

Fig. 23.22 Targeted nanoparticle "nanobot" (Davis et al. 2010)

Part II: Principle of staining

A schematic representation of the interactions between the Au-PEG-AD particles and CALAA-01 is shown in Schematic SI 2.



Scheme SI 2: Representation of the staining process of CALAA-01 by Au-PEG-AD Particles.

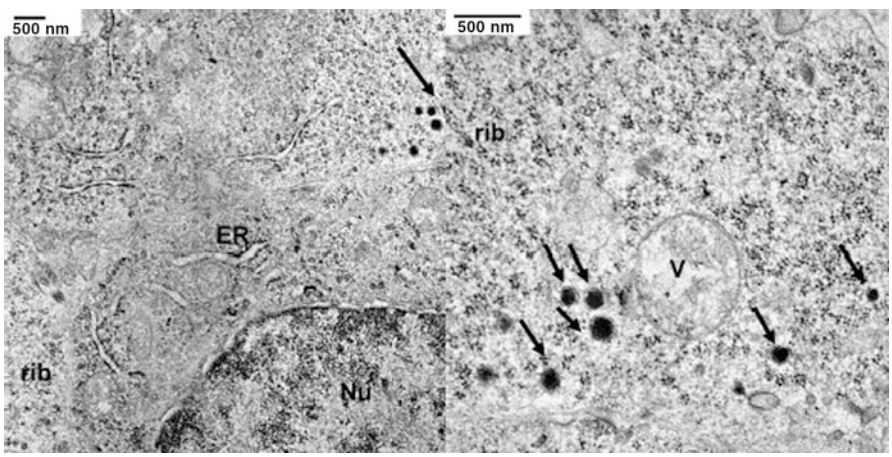


Fig. 23.23 Nanoparticle "nanobot" in tumor (Davis et al. 2010). Transmission electron microscopy confirms the existence of siRNA containing, targeted nanoparticles inside a mouse subcutaneous tumor. Figure S2: Transmission election micrographs snowed intracellular localization of siRNA- containing, cyclodextrin-based, targeted nanoparticles (*dark round objects* – the siRNA within the nanoparticle is stained by the presence of the uranyl ions that bind to the nucleic acid) inside N2A cells of the tumor subcutaneously implanted in mice (same tumor tissue used for confocal fluorescence imaging in Fig. 23.1). *Left:* The proximity of targeted nanoparticles to the nucleus shows their intracellular localization. *Right*: Expanded view of nanoparticles shown in *left panel* (Scale bar = 500 nm). *Solid arrows* point to the nanoparticles. Labeling is as follows, *Nu* nucleus, *rib* ribosome, *ER* endoplasmic reticulum, *V* vesicle

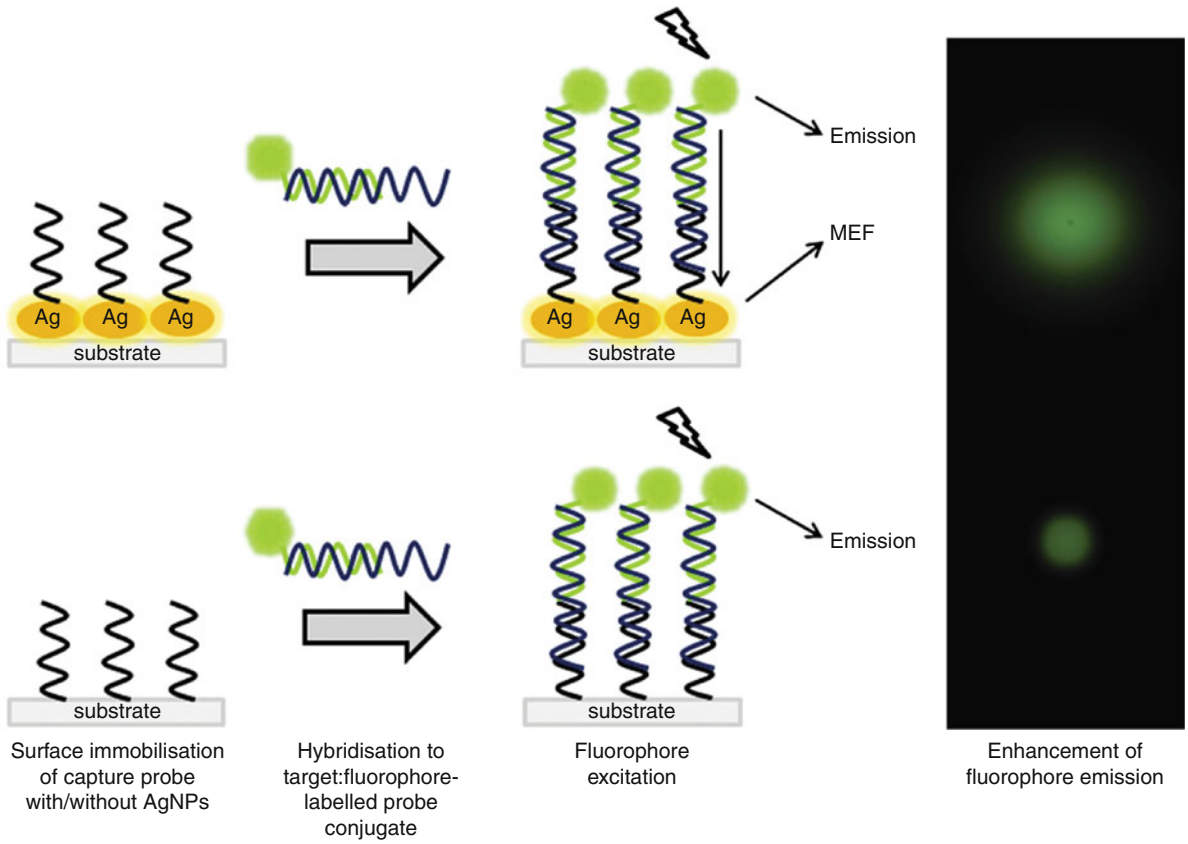


Fig. 23.24 Enhanced fluorescence using gold nanoparticles (Larguinho and Baptista 2011). Schematic configuration for a metal-enhanced fluorescence (MEF) system. Fluorescence signal enhancement may be achieved by deposition of AgNPs onto a surface, before probe immobilization. Upon hybridization with fluorophore-labeled probe, the intensity of fluorescence emission is potentiated by the AgNPs (MEF)

intended only to highlight some of these complex applications. Nanodiagnostics must be safe and effective for use in humans. Researchers should review their specific areas of interest for the most current information.

23.8 Examples of Nanomaterials Used in Diagnostics

23.8.1 Gold and Silver Nanoparticles

In a report of theoretical use of gold and silver nanoparticles in imaging techniques for proteomic and genomic detection, Larguinho and Babtista reviewed their use in a variety of settings. These nanoparticles have found application in diverse strategies, such as electrochemistry, luminescence, target labeling, and SPR-based biosensors, and may further be combined in different assembly structures to promote synergistic conditions with concomitant increase in sensitivity and versatility. They focused on the development of nanoscale devices and platforms that can be used for single molecule characterization of nucleic acid, DNA or RNA, and protein at an increased rate when compared to traditional techniques. Also, several advances were reported on DNA analysis in real time, at both high resolution and very high throughputs, suitable for biomedical diagnostics. While still in the developmental phase, their findings suggest higher sensitivities (Fig. 23.24), economy, and early diagnosis in genomic screenings for cancer, hepatitis, and diabetes (Larguinho and Baptista 2011).

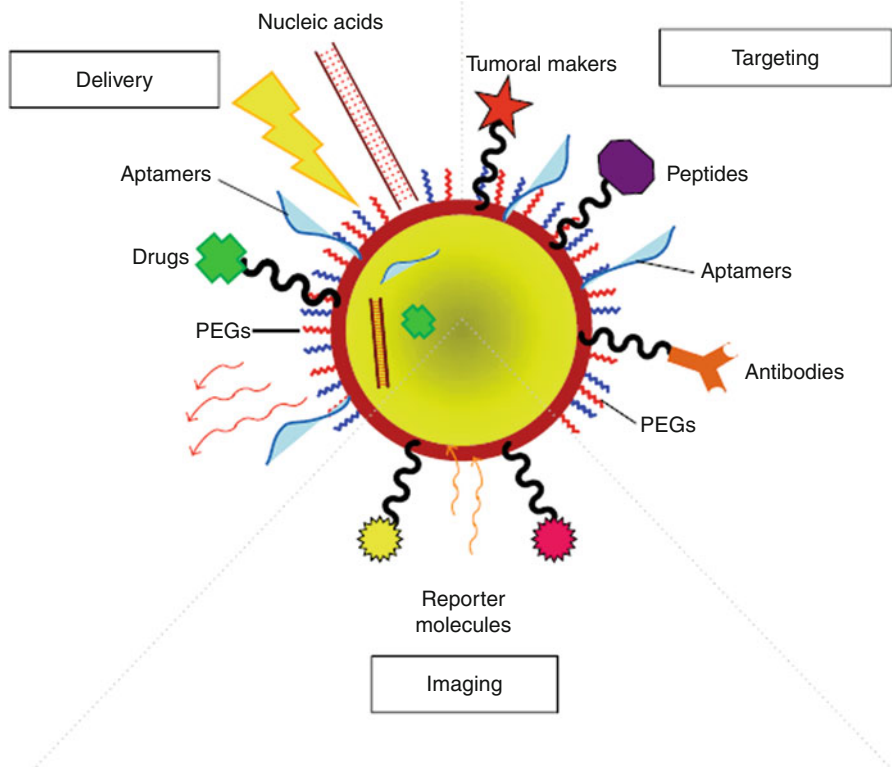


Fig. 23.25 Multipurpose noble metal nanoparticle (Conde et al. 2012). Multifunctional KP-based systems for tumor targeting, delivery, and imaging. These innovative NPs comprise nucleic acids, aptamers, and anticancer drug molecules for delivery to the target tissue. Depending on the targeting mechanism, they can be on the surface or inside the NPs. Responsive NPs/molecules can also trigger reaction upon external stimuli through the functionality of valuable tumor markers, peptides, polymers, and antibodies that can used to improve NP circulation, effectiveness, and selectivity. Multifunctional systems can carry reporter molecules tethered to the particle surface and employed as tracking and/or contrast agents

Conde et al. reviewed noble metals (primarily nanogold and nanosilver) used in the proposed treatment and diagnosis of cancer including their short- and long-term toxicities. They reported: "light absorption from biologic tissue components is minimized at near infrared (NIR) wavelengths, most noble metal NPs for in vivo imaging and therapy have been designed to strongly absorb in the NIR so as to be used as effective contrast agents. However, noble metal nanomaterials, such as nanoparticles, nanoshells, nanoclusters, nanocages, and nanorods, have showed widespread application as contrast agents for in vivo cancer imaging: those presenting a significant absorbance and scattering in the NIR region or surface-enhanced Raman scattering (SERS), or as contrast agents for computed tomography (CT), magnetic resonance imaging (MRI), optical coherence tomography (OCT), and photoacoustic imaging (PAI). Moreover, most noble metal nanomaterials are capable of combining multiple imaging modalities that can yield complementary information and offer synergistic advantages over any single imaging technique" (Figs. 23.25, 23.26). Enhanced imaging via computerized tomography (CT), magnetic resonance imaging (MRI), optical coherence tomography (OCT), photoacoustic imaging (PAI), photoacoustic tomography and surfaced-enhanced Raman scattering was reported (Conde et al. 2012).

23.8.2 Quantum Dots (QDs), Plasmonic Nanoparticles, Magnetic Nanoparticles, Nanotubes, and Nanowires

Chi et al. reviewed nanobiotechnology and a variety of materials as nanoprobes for bioanalysis for in vitro bioanalysis and diagnosis (Table 23.6). Major classes of nanoprobes include quantum dots (QDs), plasmonic nanoparticles, magnetic nanoparticles, nanotubes, nanowires, and multifunctional nanomaterials. With the advantages of high volume/surface ratio, surface tailorability, multifunctionality, and intrinsic properties, nanoprobes have tremendous applications in the areas of biomarker discovery, diagnostics of infectious diseases, and cancer detection. They highlight the distinguishing features of nanoprobes for in vitro use,

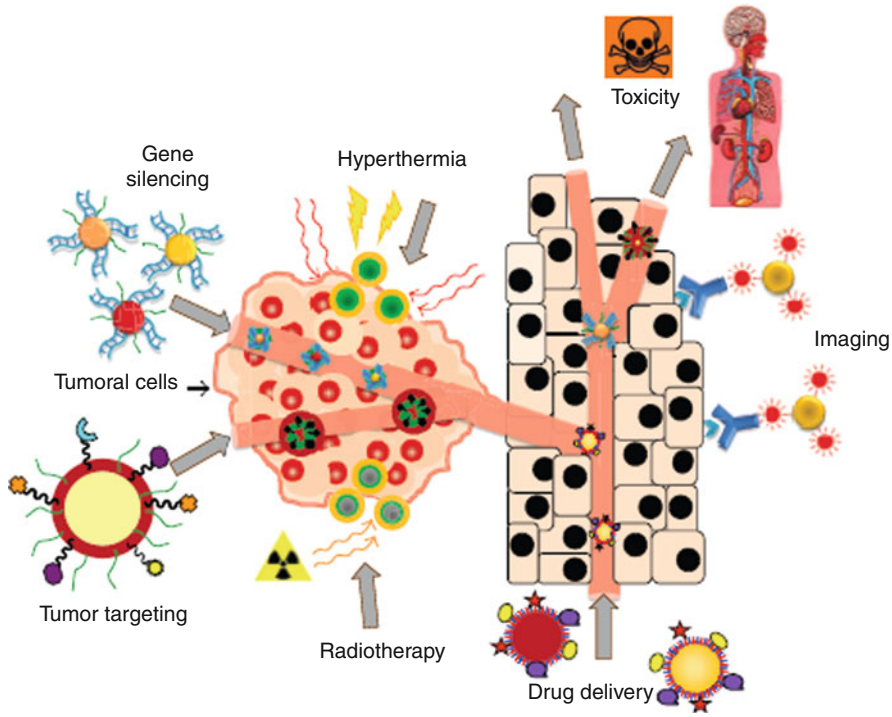


Fig. 23.26 Noble metal nanoparticles for cancer treatment (Conde et al. 2012). Noble metal NPs for cancer therapy. Once the tumor is directly connected to the main blood circulation system, NPs can exploit several characteristics of the newly formed vasculature and efficiently target tumors. Tumor cells are supplied by blood capillaries that perfuse the cells of the tissue where NPs can (1) passively accumulate or (2) anchor through targeting moieties to biomarkers overexpressed by tumor cells. NPs can act simultaneously as therapeutic agents, inducing hyperthermia, enhancing radiotherapy, silencing genes, and/or delivering drugs to induce tumor cell death, and as imaging enhancers or contrast agents, to help track the therapeutic effects in real time

such as safety, ultrasensitivity, multiplicity, and point-of-care use, which will bring a bright future in in vitro nanodiagnosis (Chi et al. 2012a).

23.8.3 Quantum Dots and Carbon Nanotubes

Tan et al. reported that "quantum dots (QDs) can be used to image cancer cells as they display superior fluorescent properties compared with conventional chromophores and contrast agents. In addition, carbon nanotubes (CNTs) have emerged as viable candidates for novel chemotherapeutic drug delivery-platforms. The unique photothermal properties of CNTs also allow them to be used in conjunction with near-infrared radiation and lasers to thermally ablate cancer cells. Furthermore, mounting evidence indicates that it is possible to conjugate QDs to CNTs, making it possible to exploit their novel attributes in the realm of cancer theranostics (diagnostics and therapy)" (Tan et al. 2011).

23.8.4 Iron Oxide

In the context of MRI imaging using superparamagnetic iron oxide nanoparticles (SPIONs), Rosen et al. discuss both passive and active uptakes of contrast media to enhance visualization (Fig. 23.28). Active techniques using iron nanoparticles combined with transferrin, RGD peptides, monoclonal antibodies, aptamers, and other targeting molecules were reviewed in a variety of oncological contexts (Table 23.10). While the pharmacokinetics and safety of many of these moieties have yet to be determined,

Table 23.6 Overview of selected nanoprobes and their potential applications of in vitro diagnostics (Chi et al. 2012a)

Nanoprobes	Physical properties	Analytical methods	Potential applications
QDs	Unique fluorescence	FRET based	Detection of DNAs, proteins, and enzymes
		BRET based	Detection of disease-related protease
Metal nanoparticles	Collective electronic excitations	Surface plasmon coupling	Single-molecule detection and the related disease diagnosis
		LSPR based	Detection of disease-related biomarkers
		SERS based	Detection and identification of biomolecules
Magnetic nanoparticles	Superparamagnetism	Magnetic capture	Bacterial detection
		Diagnostic magnetic resonance	Detection of infectious agents including bacteria, viruses, fungi, and parasites; as well as a variety of other biological targets, such as nucleic acids, proteins, antibodies, viruses, stem cells, and circulating tumor cells
		Giant magnetoresistive sensor	Multiplex detection of protein
CNTs	Resonant Raman scattering	Raman imaging	Detection and differentiation of cancer cells, detection of biomarkers
Si nanowires	Electrical properties	Field-effect device	Detection and measurement of various biological species and their interactions, including proteins, DNAs, and viruses
Multifunctional nanomaterials	Optical, fluorescent, magnetic, and/or electrical properties	Multiplexed methods	Detection and tracking of biomolecules and cells, multiplexed assay, and multimodal imaging

Table 23.7 Nanoparticles for contrast imaging of cardiovascular disease (CVD) (Godin et al. 2010)

Category	Agent (examples)	Imaging techniques
Fluorescent	Quantum dots	Fluorescence tomography
Radioactive	^{18}F CLIO, ^{111}In nanoparticles	PET, SPECT
Paramagnetic	Gd-DTPA	MRI
Superparamagnetic	Iron oxide nanoparticles	MRI
Electron dense	Gold or I-based nanoparticles	CT
Light scattering	Gold nanoshells	Optical coherent tomograpy
Photoacoustic	Colloidal nanobeacons	Photoacoustic tomography
Multimodal	Copper-CLIO	PET, MRI, NIRF
	Perfluorocarbon nanoparticles	MRI, molecular imaging

they believe that these techniques could lead to the next generation of cancer diagnostics and treatments (Rosen et al. 2011).

Diagnostic applications of nanomaterials include but are not limited to:

- In vivo near-infrared fluorescence (NIRF) imaging
- Magnetic resonance imaging (MRI)
- Positron emission tomography (PET)
- Computed tomography (CT)
- Ultrasound (US), photoacoustic imaging (PAI)
- Cancer theranostics (the combination of diagnostic and treatments) (Table 23.10)
- HIV, pathogen detection, infectious disease
- Cardiovascular disease, demonstrating the versatility of nanomaterials (Tables 23.7–23.9, Fig. 23.27)
- Heart, lung, and blood diseases
- Alzheimer's disease
- Crossing the blood-brain barrier
- Thrombosis
- Endoscopy (Hahn et al. 2011; Pan et al. 2010; Sharma et al. 2011; Shinde et al. 2011; Chi et al. 2012b; Godin et al. 2010; Buxton 2007; Brambilla et al. 2011; Bhaskar et al. 2010; McCarthy and Jaffer 2011; Niu et al. 2011)

Table 23.8 Examples of nanocarriers for cardiovascular disease therapy (Godin et al. 2010)

Nanocarrier	Example of agent	Experimental model	Outcomes
Neutral liposomes	Bisphosphonates (clodronate, alendronate, etc.)	Injured rat carotid artery	Macrophage depletion, reduced inflammation
Cationic liposomes	Chloramphenicol acetyl transferase (CAT) encoding gene (Pautler and Brenner 2010b)	Balloon injured Yorkshire pig artery, local delivery	Increased CAT expression
	Vascular endothelial growth factor (VEGF) encoding viral vector	Clinical trial, patients with 60–99% stenosis in major arteries, local delivery through catheter	Significant improvement in myocardial perfusion
Hemaglutin virus of Japan (HVJ) liposomes	Tissue factor pathway inhibitor gene	Iliac artery of hyperlipidemic rabbit following angioplasty. Local delivery through catheter.	Reduction of intimal hyperplasia
Perfluorocarbon nanoparticles	Surface bound streptokinase, α3β integrins, others	Human plasma clots, hyperlipidemic animals	In vitro fibrinolysis, theranostic in vivo
Polyelectrolyte nanoparticles (RNA or polyvinyl sulfate with polyethylene imine/DNA complex)	Gene encoding for urokinase plasminogen activator	Rat carotid artery	High transfection efficiency
Polymeric (PLA or PLGA)	AG-1295 and AGL-2043	Balloon injured rat carotid artery	Inhibition of restenosis

Table 23.9 Nanotechnology based in-vivo cardiovascular disease sensors (Godin et al. 2010)

Sensor targets	Technology	Applications
K^+, H^+ ions	Field effect transistor (FET)	Myocardial ischemia
Na^+ ions	Fluorescent nanosensors	QT syndrome, Heart failure
Ca^{2+} ions	Boron-doped silicon nanowires (SiNWs)	Multiple CVD
Nitric oxide	Single-walled carbon nanotube (SWNT)	Hypertension
oxLDL	Phorphyrinic nanosensor	Ischemia/reperfusion
Cholesterol	In_2O_3 nanowire-based FET	Acute heart attack
Blood pressure	Piezoelectric-BioMEMS	Pressure monitoring
	Chip embedded flexible packaging (CEFP)	Myocardial infarction stenosis in heart bypass
Blood flow	Piezoelectric-BioMEMS	Grafts

23.9 Nanomaterials and Cosmetics

No discussion of nanomaterials would be complete without the mention of their application in the cosmetic industry. Nanomaterials can be found in many cosmetic products including moisturizers, hair care products, makeup, and sunscreens. Their use began in 1986. In 2006, the European Union estimated that 5% of cosmetic products contained nanoparticles.

Nanomaterials used in cosmetics include but are not limited to:

- Titanium dioxide and zinc oxide (sunscreens, cosmetics)
- Organic sunscreens
- Liposomes and niosomes (delivery vehicles, shampoo, minoxidil scalp lotion)
- Solid lipid nanoparticles
- Nanostructured lipid carriers
- Nanocrystals
- Nanoemulsions
- Cubosomes
- Dendrimeres

As before, the application of nanomaterials in cosmetics could fill a textbook. Therefore only brief highlights will be presented here. Readers are encouraged to review the literature and other sources for their specific applications. An excellent starting point

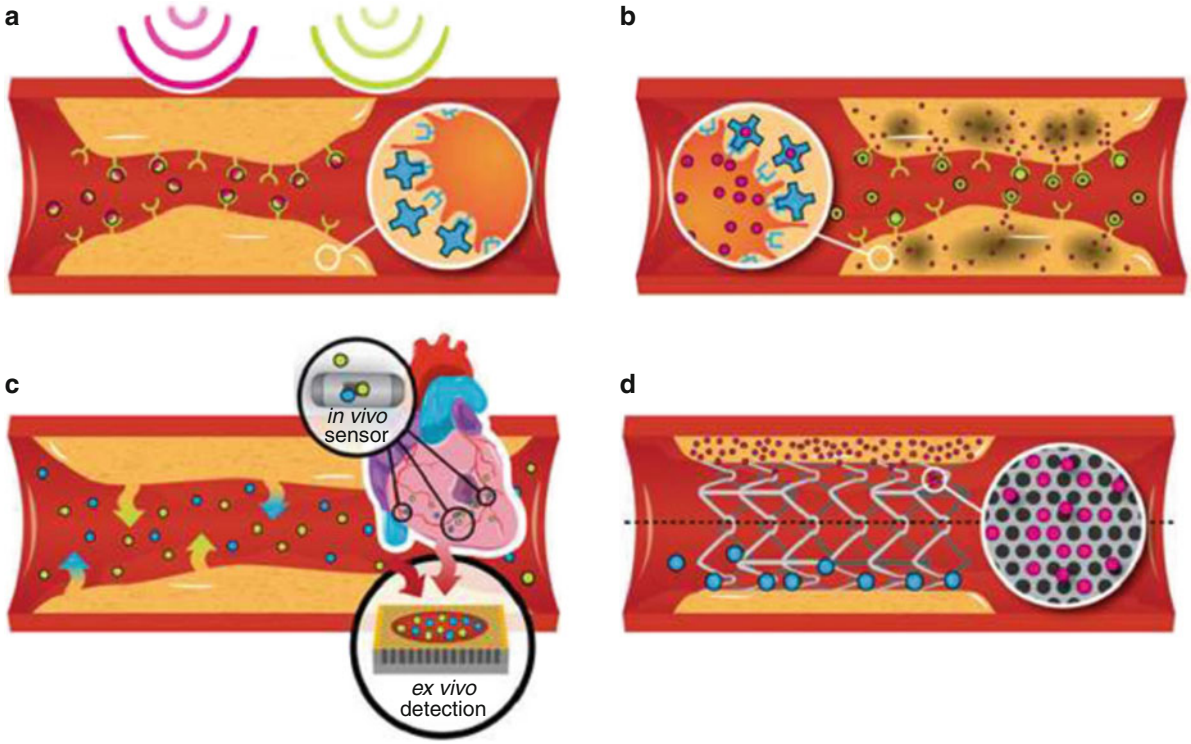


Fig. 23.27 Application of nanotechnology in the diagnosis and treatment of cardiovascular disease (Godin et al. 2010). Schematic presentation of various nanotechnological approaches for advanced CVD diagnosis and therapy: Nanoparticles for (**a**) multimodal image contrast and (**b**) improved treatment of CVD can be targeted to immune cells or the specific ligands presented on the inflamed endothelium of the atherosclerotic plaque; (**c**) in vivo sensors implanted in the pericardial region or on one of the main blood vessels and techniques for ex vivo biomarker detection; (**d**) nanostructured drug/nanoparticles eluting stents

can be found at The Institute of Nanotechnology, (www.observatorynano.eu) (http://www.observatorynano.eu/project/filesystem/files/Cosmetics%20report-April%2009.pdf) (Institute of Nanotechnology 2012). Hair follicles have been considered as reservoirs and entry points for nanomaterials. Somewhat to the chagrin of the lay press and consumer groups nanomaterials used in cosmetics appear to have a good track record to date (Morganti 2010). However, a primary concern is that there is no labeling stipulation for "nano" materials giving rise to suspicions of lack of labeling transparency. Use of nanomaterials in cosmetics suffers from toxicology limitations previously mentioned (Tables 23.1 and 23.2).

Additional confusion arises when lay and consumer reporters highlight research from one area and apply it to another; an example is from Trouiller et al., a report that received a lot of lay attention. The group found genetic anomalies in mice that were fed titanium dioxide in water (Trouiller et al. 2009). No dermal work was reported. However, they mentioned that titanium dioxide nanoparticles are used in a variety of materials from paint to cosmetics. Reference to cosmetics by others citing this work was inaccurate and confusing.

Nonetheless, consumers are entitled to transparency in labeling of any product that they use. Regulators cite the enormous volume of nanomaterial information, a reasonably safe track record, and lack of funding and resources as responses to consumer and press advocates for enhanced safety precautions.

23.10 Future Prospects

23.10.1 Handheld Diagnostic Devices

Using magnetic nanotechnology platforms, there is the promise of specific, economical, and rapid medical diagnosis either in the office or field environments. Training

Table 23.10 Cancer types and cell lines (human) used with active targeting methods directed against a variety of surface receptors (Rosen)

Targeting agent	Cell lines used for in vitro studies with this targeting agent	Cell lines used for in vivo studies with this targeting agent	Cancers that this method has been shown to be able to target
Monoclonal antibodies	LX-1, TE8, L-2981[a], C-3347[a], M-2669[a], NCI-125[a], H2981[a], H3347[a], LNCaP, WiDr, MCF-7, AU-565	LX-1, TE8, WiDr	Small cell lung carcinoma, squamous-cell carcinoma of the esophagus, lung adenocarcinoma, colon carcinoma, melanoma, non-small cell lung carcinoma, prostate cancer, mammary adenocarcinoma, breast adenocarcinoma
Nanobodies	LS174T[a], SW2[a], A431[a], HeLa[a]	LS174T[a], SW2[a], SK-OV-3[a], A431[a]	Colon carcinoma, small cell lung carcinoma, ovarian adenocarcinoma, epidermoid squamous-cell carcinoma
Affibodies	SKOV-3[a], SKBR-3[a], Capan-1[a]		Ovarian adenocarcinoma, breast adenocarcinoma, pancreatic adenocarcinoma
Aptamers	LNCaP CCRF-CEM, Ramos, Toledo, Sup-T1[a], Jurkat[a], Molt-4[a], SUP-B15[a], U266[a], Mo2058, NB-4[a], K562[a], BNL 1ME A.7R.1[a], H23[a], A549[a], HLAMP[a], NCI-H460[a], NCI-H299[a], NCI-H520[a], NCI-H157[a], NCI-H446[a], HepG2[a], Huh7[a]	BNL 1ME 1.7R.1[a]	Prostate cancer, acute lymphoblastic leukemia, B-cell lymphoma, diffuse large-cell lymphoma, T-cell acute lymphoblastic leukemia, mantle cell lymphoma, acute promyelocytic leukemia, Burkitt's lymphoma, chronic myelogenous leukemia, non-small cell lung cancer non-small cell lung cancer (adenocarcinoma), non-small cell lung cancer (large cell lung cancer), non-small cell lung cancer (large cell carcinoma), non-small cell lung cancer (squamous cell carcinoma) Small cell lung cancer, liver cancer

[a]Cell lines not tested directly with SPIONs

to operate these battery-powered systems could be minimal, enabling their use in developing countries with minimal user training (Gaster et al. 2011).

23.10.2 Personalized Medicine

The multiple utility of nanomaterials has given rise to the concepts of "nanobiotechnology" and "theradiagnostics," where targeted particles carry diagnostics and treatments combined designed for application in specific patients.

Personalized medicine is defined as the prescription of specific treatments best suited for an individual taking into consideration both genetic and other factors that influence the response to therapy. Personalized medicine is the best approach to integrate new biotechnologies into medicine in order to improve understanding of the pathomechanism of diseases, molecular diagnosis and integration with therapeutics. Nanomedicine is defined as the application of nanobiotechnology to medicine. Nanobiotechnology also makes important contributions to personalized medicine through refinement of various technologies used for diagnostics and therapeutics as well as interactions among these (Fig. 23.29).

Innovative diagnostic technologies that are increasingly utilized in personalizing treatment include novel polymerase chain reaction (PCR), direct molecular analysis without amplification, DNA sequencing, biochips/microarrays, and nanobiotechnologies. Technologies for detection of copy number variations, single nucleotide polymorphisms, and cytogenetic alterations provide basic information for personalized medicine. Molecular diagnostic technologies are used for the detection of biomarkers, which may form the common basis of diagnostics and therapeutics.

Nanobiotechnology techniques provide an analysis of DNA and protein and could dramatically improve speed, accuracy and sensitivity compared to conventional molecular diagnostic methods. Nanobiotechnology based approaches can provide more precise measurements and insight into nanoscale inherent functional components of living cells. Nanobiotechnology facilitates point-of-care diagnosis and the integration of diagnostics with therapeutics, thus advancing the development of personalized medicine (Jain 2011; Jain 2010).

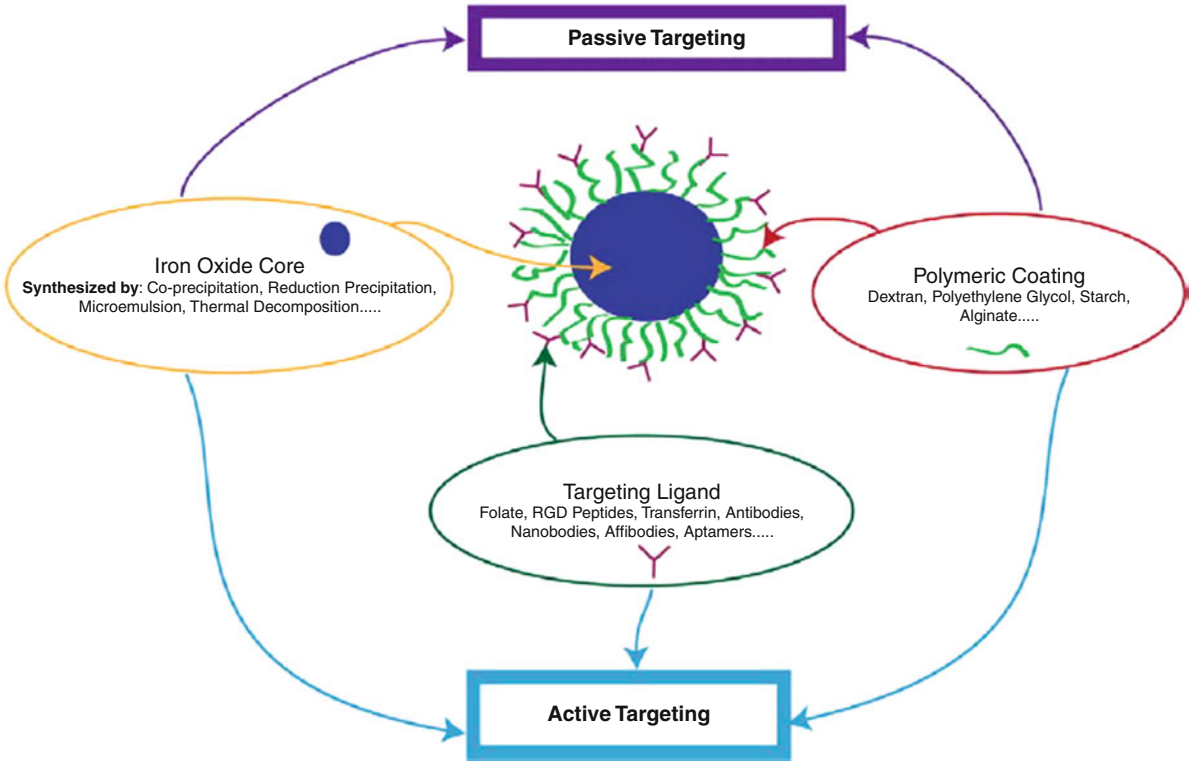


Fig. 23.28 Image showing the various components that make up active targeting–based and passive targeting–based SPIONs (Rosen et al. 2011)

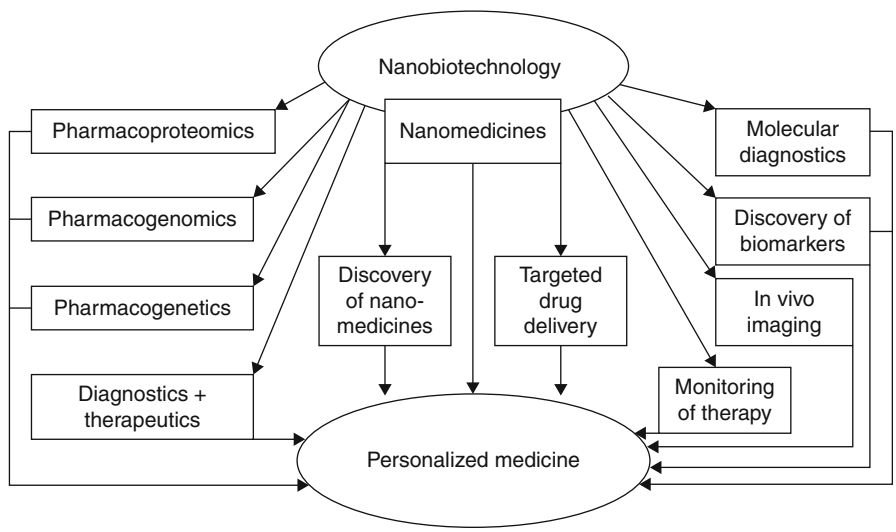


Fig. 23.29 Relationship of nanobiotechnology and personalized medicine (Jain 2010, 2011)

The Invention issue of *Time* magazine (Grossman 2011) provides some final insights on where nanotechnology will be taking direction. Fig. 23.30 shows the development of a drone hummingbird capable of all hummingbird flight motions (11 mph, right, left, forward, backward, up, down, hovering, "perching") and equipped with a camera. Drone insects are reportedly under development (Watson 2011). While still

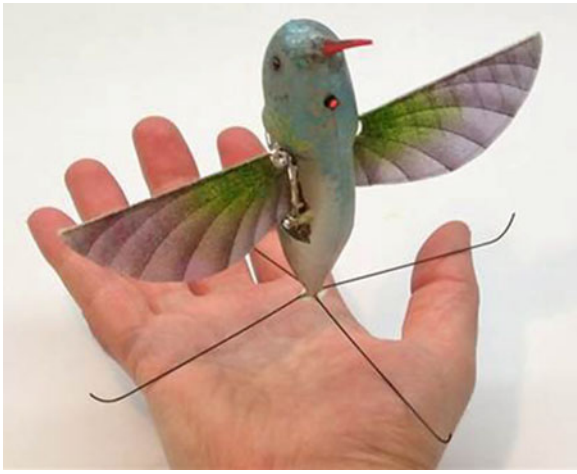


Fig. 23.30 Drone hummingbird (Grossman 2011; Watson 2011). This life-size hummingbird-like unmanned aircraft, named Nano Hummingbird, was developed by AeroVironment Inc., for the Defense Advanced Research Projects Agency. The prototype has a wingspan of 6.5 in., can fly up to 11 mph, and weighs just two-thirds of an ounce

"macrotechnology," these developments beg the question: Just how distant is the development of a true nanobot? A 22 nm, 3D TriGate transistor that will increase the performance and efficiency of computing could lend itself toward developing small, user friendly, nano diagnostic devices.

Finally, the development of a double-stranded-RNA-activated caspase oligomerizer (DRACO) molecule that is <10 nm in size. A DRACO is a genetically engineered molecule designed to trigger suicide in cells that have been invaded by a virus yet does not affect uninfected cells. It is reportedly effective against 15 viruses including rhinovirus (common cold), H1N1 influenza, and dengue fever (Rider et al. 2011).

23.11 Concluding Remarks

The purpose of this chapter was to introduce the reader to nanotechnology and its implications in a number of environments. Readers are encouraged to use these passages and references as a starting point in their own work in this rapidly expanding area. Nanotechnology represents the next technological revolution that will impact most areas of commercial application. In the 20 years prior to 2011, research in nanotechnology has grown rapidly because of predicted immense markets. Nanomaterials used in consumer products are a rapidly expanding area. Because nanomaterials have a multifunctional capability, there is a strong interest to apply them in the clinical environment as diagnostics, treatments, and in/as medical devices. Nanomaterials' variable reactivity is their great strength *and* weakness. Their application for benefit is only limited to the imagination; however, minute changes in a nanomaterial or product used in conjunction with a nanomaterial can have dramatic impact on patient safety.

Because of their variability, there is little product class information for nanomaterials. The clinical science of nanomaterials can sometimes yield variable or conflicting results because of a lack of standards and standardized testing. This has given rise to studying nanomaterials on a case-by-case basis. Progress in nano-research may become increasingly sluggish because of the lack of research standards and informatics.

Despite the current shortcomings, research in medical applications continues to expand and offers the promise of personalized diagnostics and treatments for a large variety of conditions that currently have high mortality and morbidity (e.g., cancer, heart disease, Alzheimer's). Once safe clinical use of nanomaterials has been achieved, it is ironic to consider the enormous healthcare impacts that will arise from the smallest of applications.

23.12 Additional Resources

Additional information regarding nanotechnology, and its applications, is available from:

Journals

International Journal of Nanomedicine (American Society for Nanomedicine)

Journal of Biomedical Nanotechnology (American Scientific Publishers)

Nanomedicine: Nanotechnology, Biology and Medicine (Elsevier)

Biomicrofluidics (American Institute of Physics)

Journal of Biomedical Materials Research (Wiley)

Angewandte Chemie International Edition (Wiley)

Biomaterials (Elsevier)

Nature Nanotechnology (Elsevier)

Small (Wiley)

Journal of Physical Chemistry Letters (ACS)
Nanoresearch (Springer)
Nanoethics (Springer)
Journal of Micro-Nano Mechtronics (Springer)
Journal of Nanoscience and Nanotechnology (American Scientific Publishers)

Selected Websites
www.fda.gov/ScienceResearsch/SpecialTopics/Nanotechnology/default.htm
www.azonano.com
www.epa.gov
www.nanoforum.org
www.nanotechlologied.weekly.com
http://www.mhra.gov.uk/Safetyinformation/Generalsafetyinformationandadvice/Technicalinformation/Nanotechnology/index.htm
www.nano.gov

Appendices

Appendix 1

Nanotechnology – Nomenclature

Nanosciences and nanotechnologies mean, in their concept, new approaches to the understanding and utilization of properties of the mass that critically depend on sizes, which are at the nanometer scale.

Nanoscience is the study of phenomena and manipulation of materials at atomic, molecular, and macromolecular scales, where properties significantly differ from those at larger scale.

Nanotechnologies are the design, characterization, production, and application of structures, devices, and systems controlling shape and size at the nanometer scale.

The presented definitions were formulated within the preparation of the British study "Nanoscience and Nanotechnologies: Opportunities and Uncertainties" in 2004 (An excellent reference document, however, published in 2004). It is important to define this interdisciplinary area of science and technology in order to make it different from classic disciplines of science and technology. That is the reason why the words with the prefix of "nano," like, for example, nanomaterials, nanomedicine, nanobiotechnology, nanoanalytics, nanoelectronics, and a number of others, but also nanochemistry and nanophysics, are often used, even if that can be sometimes misleading. On the other hand, many authors, institutions and companies do not use, in their fields of work done within the nanometer sizes, the prefix "nano" and that makes the identification of their activities more difficult and may even lead to not precise results of conducted research.

Nanotechnologies – Nomenclature (as of 2004 Which Includes but is Not Limited to Its Contents)

1. *Nanomaterials*
   (a) Nanopowder materials, nanoparticles, quantum dots, and nanofibers
   (b) Composite materials containing nanoparticles
   (c) Materials with carbon nanotubes or fullerenes
   (d) Thin layers, nanolayers, and nanocoatings
   (e) Nanostructural metals and alloys
   (f) Nanoceramics
   (g) Polymer nanocomposites and polymer nanomaterials
2. *Nanotechnology for the storage and transmission of information, micro- and nanoelectronics*
   (a) Nanoelectronics, materials, and equipment
   (b) Photonics
   (c) Optic materials, structures, and equipment
   (d) Magnetic materials and equipment, spintronics
   (e) Organic photonics and bioelectronics
   (f) MEMS, NEMS
3. *Nanobiotechnology and nanomedicine*
   (a) Encapsulating of drugs
   (b) Targeted transport of medicine (including genetic material)
   (c) Tissue engineering
   (d) Biocompatible and bio-analogical materials and layers
   (e) Molecular analysis and DNA analysis
   (f) Biological-inorganic interface and hybrids
   (g) Diagnostics and molecular recognition
4. *Nanotechnology for applications in sensors*
   (a) Sensors utilizing nanomaterials
   (b) Biomolecular sensors
5. *Nanotechnology in the (electric) chemical processing technologies*
   (a) Filtration, membranes, molecular sieves, and zeolites
   (b) Catalysis or electrodes with nanostructural surfaces

(c) Chemical synthesis, supramolecular chemistry

6. *Long-term research with the wide spectrum of applications*
   (a) Self-assembly
   (b) Quantum physics, quantum phenomena in nanosizes, and nanophysics
   (c) Nano- and mesoscopic systems
   (d) Chemical materials and processes – nanochemistry
   (e) Ultraprecise engineering
7. *Instruments and facilities, research, and applications of technologies*
   (a) Analytical instruments, methods, techniques, and research
   (b) Manufacture (preparation) of nanopowders (nanoparticles) and their processing
   (c) Facilities and methods for the creation of layers and coatings
   (d) Facilities and methods for the creation of objects (patterning, ECAP, fiber fabrication, etc.)
   (e) Ultraprecise machining and nanometrology
8. *Health, ecological, ethical, social, and other aspects of nanotechnologies*
   (a) Toxicity of nanoparticles
   (b) Environmental aspects
   (c) Social and ethical aspects (including regulatory issues)
   (d) Standardization
   (e) Patenting
   (f) Roadmaps and foresight
   (g) Popularization of nanotechnologies
   (h) Trade in nanoproducts

Source:
http://www.nanotechnology.cz/view.php?cisloclanku=2007100005, www.Nanotechnology.cz
http://www.nanotec.org.uk/finalReport.htm, The Royal Society of Engineers, Accessed 07 Dec 2011

Appendix 2. Uses of Nanotechnology in Medical Devices

Technology is shrinking at a rather rapid rate. As a result, more and more advancements are taking place at the cellular, molecular, and atomic levels – at the nanoscale. With scientific understanding growing, it is becoming possible to engineer the smallest devices and applications to help in a variety of fields. One of the fields that is likely to benefit greatly from nanotechnology is medicine.

Nanotechnology is especially important to medicine because the medical field deals with things on the smallest of levels. Additionally, the small nano devices that are being developed right now can enter the body and look around in ways that large humans can only dream of. Here are 25 ways that nanotechnology is revolutionizing medicine:

1. *Nanobots*: These devices have great potential for medical uses. These smallest of robots could be used to perform a number of functions inside the body, and out. They could even be programmed to build other nanobots, increasing cost efficiency.
2. *Nanocomputers*: In order to direct nanobots in their work, special computers will need to be built. Efforts to create nanocomputers, as well as the movement toward quantum computing, are likely to continue to provide new processes and possibilities for the science of medicine.
3. *Cell repair*: Damage to the cells of the body can be very difficult to repair. Cells are so incredibly small. But nanotechnology could provide a way to get around this. Small nanobots or other devices could be used to manipulate molecules and atoms on an individual level, repairing cells.
4. *Cancer treatment*: There are hopes that the use of nanotechnology could help in cancer treatment. This is because the small, specialized functions of some nano devices could be directed more precisely at cancer cells. Current technology damages the healthy cells surrounding cancer cells, as well as destroying the undesirables. With nanotechnology, it is possible that cancer cells could be targeted and destroyed with almost no damage to surrounding healthy tissue.
5. *Aging*: Nano devices could be used to erase some of the signs of aging. Already, laser technology can reduce the appearance of age lines, spots, and wrinkles. With the help of powerful nanotechnology, it is possible that these signs could be done away with completely.
6. *Heart disease*: There is a possibility that nanobots could perform a number of heart-related functions in the body. The repair of damaged heart tissue is only one possibility. Another option is to use

nano devices to clean out arteries, helping unclog those that have buildup due to cholesterol and other problems.

7. *Implanting devices*: Instead of implanting devices as we have seen in some cases, it might be possible to send a nanobot to build the necessary structures inside the body.
8. *Virtual reality*: Doctors could explore the body more readily with the help of a nanobot injection. Creating a virtual reality that would help medical professionals and others learn could help make some operations more "real" and provide practice ahead of time.
9. *Gene therapy*: Nanotechnology would be small enough to enter the body and even redesign the genome. This would be a way to alter a number of conditions and diseases. However, the human genome would need to be understood a little better for truly advanced gene therapy. However, nanobots would be qualified for swapping abnormal genes with normal genes and performing other functions.
10. *Drug delivery*: Systems that automate drug delivery can help increase the consistency associated with providing medication to those who need it. Drug delivery systems can be regulated using nanotechnology to ensure that certain types of medications are released at the proper time, and without the human error that comes with forgetting to take something.
11. *Nanotweezers*: These devices are designed to manipulate nanostructures. These can be used to move nano devices around in the body, or position them prior to insertion. Nanotweezers are usually constructed using nanotubes.
12. *Stem cells*: Nanotechnology can actually help adult stem cells morph into the types of cells that are actually needed. Studies showing how nanotubes can help adult stem cells turn into function neurons in brain-damaged rats.
13. *Bone repair*: It is possible to accelerate bone repair using nanotechnology. Nanoparticles mad up of different chemical compositions can help knit bones back together, and can even help in some cases of spinal cord injury.
14. *Imaging*: Nanotechnology can provide advancements in medical imaging by allowing a very specific and intimate peek into the body. Nano devices result in molecular imaging that can lead to better diagnosis of a variety of diseases and conditions.
15. *Diabetes*: Instead of having to draw blood to test blood sugar level, nanotechnology is providing a way for diabetics to use lenses to check their blood sugar. These nanocomposite contact lenses actually change color to indicate blood sugar level.
16. *Surgery*: We already have robotic surgeons in some cases, but nanosurgery is possible using some lasers as well as nano devices that can be programmed to perform some surgical functions. Being able to perform surgery at the smallest level can have a number of benefits for long-term medicine.
17. *Seizures*: There are nanochips being developed to help control seizures. These chips are meant to analyze brain signals and then do what is needed to adjust the brain so that epilepsy could be better controlled.
18. *Sensory feedback*: For those who have lost feeling in their body, it is possible to use nanotechnology to increase sensory feedback. Nanochips provide the opportunity for electrical impulses to be intercepted and interpreted.
19. *Limb control*: Prosthetics continue to advance, and nanotechnology is likely to help revolutionize the way paralysis is handled. There are some attempts to use nanochips that can help those who have lost limb control use their minds to send signals to provide a certain amount of motion.
20. *Medical monitoring*: You might be able to increase your ability to monitor your own body systems with the help of nanotechnology. Small nanochips implanted in your body could monitor your health and systems, and then send feedback to your computer or other device.
21. *Medical records*: In addition to monitoring your own body systems, nanotechnology can be used to send information to your health care providers and increase the efficiency of electronic medical records.
22. *Disease prevention*: Having a nano device in your body could actually help prevent diseases. With proper programming, it should be possible to help you avoid some diseases, repairing problems before they become serious issues. It may even be able to help prevent chronic diseases.

23. *Prenatal*: There are a number of ways that nanotechnology can help in terms of prenatal diagnosis. Being able to get inside the uterus and even inside the fetus without causing trauma can be beneficial to prenatal health, and nanotechnology can also help potentially repair problems in the womb.
24. *Individual medicine*: Nanotechnology is moving toward making medicine more personal. Being able to accurately work up your genome can help health providers more precisely pinpoint the proper treatments and tweak a treatment plan according to your individual needs and responses.
25. *Research*: Nanotechnology is advancing medical research, providing the tools that can help us learn more about the body and how it functions, as well as providing insight into chemistry and physics, which provide the building blocks for the body.

Source:

25 Ways Nanotechnology is Revolutionizing Medicine. Future Medica. http://mritechnicianschools.net/2010/25-ways-nanotechnology-is-revolutionalizing-medicine/, Accessed 13 Dec 2011

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Safe Chemicals/REACH

24

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24.1 Introduction

Regulation (EC) No 1907/2006 on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) initiated a significant change in the way industrial chemicals are regulated in the European Economic Area (EEA). It harmonizes the control of chemicals placed on the European market and replaces a large number of European and national regulations with a single legal framework. One of the major changes established by the REACH regulation is that the burden of proof on chemical safety is shifted from government to industry. EU manufacturers and importers are responsible for gathering information on the properties of their chemical substances, which will allow their safe handling, and to register the information in a central database run by the REACH regulator, the European Chemicals Agency (ECHA), based in Helsinki. The agency manages the databases necessary to operate the system, coordinates the in-depth evaluation of potentially hazardous chemicals, and is building a public database in which consumers and professionals can find hazard information.

A striking difference from the old regime is the requirement to provide safety information for *all* existing chemicals placed on the EU market at annual volumes above 1 tonne per annum (t/a). Prior to REACH, all chemicals first placed on the EU market before 1981 were grandfathered; that is, they could continue to be used without providing any data on intrinsic toxicological properties. These existing chemicals accounted for approximately 100,000 substances are listed in the *E*uropean *IN*ventory of *E*xisting *C*ommercial chemical *S*ubstances (EINECS). Since 1981, approximately 2,700 new substances have

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been introduced to the market and thus subject to a substantial hazard and risk assessment. Of the 2,700 new substances, 70% have been found to be "dangerous" as defined by EU Directive 67/548, because of corrosive, flammable, mutagenic, carcinogenic, developmentally or otherwise toxic, irritating, or sensitizing properties (Strategy for a Future Chemicals Policy—White Paper. COM (2001) 88 final, 27 February 2001). Companies now face the challenge to demonstrate chemical safety for approximately 30,000 grandfathered chemicals marketed in annual volumes above 1 t/a on which toxicological data is limited or does not exist. To complete this task, REACH requirements will be phased in over about a decade, first addressing high production volume chemicals produced or imported at volumes above 1,000 t/a as well as the most dangerous chemicals (i.e., classified as carcinogens, mutagens, or reproductive toxins or toxins with long-term aquatic toxicity). The registration deadline for these substances was December 2010. Chemicals produced or imported at volumes between 100 and 1,000 t/a will need to be registered by May 2013, and registration of chemicals produced or imported at volumes between 1 and 100 t/a is required by May 2018. As a result, all chemicals placed on the EU market in volumes above 1 t/a will be assessed within 10 years after REACH came into effect on January 1, 2008. In comparison, under the old regime, only 141 substances were prioritized for comprehensive risk assessment under the Existing Substances Regulation (Regulation (EEC) 793/93), and in the first decade after adoption, draft risk assessments were finalized for less than half of them.

24.2 REACH: Basic Principles

REACH is an acronym for Registration, Evaluation, Authorisation and Restriction of Chemicals. The registration is initiated jointly by all companies manufacturing or importing the same substance in annual volumes of 1 t or more. The main objective of the registration is to identify and manage risks linked to manufacturing and importing of chemicals. The REACH registration dossier consists of all available data on a substance, including proof of unambiguous substance identity, data on physicochemical properties, toxicological and ecotoxicological hazards, and environmental fate and behavior. In contrast to the old chemical legislation, REACH does not regulate chemicals based on their hazard profile alone but also on potential exposure to humans and the environment as well. Consequently, the registrant needs to evaluate if specific exposure scenarios are considered to be safe, or if a potential user needs to establish specific measures to ensure a safe use. The result of this consideration is documented by means of a Chemical Safety Report (CSR) which is part of the REACH registration dossier that is subsequently submitted to the European Chemicals Agency (ECHA). The amount of information that is needed for a valid registration depends on the annual tonnage. There are four tonnage bands requiring various types of hazard evaluation data—1–10 t/a, 10–100 t/a, 100–1,000 t/a, and above 1,000 t/a—and four corresponding annexes (VII–X) specifying the information requirements. The annual volume not only defines the amount of information that needs to be gathered but also the deadline for registration, that is, 2010, 2013, and 2018 (Box 24.1). It is important to note that these transition periods only apply to substances that have been preregistered with ECHA in accordance with Article 23 before December 1, 2008.

The second phase of the REACH process is the evaluation of dossiers performed by ECHA. The agency runs different types of evaluation ranging from a basic technical completeness check of a registration dossier to a thorough assessment of all submitted data and the validity of the ultimate risk assessments. The latter will be performed on at least 5% of all dossiers received by the agency for each tonnage band (Article 41). Evaluation may lead to the conclusion that action should be taken under the restriction or authorization procedures or that risk management action should be considered in the framework of other appropriate legislation. The ECHA reported that dossier evaluations of the 2010 registrations showed that the quality of many of the chemical safety assessments was of concern and needed to be improved by the registrant (Evaluation under REACH Progress Report 2010: (http://echa.europa.eu)). Hence, reviewing only 5% of the dossiers may potentially be a significant limitation in the REACH system.

The last phase of the REACH process is the restriction and authorization of chemicals. The aim of the authorization process is to assure that the risks from substances of very high concern (SVHC) are properly controlled and that these substances are progressively

Table 24.1 Entry for DEHP in Annex XIV to the REACH regulation

Entry Nr	Substance	Intrinsic property(ies) referred to in Article 57	Transitional arrangements		Exempted (categories of) uses
			Latest application date	Sunset date	
4.	Bis(2-ethylhexyl) phthalate (*DEHP*) EC No: 204-211-0 CAS No: 117-81-7	Toxic for reproduction (category 1B)	August 21, 2013	February 21, 2015	Uses in the immediate packaging of medicinal products covered under Regulation (EC) No 726/2004, Directive 2001/82/EC, and/or Directive 2001/83/EC

Table 24.2 Entry for phthalates in Annex XVII to the REACH regulation

Column 1	Column 2
Designation of the substance, of the group of substances, or of the mixture	Conditions of restriction
51. The following phthalates (or other CAS and EC numbers covering the substance): (a) Bis (2-ethylhexyl) phthalate (DEHP) CAS No 117-81-7 EC No 204-211-0 (b) Dibutyl phthalate (DBP) CAS No 84-74-2 EC No 201-557-4 (c) Benzyl butyl phthalate (BBP) CAS No 85-68-7 EC No 201-622-7	1. Shall not be used as substances, or in mixtures, in concentrations greater than 0.1% by weight of the plasticized material, in toys and childcare articles 2. Toys and childcare articles containing these phthalates in a concentration greater than 0.1% by weight of the plasticized material shall not be placed on the market 3. The commission shall reevaluate, by January 16, 2010, the measures provided for in relation to this entry in the light of new scientific information on such substances and their substitutes, and if justified, these measures shall be modified accordingly 4. For the purpose of this entry, "childcare article" shall mean any product intended to facilitate sleep, relaxation, hygiene, the feeding of children, or sucking on the part of children

replaced by suitable alternatives where economically and technically viable. SVHCs are substances that meet one or more of the following criteria:

- Carcinogenic, mutagenic, or toxic to reproduction (CMR), meeting the criteria for classification in category 1a or 1b according to the CLP-Regulation (EC) No 1272/2008
- Persistent, bioaccumulative, and toxic (PBT) or very persistent and very bioaccumulative (vPvB) according to the criteria in Annex XIII of the REACH regulation
- Identified, on a case-by-case basis, from scientific evidence as giving rise to an equivalent level of concern as those above (e.g., endocrine disrupters or neurotoxicants)

As described by the agency, the initial steps of the authorization process are the identification of SVHCs and their inclusion in the candidate list, and the subsequent prioritization of those substances for inclusion in the authorization list (Annex XIV to the REACH regulation). Proposals for inclusion in the candidate list are prepared by a member state or by ECHA on request of the commission. Each substance included on the authorization list is assigned a specific phase out date (Table 24.1). After this "sunset date," substances may be placed on the market only if an authorization has been granted for a specific use or the use has been exempted from authorization. The application for authorization has to be done by each individual member of the supply chain (manufacturer, importer, only representative (OR), or downstream user) and authorization will in general be granted per use and per individual user. However, if an authorization is granted within a supply chain, all members downstream from the applicant are covered by this single authorization.

The European Commission has authority for decisions regarding authorizations. The EU white paper estimates that about 1,400 substances are considered to be SVHC (Strategy for a Future Chemicals Policy–White Paper. COM (2001) 88 final, 27

February 2001). Some of these substances are likely to be banned altogether. REACH explicitly invokes the precautionary principle—that when scientific evidence suggests a substance may harm human health or the environment but the type or magnitude of harm is not yet known, the burden of proof for the safety of the substances falls on the manufacturers and importers, and interim risk management measures need to be implemented until a final assessment is achievable.

In addition to the authorization, EU authorities may impose restrictions on the manufacture, use, or placing on the market of substances causing unacceptable risks to human health or the environment. This restriction process is implemented as a "safety net" to manage risks that are not addressed by other REACH processes and if it is demonstrated that risks need to be addressed on a community-wide basis. The restriction process is initiated by member states competent authorities or the agency on request from the commission by preparing an Annex XV dossier. These restriction dossiers need to justify that the proposed restriction is the most appropriate risk management measure. All agreed restrictions will be published in Annex XVII to the REACH regulation. Any subsequent manufacture, placing on the market, or use of the substance must comply with the conditions of the restrictions. An example of an entry in Annex XVII is given below (Table 24.2). In contrast to the registration, both authorization and restriction are independent form the volume. They apply to substances manufactured or placed on the market, irrespective of quantity.

Box 24.1 REACH: Timeline.

1998	The European Commission (EC) began developing REACH
2001	EC adopted a white paper, "Strategy for a Future Chemicals Policy," detailing the proposed system
June 1, 2007	REACH came into force
June 1, 2008	Preregistration for existing ("phase-in") substances and registration for new ("non-phase-in") substances starts
October 28, 2008	ECHA publishes first candidate list (SVHC)
December 1, 2008	Preregistration for "phase-in" substances ends and registration for existing substances (that have not been preregistered) starts
January 1, 2009	ECHA publishes first list of preregistered substances
December 1, 2010	Phase 1: By this date, preregistered "phase-in" substances should have been registered when supplied at: >1,000 t/a >100 t/a and classified as very toxic to aquatic organisms >1 t/a and classified as Cat 1a or 1b (CLP) carcinogens, mutagens, or reproductive toxicants
January 3, 2011	Notification of substances to ECHA's Classification and Labeling (C&L) Inventory
February 17, 2011	ECHA publishes first Annex XIV (authorization list)
June 1, 2011	Notification of SVHC in articles if present at >0.1% (w/w) and exceeding annual volume of 1 t/a
June 1, 2013	Phase 2: By this date, preregistered "phase-in" substances should have been registered when supplied at ≥100 t/a
June 1, 2018	Phase 3: By this date, preregistered "phase-in" substances should have been registered when supplied at ≥1 t/a

(*continued*)

24.3 The Scope of REACH

The REACH regulation covers most chemical substances that are manufactured in or imported into the EU and applies to substances used alone and in mixtures, as well as in articles, if the substance is intended for release during normal and reasonably foreseeable conditions of use. The scope of REACH is defined in Article 2 of the regulation. Substances for which risks associated with manufacture and use are sufficiently covered by other legislations may be totally exempted from the requirements of REACH. Substances which fall into the following categories are fully exempt from REACH: radioactive substances within the scope of Council Directive 96/29/Euratom, substances which are subject to customs supervision, nonisolated intermediates, the carriage of dangerous substances, and waste as defined in Directive 2006/12/EC. Other substances are partially exempt from certain aspects of REACH because they fall under the scope of more specific legislation. These include substances in medicinal products, cosmetic products, medical devices, or food and feeding stuffs. In addition, some substances are specifically exempted in Annex IV, as sufficient information is known about their intrinsic properties and they are considered to cause minimum

risk, or in Annex V, because registration is deemed inappropriate or unnecessary for these substances.

Although polymers are not subject to registration and evaluation, the monomers or other substances within the polymer require registration. Moreover, there are partial exemptions of substances for specific uses such as for scientific or product- and process-oriented research and development.

Scientific research and development (R&D) under REACH means any scientific experimentation, analysis, or chemical research carried out under controlled conditions in a volume below 1 t/a. Substances in those low quantities are not subject to registration by default. But in contrast to regular substances that are subject to authorization and restriction when imported or manufactured in quantities below 1 t/a, substances used in R&D are exempted from these obligations.

In order to encourage innovation, substances used in product- and process-oriented research and development (PPORD) are exempted from the obligation to register for a period of up to 10 years if they are notified to the agency for this use. Hence, chemicals used for drug development and clinical trials may be exempted from REACH requirements. A prerequisite for using this exemption is that the substances are only to be used by a number of listed customers included in the notification and that the risks to human health and the environment are adequately controlled in accordance with the requirements of legislation for the protection of workers and the environment. The agency could decide to impose conditions to ensure that a substance will be handled only by staff of listed customers in reasonably controlled conditions and will not be made available to the general public and that remaining quantities will be recollected for disposal after the exemption period. In contrast to scientific R&D, substances used in PPORD are subject to authorization and restrictions, unless an exemption is explicitly specified in Annex XIV and XVII.

24.4 Pharmaceuticals Under REACH

Medicinal products for human or veterinary use are partially exempted from REACH. This applies to the registration, evaluation, and authorization but does not affect the obligation to communicate along the supply chain or comply with restrictions as defined in Annex XVII. Importantly, substances (active ingredients and excipients) are exempted only to the extent that they are used in medicinal products in accordance with Regulation (EC) No 726/2004, Directive 2001/82/EC, and Directive 2001/83/EC. The exemption is not applicable to quantities of substances used for other nonmedical applications like cosmetics, fermentation, or other industrial applications. It is also not applicable to chemicals in pharmaceutical production that do not end up in the finished product like starting materials, intermediates, and process chemicals.

References and Further Reading

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency.

Study Design and Statistics

25

S. W. Mittelstadt

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25.1 Introduction

The International Conference on Harmonisation (ICH) S7A guideline for safety pharmacology studies was adopted in 2000. The ICH S7A guidelines describe a core battery of studies that are to be conducted prior to first administration in humans. These studies are conducted to assess potential effects on the respiratory, cardiovascular, and central nervous systems. Due to the safety implications of results from these studies, it is recommended that such studies be conducted according to good laboratory practices (GLP).

Importantly, safety pharmacology studies are not limited to the core battery studies that are described in the ICH S7A (Guth and Siegl 2007). Pharmaceutical companies are putting additional effort into understanding potential effects on the respiratory, cardiovascular, and central nervous systems earlier in the discovery process. These studies are not conducted GLP, and they often use smaller animals or in vitro systems. The study design of these early studies varies significantly from company to company and usually do not meet the ICH requirements. Study designs depend on the company's internal data and willingness to take business risks when deciding to move compounds forward in the development process. Because of the wide variety of study designs utilized by companies early in discovery, this chapter will not attempt to characterize those studies. In contrast, as companies move into the safety pharmacology studies required by ICH guidelines, less variability is observed across companies. This chapter will specifically discuss studies required by the ICH S7A guidelines and factors that should be considered when they are being designed. Although, there is a fair amount of consistency across the core

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battery of studies, there are also decisions that must be made depending on the properties and the intended use of the drug being developed. The components of study design that must be considered are model selection, sample size, route of administration, dose selection, and data analysis.

25.2 Respiratory System

The ICH S7A guidelines state, "Effects of the test substance on the respiratory system should be assessed appropriately. Respiratory rate and other measures of respiratory function (e.g., tidal volume or hemoglobin oxygen saturation) should be evaluated. Clinical observation of animals is generally not adequate to assess respiratory function, and thus these parameters should be quantified using appropriate methodologies."

25.2.1 Model Selection

The rat is the preferred species in most drug development programs, and plethysmography is the preferred technique to measure respiratory function. In addition to the measurement of respiratory rate, most companies assess tidal/minute volume as their other measure of respiratory function (Lindgren et al. 2008). The most accurate plethysmographs are designed to directly monitor lung volume changes or airflows generated by thoracic movements. This is accomplished by isolating openings to the upper airways from the thorax and is referred to as "head-out" or "head-enclosed" chambers. The primary advantage of this technique is that the pressure changes in these chambers are a direct measure of lung airflow and accurately measure both duration and volume of each breath (Murphy 2005). In addition, this technology can be utilized for commonly used non-rodent species such as dogs and monkeys. The primary disadvantage of this technology is that restraint of the animals is required. Restraint of animals could potentially effect baseline parameters and impact the response to drugs (Ewart et al. 2010).

Whole body (barometric) plethysmography is also used to monitor ventilatory patterns. Using this method, the volume of each breath is determined indirectly from relatively minor increases in chamber pressure resulting from the expansion of inhaled gases due to a decrease in intrapulmonary pressure and to increases in intrapulmonary temperature and humidity (DeLorme and Moss 2002; Murphy 2005). The primary advantage of the technology is that animals are not restrained and are allowed to roam freely. The primary disadvantage is that it is an indirect measurement of respiratory function and has been shown to give different responses to drugs when compared to direct measurement. For example, whole body plethysmography measures an increase in tidal volume and a decrease in respiratory rate in response to inhaled methacholine. In contrast, direct measurement demonstrates only a decrease in respiratory rate (Delorme and Moss 2002). Despite the potential downside of utilizing an indirect measurement, whole body plethysmography remains a common technique used in the pharmaceutical industry to measure respiratory function because of the ability to make measurements without restraining the animals.

All of the techniques that are available for respiratory assessment in rodents are also available in the non-rodent species commonly used in drug safety assessment (dogs and monkeys) (Iizuka et al. 2010; Murphy et al. 2010). Despite their availability, large animals are not used as often as the rodent models. In general, most companies will conduct initial respiratory assessment in rats. Studies are conducted in large animals when driven by a scientific rationale. For example, effects observed in non-rodent toxicology studies could require follow-up studies further investigating respiratory effects or species differences in pharmacokinetic studies could drive a company to conduct respiratory studies in non-rodent species.

In addition to respiratory rate and minute volume, some scientists have also suggested that pulmonary resistance should be measured as part of the respiratory assessment prior to first administration to man (Murphy 2005; Murphy et al. 2001). Total pulmonary resistance defines the change in pleural, airway, or transpulmonary pressure (ΔP) required to produce a defined change in lung airflow (ΔF) and is calculated as $\Delta P/\Delta F$. In addition to the measurement of airflow described earlier, this procedure requires a pressure catheter for the measurement of pleural, airway, or transpulmonary pressure. The primary disadvantage of the technique is that the placement of a pressure catheter requires that the studies be conducted in either anesthetized animals or surgically implanted animals. A recent industry survey revealed that most

pharmaceutical companies are not including pulmonary resistance in their core battery of measurements (Lindgren et al. 2008). This is probably because compounds that are known to cause increased pulmonary resistance usually cause changes in respiratory rate, respiratory volume, or minute volume (Authier et al. 2009; Ewart et al. 2010). Although there is the potential for a drug to actually cause increased resistance (bronchoconstriction) without changes in rate or volume, published data on the frequency or severity of this are lacking. Therefore, it is considered low risk that a compound will cause adverse effects due to increased pulmonary resistance without also causing monitorable changes in respiratory rate or volume. In addition, the ability to get consistent and reliable resistance measurements in conscious animals remains difficult (Ewart et al. 2010).

25.2.1.1 Sample Size

When rodents are utilized, separate groups of animals are usually used for each dose group. The common dose group size for rodent respiratory studies is 8–10 (Authier et al. 2009; Legaspi et al. 2010). Three to four dose groups are used, allowing the assessment of a dose response. Since vehicles have been shown to impact respiration and environmental changes (i.e., noise) can also impact respiration, a vehicle control group should always be run on the same day as other dose groups.

25.3 Central Nervous System (CNS)

The ICH S7A guidelines state, "Effects of test substance on the central nervous system should be assessed appropriately. Motor activity, behavioral changes, coordination, sensory/motor reflex responses and body temperature should be evaluated. For example, a functional observation battery (FOB), modified Irwin's, or other appropriate test can be used."

25.3.1 Model Selection

Historically, most companies have assessed potential CNS effects of drugs by systematically documenting alterations in behavior of rodents. The standard method for assessing these changes has been the Irwin test (Irwin 1968) or the functional observational battery (FOB) (Moser 1989). The Irwin test was introduced to the pharmaceutical industry as a rapid psychomotor screening procedure that was commonly used in early discovery screening (Redfern et al. 2005). The FOB was first introduced as a component of neurotoxicity screening for the agrichemical industries and then adopted for use in the testing of pharmaceuticals (Trabace et al. 2000). Both assays have proven effective in assessing potential CNS effects of chemicals. The primary disadvantage is that they both require highly trained personnel to conduct the assays. Maintaining the training of personnel running these studies can be resource intensive (Mattson et al. 1996; Ross et al. 1998). In addition, the results and interpretation of these studies can be subjective (Markgraf et al. 2010).

Although the conduct of these CNS studies is most common in rodents, neurobehavioral test batteries have been developed for use in dogs (Gad and Gad 2003; Moscardo et al. 2009) and monkeys (Gauvin and Baird 2008; O'Keeffe and Liftshitz 1989). Investigators may choose to utilize a non-rodent species if the metabolism is similar to what is expected in man or if the non-rodent species is more pharmacologically relevant (e.g., monoclonal antibodies). In addition, nonhuman primates possess more similarities to man when compared to rodents, in terms of the nervous system anatomy, morphology, and functional process (Moscardo et al. 2010). Recent studies have concluded that neurologic toxicities observed in monkeys and dogs translated clinical findings better than other species (Igarashi et al. 1995; Olson et al. 2000).

Attempts have also been made to make the CNS measurements more automated thus reducing the training required for individuals running the studies and reducing subjective interpretation of the results. For example, automated assessment of motor activity by quantifying interruptions of infrared beams has been available for over a quarter century (Lynch et al. 2011; Menniti and Baum 1981). In addition, body temperature is commonly measured as part of cardiovascular telemetry studies (Lynch et al. 2008). A more recently commercialized, automated behavior analysis system that includes a locomotor activity component is the LABORAS (Lynch et al. 2011; Van de Weerd et al. 2001). Instead of using infrared photocells, LABORAS measures weight displacement and mechanical vibrations produced by the rodent and

translates the vibrations into electrical signals that the software converts into motor activity. The LABORAS system also converts the vibration patterns into additional behavioral parameters such as grooming, drinking, and feeding. At the present time, the LABORAS system is not commonly used within a regulatory environment in the pharmaceutical industry.

25.3.1.1 Sample Size

When rodents are utilized, separate groups of animals are usually used for each dose group. The common dose group size for rodent CNS studies is 6–10 (Lindgren et al. 2008). Three to four dose groups are used, allowing the assessment of a dose response. Since vehicles have been shown to impact behavior and environmental changes (i.e., noise) can also impact CNS parameters, a vehicle control group should always be run on the same day as other dose groups.

25.4 Cardiovascular System

The ICH S7A guidelines state, "Effects of the test substance on the cardiovascular system should be assessed appropriately. Blood pressure, heart rate, and the electrocardiogram should be evaluated. In vivo, in vitro and or ex vivo evaluations, including methods for repolarization and conductance abnormalities should also be considered."

The ICH S7B guidelines state, "The ionic mechanism of repolarization in adult rats and mice differ from larger species, including humans (the primary ion currents controlling repolarization in adult rats and mice is I_{to}); therefore, use of these species is not considered appropriate. The most appropriate in vivo test systems and species should be selected and justified."

25.4.1 Model Selection

The ICH guidelines clearly state that cardiovascular studies should not be run in rodents because of the differences in cardiovascular electrophysiology when compared to humans. The most common species used for cardiovascular assessment is dog. In a survey by Lindgren et al. (2008), 100% of the companies that responded utilized dogs to assess cardiovascular effects. There is no clear difference in clinical relevance when comparing dog and nonhuman primate cardiovascular studies. The primary advantage of dogs is that primate studies cost more and many companies have more experience running dog studies. Most companies will move to monkeys for scientific reasons. For example, if a drug causes emesis in dogs and appropriate systemic exposures cannot be achieved, monkeys may be an alternative. Similarly, if metabolism of the drug by nonhuman primates is most like human metabolism, monkeys may be used.

The ICH guidelines include swine as a potential species for the assessment of cardiovascular drug effects. However, the pharmaceutical industry utilizes the minipig less than dogs and monkeys for assessment of cardiovascular safety (Lindgren et al. 2008). The reason for this is probably not scientific, since minipigs have similar cardiovascular electrophysiology to humans (Laursen et al. 2011). In addition, hERG blockers (moxifloxacin, haloperidol, sotalol), β-adrenergic antagonists (propranolol, sotalol, esmolol), α_2-adrenergic agonists (medetomidine), opioid agonists (remifentamil), and dopamine induce clinically relevant cardiovascular effects in minipigs (Authier et al. 2011). The primary reason minipigs are not utilized is that most pharmaceutical companies have a larger historical data base on preclinical safety in dogs and monkeys. Therefore, companies tend to default to models where they have the most experience.

In addition to species selection, the ICH S7A guidelines state that it is preferable to use unanesthetized animals to assess the cardiovascular effects. This is due to the fact that cardiovascular effects due to neurohumoral responses that would be present in conscious animals can be blunted by anesthesia (Cox 1972). Since 1965, the technology to remotely monitor blood pressure and pulse rate by telemetry in conscious animals has been available (Sarazan and Schweitz 2009; Van Citters and Franklin 1966). The utilization of this technology shifted from academic laboratories to pharmaceutical safety assessment in the 1990s (Sarazan et al. 2011) and is presently the technology that is most utilized to conduct conscious animal studies.

In addition to telemetry, chronically instrumented dogs that are conditioned to the laboratory setting can be utilized for cardiovascular assessment. Dogs sitting quietly in a sling allow for easy intravenous dosing and blood sampling. If the dogs are appropriately trained to the sling and laboratory environment, resting heart

rates of 50–60 beats/min are common (Fossa 2008). This resting heart rate is actually lower than most resting heart rates reported in telemetry studies. The disadvantages of using animals conditioned to the laboratory environment are the time and resources required to train the animals, the time in the sling may be limited (less than 8 h of data), and nonhuman primates cannot be conditioned like dogs. Due to the practical limitations of sling trained dogs, telemetry studies will probably remain the most common method for conducting conscious animal studies.

25.4.1.1 Study Sensitivity

A significant amount of discussion has occurred in recent years around the appropriate sensitivity of nonclinical cardiovascular safety studies (Sarazan et al. 2011). The studies probably do not need to be highly sensitive if their main purpose is to protect phase 1 human volunteers from potentially life-threatening effects. This is because large effects, which would be considered life threatening, can be observed in studies with lower sensitivity. However, if the scientist wants to reduce the risk of advancing a molecule with any degree of cardiovascular liability, the sensitivity must be increased. For example, a clinical thorough QTc study is designed to have the sensitivity to detect very small changes in QTc (~5 ms). Therefore, some scientists would argue that the nonclinical studies should have a similar statistical sensitivity. Factors that impact sensitivity include, study design, number of animals, and data variability. Although extensive discussion has taken place regarding the sensitivity of cardiovascular studies, minimal discussion has taken place about the sensitivity of CNS and respiratory assays.

25.4.1.2 Study Design

The Latin square crossover design is commonly used in nonclinical cardiovascular studies testing small molecules in conscious animals (Sarazan et al. 2011). The most basic design utilizes four animals and four treatment groups (vehicle, low dose, mid-dose, high dose) administered on four different days. Daily treatments are assigned in a balanced fashion, such that each treatment group is represented on a given day and all four animals receive all treatments, none in the same order. An appropriate washout period is allowed between doses so that drug exposures return to baseline levels before the next dose. An example of a Latin square 4×4 design is shown in Table 25.1. One of the primary advantages of a Latin square design is that each animal provides information for each treatment group; thus, the number of animals required to detect the change of a specific magnitude is reduced. When using the Latin square design, there can be no treatment carryover effect that would influence responses to subsequent treatments. Carryover effects could be due to slow elimination, the presence of an active metabolite or desensitization of the receptor targeted by the drug. Drugs that have a carryover effect would not be good candidates for this study design.

Table 25.1 Example of a Latin square 4×4 design

Animal ID	Dose day 1	Dose day 2	Dose day 3	Dose day 4
No. 1	Low dose	High dose	Vehicle	Mid-dose
No. 2	High dose	Mid-dose	Low dose	Vehicle
No. 3	Vehicle	Low dose	Mid-dose	High dose
No. 4	Mid-dose	Vehicle	High dose	Low dose

In an escalating dose design, all animals receive all doses (Sarazan et al. 2011). Unlike the Latin square design, all animals receive the treatments in the same order, starting with the vehicle. The big advantage of this design is that it allows for dose modification as the study evolves, making it preferable when there is minimal preliminary pharmacokinetic, pharmacology, and toxicology data available. One concern with this design is the possibility of initial doses causing desensitization of the pharmacological effect, leading to a smaller effect in the high-dose animals. Also of concern is if the study environment is different on separate days of dosing. For example, increased noise in the facility on a dosing day could lead to higher heart rate and blood pressure. It would be difficult to separate the environmental effect from a potential drug effect, and one could conclude that a drug had a cardiovascular effect when the effect was actually due to environmental differences.

The parallel design is seldom used for stand-alone large animal cardiovascular studies. In this design, a group of animals is separately assigned to only one-dose group (Sarazan et al. 2011). The advantage of this design is that data accumulation and analysis can continue for as long as desired after or during treatment. This advantage allows the parallel design to be used for compounds such as biologics that may have extended biological effects or this design can be used when investigating the repeat-dose effects of a compound.

One disadvantage is that the number of animals required to run this type of study is much higher. If you assume that four to eight animals will be required per dosing group and if the study design includes four dosing groups, a large study of 16–32 animals will be required. The second, disadvantage for this study is that each animal no longer serves as its own control. Therefore, this design is less sensitive (Chiang et al. 2004).

25.4.1.3 Data Variability

One of the most important factors impacting the statistical sensitivity of a study is the background variability in the data. In addition to inter-animal variability that can be controlled by study design (i.e., Latin square design), there is also the reproducibility within each animal from day to day (intra-animal variability) (Chaves et al. 2006). Intra-animal variability can be reduced in animals by the quality of the surgical telemetry implants and the environment that the animals are housed (Chaves et al. 2006; Klumpp et al. 2006). It is very important to acclimate animals to the setting in which they will be tested. Not only will testing animals under a familiar setting reduce variability but it can also reduce resting cardiovascular parameters, increasing the probability of detecting drug-induced cardiovascular changes.

25.4.1.4 Animal Numbers

The most common group size across the industry is four to eight animals per dose when conducting cardiovascular telemetry studies (Lindgren et al. 2008). When designing cardiovascular studies, it is important to assess the assay sensitivity at a specific laboratory (Sarazan et al. 2011). The statistical sensitivity should be calculated based on experimental design and established variability (based on a historical database). Information about the assay sensitivity should be used prospectively by the sponsor to modify study design when indicated. Periodic recalculation of statistical power should be routinely conducted as a means to monitor drift in experimental procedures. If increases in variability (reduced statistical power) are observed, it may mean that procedures or the environment of the facility have changed over time. Assessment of those procedures may allow the investigators to reduce variability. If variability cannot be reduced, the number of animals required per dose group may have to be increased.

25.5 Route of Administration

The ICH S7A document states, "the expected clinical route of administration should be used when feasible. Regardless of the route of administration, exposure to the parent substance and its major metabolites should be similar to or greater than that achieved in humans when such information is available. Assessment of effects by more than one route may be appropriate if the test substance is intended for clinical use by more than one route of administration (e.g., oral and parenteral), or where there are observed or anticipated significant qualitative and quantitative differences in systemic or local exposure."

Since most safety pharmacology packages are designed to support first in human studies, they should use the route of administration that will be used in the phase I clinical trials. If the route of administration changes in the clinic, the safety pharmacology studies may need to be repeated. However, if an argument can be made that exposures are similar with the new route of administration, additional safety pharmacology studies may not be required. For example, if the initial safety pharmacology package was conducted with oral dosing, additional safety pharmacology studies may not be required if the clinical route changes to intravenous infusions as long as the Cmax in the clinic does not increase substantially.

25.6 Dose Selection

Prior to the ICH guidelines on safety pharmacology, most laboratories that conducted safety pharmacology studies for pharmaceutical companies were located within the discovery organization of their companies (Kinter et al. 1993). Discovery based groups tended to select the doses for safety pharmacology studies based on multiples of the efficacious dose. The primary change brought about by the ICH S7A guidelines was the recommendation that the highest dose should produce moderate adverse effects. The guidelines also state that some acute toxicities (e.g., tremors or fasciculation during ECG recording) may interfere with the ability to monitor functional changes, and these types of adverse effects need to be avoided. One option for dose selection is to use the same doses for the safety pharmacology and 28-day

toxicology studies. The reason for this is that the high dose in a 28-day toxicology study will usually cause an adverse effect after multiple days of dosing. However, the effect cannot cause a severe acute toxicity that will limit the number of days the animal can be dosed. The lack of severe acute toxicity allows the functional assessment of the CNS, respiratory system, and cardiovascular systems without interference from effects that may confound the interpretation of the results.

25.7 Summary

With the acceptance of the ICH guidelines on safety pharmacology, specific recommendations were outlined for the safety pharmacology package that is submitted to worldwide regulatory agencies. Despite the fairly detailed regulation, there are a number of important decisions that must be made based on the properties of each individual drug. The purpose of this chapter was to discuss the components of the study design that must be considered. In all cases, practical aspects (i.e., technology available may favor use of rodents) versus scientific aspects (i.e., monkeys may have similar metabolism to humans) must be taken into consideration and balanced. At the end of the day, each of these decisions must be defensible to both scientific and regulatory communities.

Conflict of Interest

The author of this chapter is currently employed at Abbott Laboratories, Abbott Park, IL, USA.

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Part 2

Pharmacokinetics

Safety Pharmacokinetics: Introduction

26

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As already stated in the introduction of the first edition, no toxicology or safety pharmacology assays are conceivable without a pharmacokinetic evaluation. If we talk about safety, we urgently need to know the exposure either of the animals used for safety studies or of the patients in clinical studies. It is common to talk about PK/PD, describing the relationship between Pharmacokinetics and pharmacodynamic efficacy, but in fact PK/Safety, describing the relationship between Pharmacokinetics and Safety, is at least as important. It is becoming ever more important since there is a clear tendency at the Regulatory Agencies to focus more and more on safety aspects—and those must always be linked to data about the individual exposures.

Pharmacokinetics per se is a relatively young and dynamic science. The basic principles—absorption, distribution, metabolism and elimination—are described in this section in detail, together with the technologies to generate data in all these aspects. Modifications in these areas together with progress in technology required an update and/or amendment to the respective chapters of the first edition. But besides these basic principles, which have already been well described, some new tendencies can be observed which have become more and more important for both scientific and regulatory reasons. One of these new approaches involves studies to evaluate the transporters which influence the pharmacokinetics of a drug and its metabolites in the body.

Until recent years few were aware of the influence of transporters, but now after only a short time, regulatory agencies are asking for details about the transporter contribution to drug disposition. For this reason, the chapters in these "cutting-edge-areas" have been completely adapted to give the most up to date information to the reader.

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Absorption: In Vitro Tests – Cell Based

27

Katharina Mertsch

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27.1 Introduction

In the pharmaceutical industry, we observed a change in paradigm during the 1990s: project teams focused their search for potent compounds not exclusively on potency and specificity—they added pharmaceutical, biopharmaceutical, pharmacokinetic, metabolic, and toxicological characteristics for the most interesting compounds as well. The motivation to characterize compounds for a set of pharmacokinetic and toxicological parameters was driven not only by economic reasons. The number of hits from combinatorial and other chemical libraries raised the necessity to increase quality of nominated drug candidates to reduce the attrition rate in preclinical and clinical stages of project development. Nearly all pharmaceutical companies introduced an expanded assessment for drugability and developability of compounds at the stage of lead selection and lead optimization (Borchardt et al. 1998).

The integration of pharmaceutical, pharmacokinetic, and toxicological criteria into discovery and development of compounds at the stage of compound lead nomination resulted in a higher percentage of successful clinical drug candidates. Most important criteria for developability and drugability of a compound include pharmacokinetic parameters like permeability, efflux potential, metabolic stability, metabolic profiling, protein binding, metabolic finger printing, inhibition and induction of major metabolizing enzymes, and safety aspects like Ames, HERG interaction potential, Irwin screening, Micronucleus tests, IC_{50} determination with important cells, and others.

This chapter will focus on one part of early ADME (adsorption, distribution, metabolism, excretion) strategy: the absorption and efflux screening.

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Since many projects focus on the most accepted route of administration—the oral one—assessment of oral bioavailability potential for a compound is a very important part in early ADME screening.

In vivo different factors play a crucial role during absorption processes: disintegration of the dosage form, dissolution of the compound in different parts of the gastrointestinal tract (GI), metabolism in the GI tract and in the liver (so-called first-pass), permeation across the intestinal mucosa, and clearance. There is no integrative in vitro model being able to cover all aspects if in vivo absorption. Therefore, an approach to divide the aspects into different test systems has been used: HTS methods for solubility testing, drug partitioning into membranes between aqueous and lipid phases, metabolic stability, permeability, CYP-inhibition and induction, efflux, protein binding, and of course in silico methods and Lipinskis rule of 5 are used prior first animal experiments in pharmacokinetics. Due to the in vivo complexicity, an in vitro prediction of absorption is only possible taking all factors of in vitro experiments together into account.

27.1.1 Absorption in the Gastrointestinal Tract

Absorption of compounds in the gastrointestinal tract occurs mainly in the small intestine with three different regions (duodenum, jejunum, and ileum) and in the large intestine (colon). In the small intestine, the uptake surface is increased by folds (3 times), villi (30 times), and microvilli (600 times). In the colon, folds and villi are absent (Daugherty and Mrsny 1999a, b).

The major physiological role of the GI—to provide a protection against pathogens and allow at the same time the permeation of nutrients and vitamins—is supported by the brush border structure of microvilli and glycocalyx. They form an enzymatic and physiological barrier. Mucus secreted by goblet cells and subepithelial glands interacts with compounds and can bind compounds (Larhed et al. 1998). During the passage through the GI, a compound passes regions with different pH: from slight acidic to slight basic impacting solubility and stability. The protection against pathogens in the GI is provided by lymphoid cells producing antibodies and forming a part of the immune system called the gut-associated lymphoid tissue (GALT, Mestecky and Mc Gee 1987). In the small intestine, lymphoid cells form structures called Peyers patches.

Peyers patches are covered by epithelial cells which can differentiate into M cells. M cells play an important role in the immunologic surveillance of the gut and are involved in specific functions like transport of particles, antigens, and macromolecules. M cells seem to be involved in the absorption of intact proteins (Walker and Sanderson 1992). Peyers patches have been studied for uptake of macromolecules. Their proximal vicinity to immunocompetent cells is another hurdle for industrial use as a preferred uptake route (Daugherty and Mrsny 1999a, b; Neutra 1998). M cells have been used to study uptake of lectins (for overview, see Daugherty and Mrsny 1999). However both cell types—Peyers patches and M cells—clearly are limited in their use as a preferred uptake route due to a small surface area they are covering and the limited capacity of absorbed molecules.

Epithelial cells covering the Peyers patches form the barrier in the intestine.

27.1.2 Mechanisms of Control and Barrier Function of Epithelial Cells

The main functions of the GI—digestion and absorption of nutrients, vitamins, and cofactors as well as movements of ions and water—need a precise mechanism of biochemical and physiological control to maintain barrier functions. The cells in the intestine are characterized by high enzymatic activity (lumen and wall), low permeability and typical resistance (between cells, tight junctions are formed characterizing the very tight barriers in the organism), efflux pathways back into the gut lumen, and first-pass metabolism. The barrier function of the gut is a crucial prerequisite for a normal function of intestine. Impairments lead to diarrhea and other serious consequences.

Epithelial cells in the intestine form tight monolayers with tight junctions between polarized cells. Tight junctions of epithelial intestinal cells are studied extensively and contain among others ZO-1, occludin, claudin-1, and claudin-2. Tight junctions and complexes of adherence junctions in the membrane are closely connected to cytoskeleton. Studies with CACO-2 cells showed that localization of ZO-1 in

the zonula occludens of tight junctions depends on Ca^{++} and Mg^{++} concentrations in the medium (Anderson et al. 1989). CACO-2 cells cultured in serum-free medium did not differ from cells cultured in serum-containing medium concerning brush border enzymatic activity, but the transepithelial electrical resistance (TEER) was significantly lower in cells grown without serum reflecting an impairment of tight junctions (Hashimoto and Shimizu 1993; Jumarie and Malo 1991). Cells grown without serum show also a decreased transport of glucose, alanine, and Gly-Gly (Hashimoto and Shimizu 1993). This might be related to incomplete differentiation and different expression of receptors and transporters without serum.

Biochemical part of the barrier contains alkaline phosphatase, γ-glutamytranspeptidase, dipeptidyl-peptidase IV, aminopeptidase *N*, endopeptidase 24-11 and disaccharidases, saccharidase, isomaltase, and lactase. Among metabolizing enzymes, phase I enzymes CYP 1A1 and 3A4 and phase II enzymes glutathione-S-transferase, sulfotransferase, and glucuronidase are present.

Uptake and efflux transporters complete the equipment of brush border membrane cells in performing controlled permeation of compounds.

27.1.3 Routes of Permeation Across Intestinal Cells

Compounds can cross intestinal cells by different routes (Fig. 27.1):

Transcellular passive diffusion (drug absorbed from GI tract mainly in unionized form)

Paracellular diffusion (size and charge dependent)

Transcellular diffusion by endocytosis

Transcellular diffusion using lipid particles

Paracellular passive diffusion modulating tight junctions

Carrier-mediated diffusion and active transport

The surface area of brush border membranes is 1,000-fold larger than paracellular surface area (Pappenheimer and Reiss 1987). Therefore, the probability for transcellular permeability is much higher than for paracellular permeability. Indeed, lipophilic drugs with rapid and complete absorption have a high probability for passive transcellular route. Hydrophilic drugs tend to pass cellular membranes via water-filled pores in the paracellular pathway (for review, see Lee et al. 1991). However, there is also a part of hydrophilic molecules passing membranes by transcellular route (Nellans 1991). The paracellular pathway is used by some positively charged compounds whereas transcellular pathway is preferred with unionized compounds.

Regulation of tight junctions seems to be an attractive way of increasing bioavailability. There have been attempts to induce a transient damage of the intestinal mucosa and to enhance drug uptake. Surfactants, detergents, unsaturated cyclic ureas, fatty acids, bile acids, and chelating agents were used with more or less serious consequences of transient entry of pathogens and lack of control concerning repair mechanism in the gut. None of the agents used so far resulted in an approval by the FDA for drug absorption support due to the risk of local epithelial trauma (Daugherty and Mrsny 1999).

Other interesting approaches to enhance drug absorption used cyclodextrins. They act as solubilizers of drugs or alter the physical structure of administered proteins.

Transcytosis through epithelial cells is of limited capacity.

Several processes limit the permeability: efflux and metabolism in the intestinal cells as well as high lipophilicity.

Receptor-mediated permeation offers an attractive pathway and has been studied for many drugs and compounds under evaluation (see Chap. 27.2.6).

The old view that the majority of compounds enter the organism by passive diffusion is no longer valid, taking into account the complexity of intestinal membranes and number of transporters discovered during the last 10 years.

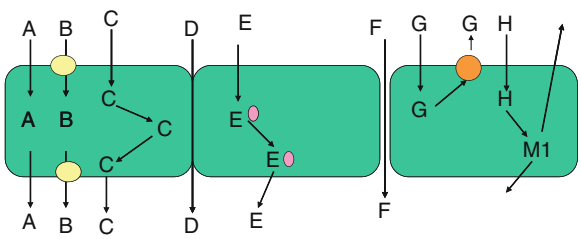

Fig. 27.1 Drug permeation across intestinal cells. A-transcellular passive diffusion. B-carrier-mediated transport. C-transcellular diffusion by endocytosis. D-paracellular passive diffusion. E-transcellular diffusion by lipid particles. F-paracellular passive diffusion via modulation of T.J. G-efflux. H-metabolism

27.1.4 In Vitro Models to Predict Membrane Permeability

As mentioned above due to the increased number of hits in several projects, it is not feasible to test all compounds in in vivo experiments. Even cassette dosing does not solve the issue of limited in vivo capacities. In situ models like rat perfusion or inverted out gut segments have also only a limited capacity of throughput. They are used to solve special issues in projects.

Many different models have been introduced to assess permeability of a compound: human intestinal cells (Hidalgo and Borchardt 1990; Artursson and Karlsson 1991), intestinal mucosal tissue or intestinal segments (Smith et al. 1998; Fisher and Parsons 1949), in situ models with perfused intestinal mucosa (Lennernäs et al. 1996); Griggiths et al. 1996), and cellular models (T-84, HT-29, TC7, and others, Zweibaum et al. 1985; Meunier et al. 1995).

The majority of permeability screening is based on cellular assays mimicking intestinal adsorptive cells: CACO-2 cells (human colon carcinoma) and the CACO-2 clone TC7, HT-29, T84, IL-6, or MDCK cells (Madine Darby Canine Kidney). In these widely used and accepted cellular assays, compounds are classified into three classes of permeability: high, medium, and low. Moreover, the absorption potential of compounds within a chemical series can be compared concerning their apparent permeability coefficients (Artursson 1991; Hillgren et al. 1995; Artursson and Borchardt 1997).

CACO-2 are characterized by easy handling and at the same time resemble morphological and biochemical characteristics of intestinal cells.

Among 20 cell lines tested, CACO-2 cells were the only once to differentiate spontaneously to intestinal enterocytes (Chantret et al. 1988). They resemble many characteristics of small intestinal epithelial cells (Hidalgo et al. 1989) and are still the most widely accepted model in industry and academia (Artursson and Karlsson 1991; Artursson et al. 1996; Audus et al. 1990; Gan and Thakker 1997; Artursson and Borchardt 1997 and many others). Reduced demands for compound volumes in cellular assays allow for screening (Stevenson et al. 1995; Kuhfeld et al. 1994). CACO-2 cells predict permeability with reasonable accuracy and are used in many different test designs.

Their growth is characterized by the sequence of proliferation, confluency, and differentiation. Morphologically, they reveal typical brush border cells with columnar shape and microvilli, polarization, cobblestone morphology with flower-like clusters (Pinto et al. 1983; Hidalgo et al. 1989), presence of tight junctional and adherence junctional complexes (Daugherty and Mrsny 1999), and biochemical features of intestinal cells and typical transporters expressed at permeability controlling membranes. Among biochemical markers of intestinal cells, lumenally expressed sucrase isomaltase, alkaline phosphatase, and aminopeptidase N increase in activity after proliferation phase with reaching confluency (Pinto et al. 1983; Zweibaum et al. 1983). Distribution of proteins to polarized membranes in intestine is a typical marker of differentiation. In CACO-2 cells, sorting to apical membranes involves trans-Golgi network (for sucrase isomaltase) or additional routes (aminopeptidase N and dipeptidylpeptidase IV; Matter et al. 1990).

Other polarization factors characterizing CACO-2 cells are growth factor receptors (Hidalgo et al. 1989), bile acid transporters (Hidalgo and Borchardt 1990; Wilson et al. 1990), glucose transporters (Blais et al. 1987; Mahraoui et al. 1994), lipoproteins (Traber et al. 1987), and neutral amino acid transporters (Hidalgo and Borchardt 1990). Additional enzymes and transporters like dipeptide transporter (Dantzig and Bergin 1990), PEPT1 (Saito and Inui 1993; Thwaites et al. 1994), vitamin B12 carrier (Dix et al. 1990), nucleoside transporters (Ward and Tse 1999), P450 enzymes (Boulenc et al. 1992), sulfotransferase (Baranczyk-Kuzma et al. 1991), and UDP-glucuronyltransferase (Peter and Reolofs 1992) complete the biochemical and enzymatic barrier of CACO-2 cells.

Cytosolic enzymatic activities (chymotrypsin-like, trypsin-like, cucumsin-like) were detected in CACO-2 cells at higher activities than in colonic and rectal mucosae (Bai 1995). These enzymes contribute to first-pass effect in CACO-2 cells. At confluency in CACO-2 cells, glutathione-S-transferase isoenzymes were detected with high activity. Interestingly, in non-confluent cells, the placental π-form, characteristic for colonic cells, was found, whereas in confluent, differentiated monolayers, the μ-form was active being characteristic for intestinal cells (Peters and Reolofs 1989). Cytochrome P450 enzyme 1A1 was present and highly inducible in CACO-2 cells whereas CYP 1A2 was not active (Boulenc et al. 1992; Rosenberg and Leff 1993). Phenol sulfotransferase was expressed in mature cells (Baranczyk-Kuzma et al. 1991).

The major detoxification enzyme CYP 3A is expressed to a different degree in CACO-2 cells and clones: only clone MTX of HT-29 cells and TC-7 of CACO-2 cells exhibited significant activity of CYP 3A (Boulenc et al. 1992; Carriere et al. 1994). CACO-2 cells express antioxidant enzymes like superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase (Grisham et al. 1990; Baker and Baker 1992). Induction and expression of UDP-glucuronosyltransferase (UGT1A1) and glutathione transferase GST A1 were studied in CACO-2 cells (Svchlikova et al. 2004).

Efflux pumps from the ABC-transporter family are expressed: multidrug resistance protein MDR1 (=Pgp), ABCG2 (=BCRP), MRP1, and MRP2 (Cordon-Cardo et al. 1990; Hunter et al. 1993 and others).

In 2008, 10 different laboratories compared mRNA expression of 72 drug and nutrient transporters using RT PCR (Hayeshi et al. 2008). Rank order of top 5 expressed genes was HTP1 > GLUT3 > GLUT5 > GDT1A > OATP-B. Tests for functionality showed differences between laboratories for atenolol permeability (paracellular), whereas Gly-Sar uptake was shown only in five labs (PEPT1); efflux of bromosulfophthalein was shown only in three labs (MRP2 functionality). For MDR1 (talinolol) and PEPT1, functionality and mRNA expression correlated well; on the other hand, for OATP-B and MRP2, there was no correlation between functionality and mRNA. Concerning rank order of clinically most important transporters, the study was in agreement with Sun et al. (2002) and Calvagno et al. (2006): OATPB > MRP2 > PEPT1 > MDR1.

It is important to note that many transporters showed in this study a several fold variability, for example, SGLT1, PAT2, MCT3, GLUT5, and IBAT showed several hundred fold variability among the labs, NTCP, MRP3, GLUT3, BCRP, OCTN1, MRP5, PEPT1, OAT2, ENT1, MRP2, and OST alpha and even MDR1 more than tenfold variability. A very careful characterization and validation of the in vitro model used is therefore a prerequisite for an acceptable in vitro in vivo correlation as pointed out for all assays in this chapter.

In summary, CACO-2 cells express many enzymes and transporters characteristic for intestinal cells involved in drug metabolism. The major drug-metabolizing enzyme, CYP 3A, is active only in selected clones pointing to polyclonal origin of CACO-2 and the necessity to characterize CACO-2 cells extensively for growth and experimental conditions used for given experiments.

While in the 1990s the CACO-2 cell model has been widely characterized and used for different purposes, Artursson and Borchardt (1997) raised in a review several questions to modify existing technologies. They discussed several questions which have been solved in the meantime in industry: small amounts of compounds for permeability experiments and the need for miniaturization of permeability tests, developing routine methods for testing compounds with low solubility, new cell growth and permeability test equipment, introduction of generic LC-MS analytical methods, and acquisition and storage of data in a time frame consistent with high throughput pharmacological screening. Today, we use less than 40 µl of a 10-mM solution of the test compound; the permeability tests consider solubility of the compound in automated solubilization steps leading to prepared compound plates all over the world at different company sites. Many companies use 24-well plates or even 96-well plates for permeability experiments. Pipetting robots (TECAN and others) have been designed to perform all experiments automatically. Cell feeding robots have been introduced. Data storage and handling can be performed at different sites of a company at the same time. Screening of libraries was used to get an overview on a large amount of compounds, but in the meantime, strategies have changed, and the trend is rather to support teams by secondary assays and sometimes sophisticated procedures differing from the high throughput method.

The experimental procedures which will be presented now consider only manual use. Protocols using robotic systems are not described due to great differences in the robotic systems. Nevertheless, many details refer to the use in an industrial environment.

27.2 Cellular In Vitro Methods to Predict Permeability

27.2.1 Starting and Maintaining CACO-2 Cells

PURPOSE AND RATIONALE

CACO-2 cells from ATCC at passages 20–50 are used. CACO-2 cells are stored in cryovials in liquid nitrogen. Original cryovials from ATCC are handled as described in the ATCC procedure (see also

Chen et al. 2002). This ensures constant cell source for many years once cells have been splitted, grown under the same conditions, and then frozen in liquid nitrogen.

PROCEDURE

Cryotubes containing 2 Mio cells/ml are taken from liquid nitrogen tank and placed in a 37°C water bath for approximately 5 min. After thawing up, the suspension is transferred to a 50-ml centrifuge tube containing complete cultivation medium at 37°C. As cell seeding/feeding, medium DMEM/GlutaMAX I is used (Gibco, high glucose content, HEPES 25 mM) with following supplements:

- 1% NEAA (nonessential amino acids)
- 10% FBS (fetal bovine serum)
- 40 µg/ml gentamicin (antibiotic additive)

The suspension is mixed carefully and then centrifuged at 150 g for 5 min. The cell pellet is resuspended after discarding freezing medium in cultivation medium and placed in a 175-cm^2 cultivation flask containing 80 ml complete medium. Cells are cultivated at 37°C, 95% humidity, and 10% CO_2.

Medium change is performed every 3 days. After 10 days, cells are splitted.

Cell splitting starts with washing the attached cells with 20 ml PBS for 2–4 min. After removal of PBS, trypsin/EDTA is added (20 ml) for 2 min. After removal of trypsin/EDTA, 2 ml of trypsin/EDTA are added and incubated for 5 min at 37°C with gently shaking the flasks. Trypsinization is stopped when cells start to detach by suspending the cells in 20 ml complete medium while trypsinization procedure stops. After a centrifugation step (to remove trypsin) at 150 g for 5 min, cells are resuspended in complete medium and splitted to three flasks of 175 cm^2 (2.4–2.6 $\times$ 10^6 cells per flask).

After 7 days of cultivation with medium changes every 3 days (confluency 80–90%), cells are trypsinated and prepared for use in 24-well plates.

EVALUATION

To ensure a constant and comparable viability, cells are counted, and cell viability is determined with trypan blue exclusion method. Results are compared from 1 week to the next.

MODIFICATION OF THE METHOD

Several modifications of the method are described in the literature (Artursson and Karlsson 1991; Hidalgo et al. 1989 and many others). Modifications include cell culture medium, time of cultivation and frequency of medium change, variations of trypsinization methods, and others. In an industrial environment, cell cultivation methods are maintained over many years constant to reduce variability and ensure constant results in quality assessment protocols. Additionally to quality control parameters like TEER and permeability markers, expression levels of major enzymes and transporters are checked.

27.2.2 Growth of CACO-2 Cells on 24-Well Plates

PURPOSE AND RATIONALE

Transport studies are performed at 24-well or 96-well formats. Cells grown in 175-cm^2 flasks are moved to filter inserts and after 21 days of feeding and cultivation used in transport studies. The following method applies for use of 24-well filters of BD Falcon TM HTS 24-Multiwell Insert System. Alternatively, Costar 24-well filter systems could be used (non-coated, Transwell system). In many pharmaceutical companies, cell permeability tests and feeding are performed with automatization equipment.

PROCEDURE

Trypsinization is performed as described above. Cells in the suspension are counted and prepared in a suspension containing 4.2–6.7 $\times$ 10^4 cells/cm^2. To each apical well of the 24-well plate, 400 µl of the cell suspension are added apically. The Feeder Tray contains 40 ml of complete medium (feeding cells from the basolateral side).

Every 3 days, medium is changed. Cells are cultivated in an incubator at 37°C, 95% humidity, and 10% CO_2.

After 21 days of cultivation and control measurements of monolayers cell density, the permeability experiment can be performed.

EVALUATION

Control measurements of the monolayer include measurements of transepithelial resistance with an Endohm Meter (World Precision Instruments, New Haven) and permeability measurements of mannitol and polyethylenglycol 4000 (marker for low permeability) and of metoprolol (marker for high

permeability), for example. Permeability experiments can start if the resistance measured is >250 Ohm/cm^2. Permeability coefficient of mannitol, a standard permeating by passive diffusion, should be in a defined range before permeability experiments start. Several laboratories start permeability tests if permeability coefficient for mannitol is below 1 × 10E-06 cm/s.

MODIFICATIONS OF THE METHOD

Modifications of the method include manual or automated cell feeding in 24-well plates, media modifications, possible coating of filter surfaces, and pore size of filter membranes. Other modifications include quality assurance criteria (TEER, permeability values).

CRITICAL ASSESSMENT OF THE METHOD

The decision for selection of filter support membranes, coating, pore size, and medium additives depends on the design of the study. In an industrial environment, manual coating is not performed due to labor intensity; precoated filters are cost intensive. It is known that in vivo cells are growing on an extracellular matrix and that in vitro mimicking these conditions by coating with collagen I, II, III, or IV improves cell attachment, spreading, migration, and time to reach confluency (Hidalgo et al. 1989; Basson et al. 1992). On the other hand, decision for experimental procedures is also driven by factors like costs, labor intensity, and time.

Different filter supports have been tested: nitrocellulose (NC), polycarbonate (PC), aluminum oxide (AO), and polyethyleneterephtalate (PET). NC filters have shown reduced nonspecific binding compared to AO filters but seem to interact with marker PEG and steroids (Nicklin et al. 1992). AO filters displayed only half of permeability of PC for taurocholic acid. Reproducibility of binding and transport experiments was improved with PC filters (Hidalgo et al. 1989). Pore size is an important factor which needs to be considered and tested: CACO-2 cells migrate through pores >1 μm (Tucker et al. 1992; Hilgers et al. 1990). PET filters are translucent and allow microscopic observation as well as staining procedures.

Cells grown on PC showed lower TEER and about threefold higher permeabilities for a series of thrombin inhibitors (Walter et al. 1995).

CACO-2 cells display a heterogenicity from batch to batch and as a function of time and culture conditions due to different selection pressure (Vachon and Beaulieu 1992; Wilson et al. 1990; Jumarie and Malo 1991; Karlsson et al. 1994). Therefore, the cells have to be characterized carefully for those parameters important in a study design. Not only TEER and permeability of marker compounds are important characteristics of the system (for review, see Delie and Rubas 1997) but also expression of enzymes, transporters, and proteins as a function of time and experimental conditions. Delie and Rubas (1997) point in their review to importance of passage number of CACO-2: several authors described changes in morphology, TEER, proliferation, and permeability characteristics with increased passage number of cells (Walter and Kissel 1995). In an industrial environment where a test system is used over many years and necessarily data need to be compared within and between projects over a long time, a test system needs to be robust and well controlled with quality standards and cell cultivation standards (passage number etc.).

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EXAMPLES

Examples of CACO-2 cultivation for use in permeability tests are given in Artursson and Karlsson (1991), Artursson (1991), Hilgers et al. (1990), Stevenson et al. (1995), Walter et al. (1995), and Wilson et al. (1990).

27.2.3 Permeability Assay Conditions

PURPOSE AND RATIONALE

Permeability of compounds across a cell layer is measured in order to determine the absorption potential of a compound or a chemical series and select compounds for in vivo studies. Apparent permeability coefficients can be used to compare compounds within a series for ranking. Between different laboratories, comparison of compounds should be done only on the basis of classification (high, medium, low) since permeability coefficients can differ between the labs (Artursson et al. 2001; see "Critical Assessment of the Method").

PROCEDURE: PERMEABILITY EXPERIMENT

The design of the 24-well plates containing filter supports is shown in Fig. 27.2.

As experimental buffer for washing and permeability assay HBSS, pH 7.4 is proposed.

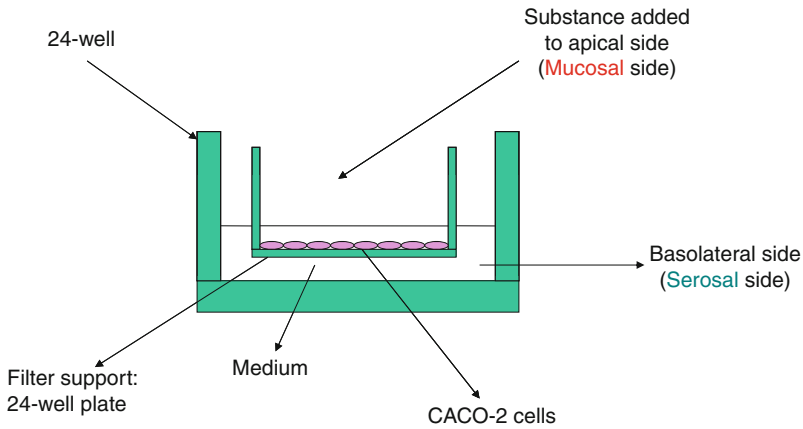

Fig. 27.2 Filter insert system for permeability studies

Compound dilutions should be performed from 10 mM compound stock solutions in DMSO at the day of experiment. Compounds should be taken from microwell plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5%. Alternatively, compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5%.

Compound concentration should be chosen on the basis of solubility data. Compound concentrations of 50 μM should not be exceeded in routine tests. Concentrations lower than 6.25 μM should be avoided due to possible analytical limitations.

As an example, four different categories of compound concentrations could be used due to differences in projects and compound characteristics: 50, 25, 12.5, and 6.25 μM.

As assay time of 1 h is proposed.

In an industrial environment, very often, one-time-point determinations are performed in triplicate using incubator conditions (37°C, 1 h) with shaking or non-shaking conditions.

Samples are collected in triplicate from both the basolateral and apical compartments after 1 h.

Triplicate samples are also taken from the dosing solution.

PROCEDURE: ANALYTICAL METHODS

All analytics should be performed on LC-MS systems: in industrial labs, very often, ion traps or quadrupole systems are used. Single ion monitoring in either positive or negative mode (compound dependent) is used in HTS conditions. Local LC conditions (injection volume, mobile phase gradients) should be applied to accommodate variations in LC equipment. The selected LC column stationary phase can be used routinely in different labs (e.g., C-18; phase selection is compound dependent).

Mobile phase consists of varying amounts of ammonium acetate (5–10 mM) or formic acid and acetonitrile.

All samples should be kept in a stack cooler during injection routine. According to limited solubility of compounds in projects, compounds could be screened at different concentrations (as described above). Analytical samples should be run than at either 6.25, 12.5, 25, or 50 μM, depending on solubility results of plate/project.

Dosing and apical samples will be diluted routinely to a final concentration of not more than 2.5 μM (depending on linearity of LC-MS measurement range and MW of compound).

One injection per sample should be done (for the procedure described, routinely, ten samples will be acquired per compound: buffer blank, 3-basolateral 1 h, 3-apical 1 h, and 3-dosing solution).

EVALUATION

Apparent permeability coefficients (Papp in cm/s) are calculated according to the following equation:

$$\mathrm{Papp(cm/s)} = (\mathrm{dQ} \times \mathrm{V})/(\mathrm{dt} \times \mathrm{S} \times \mathrm{C_0})$$

where S is the surface area of the filter in cm^2, $\mathrm{C_0}$ is the initial concentration of the compound on the donor

Table 27.1 In vitro Papp (cm/s) values corresponding to 20% and 80% in vivo permeability data from sigmoidal correlation curve with standard compounds

% Human absorption	Rating	Laboratory 1	Laboratory 2	Laboratory 3
20%	Low	*<3.2 × E-07*	<0.3 × E-06	*<8 × E-07*
20–80%	Moderate	3.2 × E-07 to 1.25 × E-06	0.3 × E-06 to 3.9 × E-06	8 × E-07 to 6 × E-06
80%	High	*>1.25 × E-06*	>3.9 × E-06	*>6 × E-06*

side, dQ/dt is the amount of compound transported during the 60 min experiment, and V is the volume of the receiver chamber.

In industrial labs, standardized worksheets are used, and results automatically appear in databases (globally or at a certain site of the company). Results are then given, for example, as mean Papp (single point, cm/s) with standard deviation ($n = 3$), absorption rating (low, medium, high), mean % transport/h, mean % mass recovery, and mean % remaining donor.

Mean % mass recovery is calculated from the sum of the basolateral and apical quantities of compound at 1 h as a % of the original dosing solution at 0 h.

Mean % remaining donor is calculated as quantity of compound remaining in the apical compartments at 1 h as a % of the original dosing solution at 0 h.

Each recovery should be individually checked to see if it falls within a predefined threshold. The partial recovery is calculated as the sum of the percent transport and the percent remaining donor (peak area donor 1 h + receiver/donor 0 h). As quality assurance criteria, for example, partial recovery >65% and partial recovery <125%, could be accepted, and then, the result value is included in the calculation of the average transport/h or the average Papp/single point.

If the recovery for two or more wells falls outside of 65–125% range, then no result can be reported, and experiment is usually repeated.

The assay is evaluated in all laboratories by correlating in vivo absorption data of standard compounds with in vitro permeability values (see Artursson and Karlsson 1991). Since CACO-2 cells differ due to cultivation procedures and possible selection of populations/clones of cells, this correlation curve should be established in every laboratory. It is not possible to use a curve from literature for prediction of in vivo permeability from in-house in vitro data. Every laboratory should use its own curve. A comparison of Papp values from different labs for compound ranking is not possible (Artursson et al. 2001). Therefore, many industrial labs use a classification of compounds into three classes of permeability: high, medium, and low. This classification is very well comparable between different laboratories. Test compounds analyzed in different labs should be classified into the same class. On the basis of correlation curve between in vivo absorption and in vitro permeability data, a sigmoidal curve can be calculated, and two important thresholds are usually set: at in vitro Papp values corresponding to 20% in vivo permeability and to 80% (Artursson et al. 2001; Bailey et al. 1996 and others). Other labs use 90% threshold values according to a FDA proposal. Compounds with in vitro Papp below the first threshold value (at 20% in vivo permeability from standard curve) are classified as low permeable, compounds with Papp values above the higher threshold are classified as high permeable, and compounds with permeability values between the two thresholds are classified as medium. An example of different permeability values in laboratories is given in the table below (Table 27.1).

Due to a high throughput in industrial laboratories, very often, so-called mean Papp single point is calculated from three filters with standard deviation meaning that only one time point (60 min) is used for calculations. Alternatively or in addition, mean % transport/h can be calculated from three filters.

If two or more filters do not meet acceptance criteria, compound assay should be repeated (exception: compounds with unusual features causing, e.g., a discrepancy in mass balance due to high plastic binding).

Standard markers should be included in all experiments. Usually, marker compounds for different permeability classes are used like metoprolol for high permeability and radioactive mannitol for low permeability. Quality assurance criteria define accepted upper permeability values for mannitol (in the case of mannitol, many laboratories use 1.0 × E-06 cm/s). Permeability values higher than upper limit should lead to rejection of the test.

Integrity control of the monolayer is based on measurements of both TEER (transepithelial resistance measurements) and permeability for a standard marker (radioactive mannitol or Lucifer yellow as nonradioactive alternative).

TEER is usually measured at the beginning (0 h) and end of the assay (1 h). Monolayers with TEER values below an acceptance value should be rejected (e.g., TEER <250 Ohm/cm^2 at 0 h could be used as criterion to exclude cell monolayers from experiment). TEER acceptance criteria should also be defined (e.g., +/− 30% change in TEER at 1 h from TEER value at time zero means that the experiment cannot be accepted).

Again, quality criteria should be defined in every lab independently: while Papp values (upper permeability criteria) and TEER limits cannot be transferred from one lab to another one, recovery data (% of start solution) are comparable.

Additional comments are usually included like leaky monolayer, solubility below 6.25 μM, variability in data.

Recently, in some industrial labs, the blind probe test has been introduced: five selected standards are analyzed in permeability tests at different sites, and results are compared (rating, recovery). The qualification and classification of the compounds to the criteria low permeability (<20%), moderate (20–80% permeability), or high (>80% permeability) should not differ among different labs. This blind probe tests can be used as quality assurance if repeated several times a year.

MODIFICATIONS

Modifications include all details described in the method above (time of permeability experiment, concentration ranges, shaking, quality criteria, and acceptance parameters).

Modifications to the analytical procedure presented are of course necessary if different equipment is used or if a compound class cannot be analyzed with the standard procedure proposed.

CRITICAL ASSESSMENT OF THE METHOD

In vivo as well as in vitro permeability is reduced due to the aqueous boundary layer or unstirred water layer (UWL). Shaking can reduce the UWL using different shaking frequencies (Hidalgo et al. 1991; Karlsson and Artursson 1992). However, the experimental conditions need to be tested carefully to avoid damage of monolayers. In side-by-side diffusion systems, the UWL was 52 μm (Karlsson and Artursson 1992). In vivo thickness of UWL was found 40 μm (Strocchi and Levitt 1991).

TEER, normalized to surface, was 188–221 Ohm/cm^2 in CACO-2 cells and 78–125 Ohm/cm^2 in colon (Rubas et al. 1996). The presence of villi and crypts in vivo with a higher surface and a different cellular composition (goblet cells, M cells, higher permeability in crypts) compared to a cell monolayer might lead to higher permeability. Tanaka et al. (1995) compared permeability of FITC-Dextran (MW 4000) in CACO-2 cells and rat jejunum and colon: the permeability was tenfold decreased in CACO-2 compared to jejunum and fivefold lower than in colon. TEER in CACO-2 was 470 Ohm × cm^2 in this study, 40 Ohm × cm^2 in rat jejunum, and 80 Ohm × cm^2 in rat colon. Permeability of standard markers like PEG was compared between CACO-2 cells and colon (Artursson et al. 1993). The conclusion for cellular studies is to select cells with acceptable TEER and permeability values and to perform quality assurance tests on a regular basis.

Several authors compared permeability of CACO-2 with in situ perfused intestine or everted intestinal rings (Lennernäs et al. 1996; Rubas et al. 1993; Jezyk et al. 1992). Their conclusion is that CACO-2 cells with TEER below 300 Ohm/cm^2 give permeabilities for hydrophilic drugs comparable to intestinal tissue. The trend for hydrophilic compounds concerning permeability was CACO-2 > colon > small intestine.

Further limitations of cellular layers in comparison to in vivo conditions are absence of mucus and different expression of enzymes (lack of CYP 3 in CACO-2) and transporters. There have been attempts to introduce mucus-secreting cells (HT29) to the CACO-2 permeability system (Wikman-Larhed and Artursson 1995; Behrens et al. 2001; Pontier et al. 2001). Cells with a higher permeability than CACO-2, for example, IEC-18 cells (Ma et al. 1992; Duizer et al. 1995) or immortalized cells 2/4/A1 (Milovic et al. 1996), have been studied. These attempts were only partly successful: in coculture systems, cells did not mix as in vivo, goblet cells produced not enough mucus, and permeabilities for standards increased more than expected.

However, these systems need further evaluation. For an industrial environment, coculture systems need to be robust and reliable being at the same time

easy to handle. On the other hand, convincing advantages have to be shown to change the monolayer system to a coculture model or to change the cell system used. In many companies, CACO-2 cells are used since many years, and an immense database is available.

Another aspect to consider is the effect of food, increasing or decreasing uptake characteristics of a compound. Diet, physiological status, and diseases influence permeability as well.

Prognosis of a compound's permeability should be made, stressing limitations of the model. There is no bioavailability prognosis from in vitro data—a cellular assay can provide only permeability potential through a biological membrane. The membrane, in most cases CACO-2 cells, is very similar to what we observe in vivo in the small intestine and resembles many characteristics to in vivo enterocytes. CACO-2 cells can be used for prediction of different pathways across intestinal cells. Best correlation occurs for passive transcellular route of diffusion. Passive paracellular pathway is less permeable in CACO-2, and correlations are rather qualitative than quantitative for that pathway. CACO-2 cells are an accepted model for identification of compounds with permeability problems, for ranking of compounds, and for selection of best compounds within a series. Carrier-mediated transport can be studied as well using careful characterization of transporters in the cell batch or clone as a prerequisite for transporter studies.

There are advantages of cell culture models in permeability testing which should be exploited adequately. Differentiated human cells with similarities to human intestinal epithelia can be used for screening procedures, tests for absorption enhancers and sensitive cytotoxicity screening, uptake and secretion studies, as well as functional bioassays to study proteins, transporters, or enzymes generating reproducible data. Mechanistic studies are increasingly introduced to clarify issues and enable studies for structure-activity relationship for a selected issue.

In the last decade, knowledge about transporter expression and impact on a drug's permeability and/or absorption has increased. Therefore, additional tools like the BDDCS might be helpful during drug development. BDDCS (Biopharmaceutics Drug Disposition Classification System) was proposed by Les Benet modifying BCS (Biopharmaceutical Classification System) by introducing metabolism and transporters in addition to solubility and permeability of a compound in order to predict in vivo pharmacokinetic behavior. While BCS is a system to determine probability of a biowaver for a given drug, BDDCS could be used a guidance for probability of drug-drug interactions and transporter-related interactions.

For class 1 compounds, having high solubility and permeability and showing high metabolism, interaction with transporters in intestine and liver is rather minimal (Benet 2009). Class 2 compounds with low solubility, high permeability, and high metabolism have a high probability of interacting with efflux transporters in the gut and uptake as well as efflux transporters in the liver. For class 3 compounds with high solubility but low permeability and low metabolism uptake transporters are important determinants for intestinal absorption and uptake into the liver. However, due to poor permeability, efflux transporters might also be involved in elimination. And finally, class 4 compounds need uptake and efflux transporters due to their low solubility, poor permeability, and poor metabolism (Shugarts and Benet 2009).

In 2011, Benet and Oprea published an extensive list of drugs and metabolites (927 drugs and 30 active metabolites) and correlated the pharmacokinetic data with physicochemical properties like log P, log D-7.4, polar surface area, and number of hydrogen bond acceptors and donors. The goal is to use BDDCS and physicochemical properties to predict drug disposition characteristics of novel compounds (Benet et al. 2011).

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EXAMPLES

Examples of CACO-2 permeability tests are given in Artursson and Karlsson (1991), Artursson (1991), Hilgers et al. (1990), Stevenson et al. (1995), Walter et al. (1995), and Wilson et al. (1990). Additionally, CACO-2 has been used in testing excipients (Saha and Kou 2000) and applying simulated intestinal fluid (Ingels et al. 2004).

27.2.4 Efflux Experiments Using CACO-2 Cells

RATIONALE

Multidrug resistance (MDR) is an important and common cause of drug resistance in cells. MDR is mediated by the increased expression of energy-dependent drug efflux pumps from the ABC-transporter family (Borst et al. 1999). Efflux transporters like P-glycoprotein (P-gp or MDR1), encoded by the MDR1 gene; multidrug resistance-associated proteins (MRP1 and 2); lung resistance protein (LRP); and breast cancer resistance protein (BCRP = ABCG2) are efflux transporters present in the gastrointestinal tract (Ambudkar et al. 1999; Borst et al. 2000; Jonker et al. 2000; Doyle et al. 1998). For review, please refer to Schinkel and Jonker (2003).

MDR1 is a 170-kDa transmembrane protein member of the ATP binding cassette (ABC) transporter family. It is localized at the apical secretory surface of various tissues (e.g., liver, kidney, gastrointestinal tract, blood-brain barrier) where it mediates the active transmembrane transport of a variety of lipophilic substrates, which tend to be large, aromatic, and amphiphilic. MDR1 can extrude/exclude a wide range of structurally diverse drugs (Ambudkar et al. 1999). MDR1 limits the oral absorption of a number of drugs by transporting them back from the intestinal cells into the gut lumen. Recent studies have revealed the overlapping substrate specificity of CYP3A4 and MDR1, and many substrates of CYP3A4 are also

substrates or inhibitors of MDR1. The functional expression of MDR1 has also been shown in CACO-2 cells.

MDR1 and members of the MRP family have substantial substrate overlaps (Borst et al. 2000).

MRP1 and MRP2 have substrate specifity overlaps for amphiphilic anions, glutathione, glucuronate, or sulfate conjugates (König et al. 1999). The transporter cMOAT (canalicular multispecific organic anion transporter, i.e., cMRP or MRP2), responsible for the active excretion of amphiphatic anionic conjugates formed by phase II conjugation into bile, is also found in the intestine.

For MRP2, the following substrates have been identified and characterized so far: glutathione disulfide, leukotrienes (C4, D4, E4, *N*-acetyl E4), glutathione conjugates (DNP, BSP, metals Sb, Bi, As, Cd, Cu, Ag, Zn), glucuronide conjugates (bilirubin, T3, p-nitrophenol, grepafloxacin), bile acid conjugates (glucuronides, sulfates), and organic anions (folates, methotrexate, ampicillin, ceftriaxone, cefadoxine, grepafloxine, pravastatin, temocaprilate).

BCRP is an ABC half-transporter and is expressed at very high levels in the intestine, liver, kidney, and blood-brain barrier. The identified list of substrates and/or inhibitors is constantly growing: mitoxantrone, methotrexate, topotecan, doxorubicin, flavopiridol, SN-38, Lysotracker green, BBR 3390, BODIPY-prazosin, rhodamine 123, pheophorbide A, PhPI, estrone sulfate, estradiol-17 beta-glucuronide, cimetidine, and dipyridamole. As inhibitors, GF 120918, Ko143, nelfinavir, and fumitremorgin C were identified (Doyle et al. 1998; Litman et al. 2000; Poguntke et al. 2010).

In the small intestine, expression data for MDR1, ABCG2, MRPs, and LRP have been detected (Kool et al. 1997; Doyle et al. 1998; Fromm et al. 2000; Maliepaard et al. 2001).

In 2008, ten different laboratories compared mRNA expression of 72 drug and nutrient transporters using RT PCR (Hayeshi et al. 2008). Concerning efflux transporters, efflux of bromosulfophthalein was shown only in three labs (MRP2 functionality). For MDR1 (talinolol), functionality and mRNA expression correlated well; on the other hand, for MRP2, there was no correlation between functionality and mRNA. Concerning rank order of clinically most important transporters, the study was in agreement with Sun et al. (2002) and Calcagno et al. (2006): OATPB > MRP2 > PEPT1 > MDR1. Interestingly, in this comparison, BCRP was expressed at the same amount as MDR1, although in previous papers, expression levels seem to be higher (see citation in Hayeshi et al. 2008, reference in Chap. 1).

Efflux studies are very often performed in cellular systems which are tight enough to perform flux and efflux studies (CACO-2, MDCK). Taipalensuu and colleagues were the first to study expression and quantitative relationship in detail for CACO-2 cells in comparison to jejunal levels (2001).

Jejunal transcript levels of the different ABC transporters are spanning a range of three log units with rank order: BCRP ~ MRP2 > MDR1 ~ MRP3 ~ MRP6 ~ MRP5 ~ MRP1 > MRP4 > MDR3 (Taipalensuu et al. 2001). Transcript levels of 9 of the ABC transporters correlate well between CACO-2 cells and jejunum, only BCRP exhibits a 100-fold higher expression in the in vivo system (Taipalensuu et al. 2001).

Many laboratories use CACO-2 cells as a standard method for assessment of efflux. Since the standard CACO-2 cell assay is very well established, easy to use, reproducible, and reliable, the corresponding efflux assay can give valuable and helpful data for project support in a screening approach. A prerequisite for the interpretation of efflux data is a characterization of efflux transporters present in the system used and a set of standard efflux markers checked regularly (like digoxin for MDR1).

Moreover, while for identification of inhibitory characteristics vesicle studies are well accepted and used, cellular efflux studies are recommended by the FDA and the International Transporter Consortium White paper also for compounds in development to justify clinical drug-drug interaction studies (see Giacomini et al. 2009 and FDA Draft Guidance 2006).

PROCEDURE

As experimental buffer for washing, permeability, and efflux assay HBSS, pH 7.4 is proposed.

Compound dilutions should be performed from compound stock solutions in DMSO, 10 mM at the day of experiment. Compounds should be taken from microwell plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5%. Alternatively,

compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5%.

Compound concentration should be chosen on the basis of solubility data. Compound concentrations of 50 μM should not be exceeded in routine tests. Concentrations lower than 6.25 μM should be avoided due to possible analytical limitations.

As an example, four different categories of compound concentrations could be used due to differences in projects and compound characteristics: 50, 25, 12.5, and 6.25 μM.

In contrast to permeability tests, compounds are added not only to apical compartment (to determine flux A-B) but in an additional experiment to basolateral compartment (efflux B-A). As assay time of 2 h is proposed.

Further experiments are usually performed if efflux is observed increasing concentrations (depending on compound solubility) or determining inhibitor effects.

Care should be taken when performing additional experiments to determine active efflux. Usually, flux and efflux assays are performed at 4°C in comparison to 37°C. Since membrane fluidity is changing at 4°C compared to 37°C, results have to be interpreted with caution (Seelig et al. 2005).

As for the standard CACO-2 assay, very often, one-time-point determinations are performed in triplicate using incubator conditions (37°C, 2 h).

Samples are collected in triplicate from both the basolateral and apical compartments after 2 h. Triplicate samples are also taken from the dosing solution. Analytics are performed as described in Sect. 27.2.3.

EVALUATION

Apparent permeability coefficients (Papp in cm/s) are calculated according to the following equation:

$$\mathrm{Papp(cm/sec)} = (\mathrm{dQ} \times \mathrm{V})/(\mathrm{dt} \times \mathrm{S} \times \mathrm{C_0})$$

where S is the surface area of the filter in cm^2, C_0 is the initial concentration of the compound on the donor side, dQ/dt is the amount of compound transported during the 60-min experiment, and V is the volume of the receiver chamber.

Ratio of B-A/A-B is calculated and compared to the ratio of standard compounds.

As standards, digoxin, etoposide, or paclitataxel (all for MDR 1); estradiol-17 beta-glucuronide or furosemide (for MRP2); and methotrexate or estrone sulfate (for ABCG2) can be used as substrates. Inhibitors being used as validation of the test assay might be PSC 833 or elacridar for MDR1, Ko 143 or fumitremorgin C for ABCG2, and probenecid for MRP2. Efflux ratio of the compound is compared to ratio of standards, and inhibitors could help to identify the transporter involved in the efflux.

MODIFICATIONS OF THE METHOD

Modifications include all details described in the method above (time of permeability experiment, concentration ranges, shaking, use of standards, quality criteria, and acceptance parameters). Since in CACO-2 cells efflux for standard compounds varies due to heterogenicity and batch variations, several teams used selected CACO-2 cells. Cells set under selection pressure with vincristine (Eneroth et al. 2001) or digoxin (Tanaka et al. 2000). Other teams selected subclones of CACO-2 cells with higher expression rates of MDR1 (Horie et al. 2003).

Other teams prefer the use of overexpressing cell lines. In this case, a careful characterization of the test system and a validation of the assay using substrates and inhibitors mentioned is a prerequisite for correct identification of compounds as substrates and/or inhibitors in vitro.

CRITICAL ASSESSMENT OF THE METHOD

Cellular efflux assays have a lower throughput as binding studies or competition studies. The calcein assay used very often to identify MDR1 substrates can offer interactions only with one binding site of MDR1. Consequently, the test fails in identification of those substrates or inhibitors interacting with the second binding site, with both or with a so-called interaction site (Litman et al. 2001). Two very important studies have been performed by Schwab et al. (2003) and Polli et al. (2001) comparing several different MDR1 assays for identification potential toward known substrates/inhibitors of MDR1. Both studies conclude that HTS assays such as calcein or rhodamine 123 assay are not sufficient to detect MDR1 interacting compounds. ATPase assay can provide additional information but fails also for some known MDR1 interacting compounds. In the ATPase assay, colchicine, digoxin, etoposide (Schwab et al. 2003), cyclosporin A, GF120918, doxorubicin, and vincristine (Polli et al. 2001) were not identified. Inhibition (or competition) assays such as calcein assay failed to identify

colchicine, digoxin, etoposide (Schwab et al. 2003), vincristine, taxol, and others (Polli et al. 2001). Rhodamine 123 assay again did not identify colchicine, digoxin, etoposide, ranitidine, and others (Schwab et al. 2003). The rank order for compounds in MDR1 efflux (based on a comparison of efflux ratio B-A/A-B in both studies) is different between both test systems used (MDR1-MDCK cells in Polli's study and LLC-MDR1 and LLC-mdr1a cells in Schwab's study). Compounds with high intrinsic permeability (midazolam, nifedipine) could overcome efflux and were not identified as MDR1 interacting compounds (Schwab et al. 2003). Verapamil was not effluxed due to high membrane partitioning, too (Polli et al. 2001). Both studies conclude that it is not sufficient to rely on one HTS assay. The authors propose to perform several assays in a cascade strategy: first, HTS assays using fluorescent readouts (calcein, rhodamine) followed by ATPase assay and/or cellular transport assays. However, following this strategy, etoposide, colchicine, and digoxin would have been not identified as interacting MDR1 substrates. Therefore, at the stage of early drug candidate identification, efflux assays using well characterized cell systems are very useful. Given that the rank order of effluxed compounds was different (taking ratio as comparison), it is necessary to stay in one efflux system for comparative studies and to check in overexpressing cell systems such as MDCK and LLC-PK1 protein sequence and molecular weight of transfected efflux transporters. Other efflux transporters should be checked as well because due to transfection, they could be over- or underexpressed or be expressed in a different glycosylation form. FDA Draft Guidance for Industry and the International Consortium White paper recommend bidirectional transport assays using polarized monolayers with expression or overexpression of efflux transporters (Giacomini et al. 2009; FDA Draft Guidance, 2006).

ABCG2 (BCRP) is able to establish only a small concentration gradient which complicates the identification of ABCG2 substrates (Poguntke et al. 2010). Moreover, due to the different partly overlapping binding sites and electrostatic interactions with potential substrates (electrostatic funneling), for ABCG2, the use of a combination of assays is recommended to identify substrates or inhibitors, such as ATPase assay, vesicle inhibition assays, and cellular assays in CACO-2 and/or overexpressing cells (review of Poguntke et al. 2010).

Please pay attention to the critical assessment part in efflux inhibition studies (Sect. 27.2.5).

An international Pgp working group including 22 pharma companies and CROs was the pioneer for validation of the first efflux transporter in an interlaboratory comparison: MDR1. Different assays were compared (vesicles, cells—CACO-2, MDR1-MDCK, MDR1-LLCPK1) and analyzed statistically. C. Lee presented at the AAPS Workshop on Drug Transporters 2011 first results showing a very high variability of IC50 values (about 200-fold variation). That makes it difficult to establish risk factors for in vitro in vivo correlation even for the best studies transporter Pgp. Determination of drug-drug interaction risks (resulting in clinical studies) needs a lot more validation in industry and CROs for Pgp and for other clinically relevant transporters as well.

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EXAMPLES

Examples of efflux studies using CACO-2 and other cells expressing efflux transporters are given in the studies of Schwab et al. (2003), Polli et al. (2001), and Pachot et al. (2003 and others).

27.2.5 Efflux Inhibition Experiments Using CACO-2 Cells

RATIONALE

Once a compound is identified in an efflux assay, it is sometimes of importance to discriminate between efflux and inhibition potential and to determine the efflux transporter responsible for efflux of the compound.

Concerning selectivity of transporter inhibitors interacting efficiently with only one efflux or uptake transporter, there was clear progress during the last years. Cellular inhibition assays are still used very often as a first step to characterize efflux and/or identify the efflux transporter involved (Table 27.2).

The list of substrates and inhibitors for MDR1 is extensive due to the fact that we are aware of different binding sites (two binding sites minimum) and interacting sites (capacity of competition or inhibition at an additional site of MDR1, see Litman et al. 2001).

Two published studies from Roche (Schwab et al. 2003) and Glaxo (Polli et al. 2001) showed limitations in studying MDR1 by simplified HTS methods: several compounds known as substrates or inhibitors of MDR1 were not clearly identified, others were identified only by one or two of the test methods (either ATPase assay or calcein efflux or rhodamine efflux or cellular efflux studies).

Best prediction is still achieved with cellular efflux assays comparing permeability in A-B and B-A directions. However, cellular efflux assays do

Table 27.2 Frequently used efflux standard substrates and inhibitors

Efflux transporter	Compound	Substrate (S)/inhibitor (I)
MDR1 (Pgp)	Digoxin	S
	Paclitaxel	S
	Cyclosporin A	S/I (multiple interactions)
	GF 120918	I (interacts with BCRP)
	Verapamil	S/I (multiple interactions)
	Zosuquidar	S/I
	Vinblastine	S/I (interaction with MRP)
	PSC833	S/I
ABCG2 (BCRP)	Fumitremorgin C	I
	Ko143	I
MRP2	Probenecid	I (interacts with other MRPs and OATPs)

not have high throughput capacity and can be applied only for a limited number of compounds. Please refer also to critical assessment part of Sect. 27.2.4.

PROCEDURE

The conditions for inhibition assays using CACO-2 cells are as described above for efflux assays.

In contrast to permeability tests, compounds are added not only to apical compartment (to determine flux A-B) but in an additional experiment to basolateral compartment (efflux B-A). As assay time of 2 h is proposed. At the same time, an inhibitor is added, and results of permeability and efflux assays are compared to results obtained without inhibitor. Additionally, a selection of inhibitors covering different efflux transporters is used. After a preincubation period used in many studies (e.g., 15 min), test compounds are added and flux/efflux studies performed.

As for the standard CACO-2 assay, very often, one-time-point determinations are performed in triplicate using incubator conditions (37°C, 2 h).

Samples are collected in triplicate from both the basolateral and apical compartments after 2 h. Triplicate samples are also taken from the dosing solution. Analytics are performed as described in Sect. 27.2.3.

EVALUATION

Apparent permeability coefficients (Papp in cm/s) are calculated according to the following equation:

$$\mathrm{Papp(cm/s)} = (\mathrm{dQ} \times \mathrm{V})/(\mathrm{dt} \times \mathrm{S} \times \mathrm{C_0})$$

where S is the surface area of the filter in cm^2, C_0 is the initial concentration of the compound on the donor side, dQ/dt is the amount of compound transported during the 60-min experiment, and V is the volume of the receiver chamber.

Ratio of B-A/A-B is calculated and compared to the ratio of standard compounds. In addition, B-A/A-B ratio determined in the inhibition experiment is compared to efflux ratios determined without inhibition. Given that efflux of a compound cannot be inhibited by an inhibitor, one should conclude that more than one efflux transporter are involved in the efflux. Adding two or more inhibitors at the same time provides maximal inhibition.

As a standard inhibitor, cyclosporin A is used very frequently; as also PSC833, Zosuquidar, or GF120918, all for MDR 1); fumitremorgin C or Ko143 for ABCG2; and probenecid for MRP2.

MODIFICATIONS OF THE METHOD

Modifications for efflux inhibition studies are as frequent as efflux studies and include all parts of the method (permeability time, inhibitors, determination of Papp at single time point versus several time points).

Tang et al. (2002a, b) used [3H]-vinblastine as a substrate for MRP2 studies (Evers et al. 1998). Inhibitors were cyclosporin A (25 μM) for MRP2 and MDR1 (Tanaka et al. 2000; Nies et al. 1998; Smith et al. 1998), GF120918 (2 μM) for MDR1 (Hyafil et al. 1993; Utsunomiya et al. 2000), and MK571 (50 μM) for MRP2 (Walle et al. 1999).

CRITICAL ASSESSMENT OF THE METHOD

In Chap. 27.2.4, dealing with efflux studies, it was discussed that rank order of effluxed compounds was different in different test systems (taking ratio as comparison). The conclusion was to stay in one efflux system for comparative studies and to check in overexpressing cell systems such as MDCK and LLC-PK1 protein sequence and molecular weight of transfected efflux transporters. Other efflux transporters should be checked as well because due to transfection, they could be over- or underexpressed or be expressed in a different glycosylation form. It was noted that animal cells transfected with a human gene express a protein migrating to a lower molecular weight (Evers et al. 1996). Pig kidney epithelial cells LLC-PK1, when overexpressed with MDR1, expressed a protein with a MW at 120 kD.

Tang et al. (2002a, b) detected in MDCK-MRP2 overexpressing cells, in MDCK-WT, and in CACO-2 cells 2 bands (cross-reaction of the MRP2 antibody): at 150 and 190 kD. In MDCK-MRP2 cells, only the band at 150 kD was overexpressed. The authors discuss possible differences in glycosylation as a reason for this phenomenon. In the same study, it was found that Michaelis-Menten constants Km and Vmax differed between CACO-2 and MDCK-MRP2 cells for vinblastine. Similar observations were reported by Soldner et al. (2000) for losartan and by Lentz et al. (2000) for vinblastine in MDR1 studies. Affinities for the inhibitors cyclosporin A, MK 571, vincristine, and etoposide were lower in CACO-2 cells compared to MDCK-MRP2 (Tang et al. 2002a, b). On the other hand, reserpine had no affinity in MDCK cells at all, and daunorubicin showed lower affinity in MDCK-MRP2 compared to CACO-2. Summarizing all data, authors discuss as a possible reason for those differences between CACO-2 and MDCK cells a different lipid composition of the membrane which could lead to a different orientation of MRP2 and MDR1. Additionally, drug partitioning into membranes may be different resulting in differences in substrate/inhibitory specificity and binding kinetics (Romsicki and Sharom 1999; Ferte 2000; Tanaka et al. 1997; Tang et al. 2002a, b).

Moreover, since in the Schwab study, differences in the human MDR1 and mouse mdr1 were detected concerning substrate recognition (ritonavir and saquinavir were negative in mdr1a cells, and verapamil, terfenadine, and quinidine were less active in mdr1a than in MDR1), differences occurred between porcine brain endothelial efflux and LLC-PK1-MDR1 (Vinblastine) in the future efflux systems containing animal transporters have to be considered, too. In addition, we have to keep in mind that in rodents, two efflux transporters correspond to human MDR1: mdr1a and mdr1b which have both different substrate specificities (for cyclosporin A, terfenadine, verapamil, vinblastine). And, complicating the story even more, some compounds have been identified in mdr1a as strong substrates for mdr1a but were not good substrates for human MDR1. Keeping in mind that in pharmacokinetics, studies are performed in rats and mouse before going into dogs or mini pigs PK parameters could be different between the species due to different substrate specificity of transporters.

However, for MDR1, ABCG2, and MRP2, we have a panel of studies available, and we are aware of complications and considerations. There are several models addressing binding sites and modulatory sites. Concerning in vitro in vivo correlation of results and calculation of risk factors for drug-drug interaction studies, we have to expand knowledge and add more validation studies in vitro as well clinical studies (see also Sect. 27.2.4).

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EXAMPLES

Examples of efflux studies using efflux inhibitors are given in Tang et al. (2002), Tanaka et al. (1997), and others.

27.2.6 Transporter Uptake Studies Using CACO-2 Cells

RATIONALE

Many uptake transporters as well as efflux transporters have been described in the GI and especially in the small intestine. They allow uptake of ions, amino acids, peptides, nucleic acids, sugars, organic acids, vitamins, cofactors, and nucleosides. On the other hand, efflux transporters ensure protection of the organism from unwanted pathogen or compound delivery. Subsequently, cellular systems have been used to study uptake in mechanistic studies in more detail. Additionally, inhibition studies provide hints on potential drug-drug interactions.

CACO-2 cells resemble many features of enterocytic intestinal cells. In gene chip analyses, 38% of 11,559 genes were detected in 16-day-old CACO-2 cells. One hundred and seventy of those genes were transporters and channels, and 443 were transporters, channels, and metabolic enzymes (Sun et al. 2002). In human duodenal probes, 44% of the total of 12,559 gene sequences were detected. Permeability values between CACO-2 and duodenum which were compared resulted in good correlation for passively absorbed drugs whereas correlation coefficients for actively absorbed drugs were lower in CACO-2 than in duodenum.

Nevertheless, several uptake transporters have been studied successfully in CACO-2 cells as possible drug delivery systems. CACO-2 cells are a suitable model for uptake studies given that they are characterized for the studied transporter (Anderle et al. 2003). Whereas permeability values for actively transported compounds may differ substantially from in vivo situation, a ranking of compounds for higher or lower permeability seems to be possible.

PROCEDURE

As experimental buffer for washing and permeability assay HBSS, pH 7.4 is proposed. For uptake studies, pH 6.5 might be required for those transporters which need a pH gradient (PEPT1).

Compound dilutions should be performed from compound stock solutions in DMSO, 10 mM at the day of experiment. Compounds should be taken from microwell plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5%. Alternatively, compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5%.

Compound concentration should be chosen on the basis of solubility data. Uptake experiments should be performed using Petri dishes or filter supports. In the case of filter supports, a permeability experiment has to be performed at the same time to control basolateral amount of compound.

Uptake studies for peptides have been performed by adding the compound to the apical side of the filter (pH 6.5 for optimal function of oligopeptide transporter). The pH at the basolateral side was 7.4. After a preincubation period (10 min, 37°C), cells should be washed and incubated for 15 min (or for a shorter time period) with the compound under evaluation. After incubation period medium is removed, cells are washed three times with ice-cold pH 7.4 buffer to stop further uptake and to remove unbound compound (for details, see Tamura et al. 1996). Cells should be scraped and dissolved in ice-cold buffer. If a radioactive compound is studied, cells and filters are dissolved in a Ready-Safe scintillation cocktail, and radioactivity is determined in a liquid scintillation counter. Alternatively, for nonradioactive compounds, LC-MS or HPLC detection is recommended.

An alternative is a time-dependent study where cells are scraped and uptake is stopped at different times over 15 min. In both cases (in time-dependent studies and one-time-point determinations), protein content is used to express uptake in nmol/mg protein.

In another study, uptake of biotin was studied with confluent CACO-2 cells cultured on 12-well plates (Balamurugan and Said 2003). Labeled and unlabeled biotin was added to cell monolayers, and reaction terminated after 3 min by adding ice-cold buffer (2 ml). Cells were rinsed twice in ice-cold buffer, digested with 1 ml of 1 N NaOH, neutralized with HCl, and then counted for radioactivity. Protein content of cells was determined with a Bio-Rad kit (Richmond, US).

A comparable uptake assay to study uptake of peptides and compounds via peptide transporter was described by D'Souza et al. (2003).

EVALUATION

The uptake is expressed in nmol/mg protein and compared to standard drugs. In addition, time-dependent uptake experiments provide uptake kinetic parameters.

MODIFICATION OF THE METHOD

Modifications are used to study different uptake transporters. Inhibition studies provide information about transporters involved in uptake. An alternative are cells overexpressing uptake transporters or oocyte studies with one expressed uptake transporter.

CRITICAL ASSESSMENT OF THE METHOD

An uptake assay using CACO-2 monolayers grown on filter dishes needs to be combined with permeability studies to assess the amount of compound permeating during the short time of uptake. Studies with cells grown on Petri dishes or multi-well plates have the disadvantage that they may be performed with cells not fully differentiated. Uptake and efflux transporter expression increases with differentiation. Moreover, some transporters require pH gradients and the three-dimensional filter geometry to function with optimal activity.

Transporter studies have been performed with CACO-2 cells for many different transporters of the Solute Carrier system (SLC). The use of transporters as drug targeting systems to increase bioavailability is discussed since several years. Many transporters from the SLC family are being evaluated for their drug targeting use. The following part of the critical assessment will deal with SLC transporters identified in the intestine and their possible role in respect to drug targeting.

27.2.6.1 Uptake Transporters in the Intestine

Figure 27.3 illustrates the most important transporters and enzymatic systems identified in enterocytes.

Subsequently, several of the transporters have been used as drug delivery systems.

27.2.6.2 Amino Acid Transporters

Several uptake amino acid transporters have been identified at the apical side of brush border membrane enterocytes: heterodymeric amino acid transporters (HAT system) with apical transporters LAT1-4F2hc and b0. + AT and the systems PAT, B0, Beat/Taut, A, and B0+. At the basolateral side, the HAT transporters y + LAT1-4F2hc, LAT2-4F2hc, and ASC1-4F2hc are expressed together with other systems: A, y+, XAG-, and Tat (for overview, see Steffansen et al. 2004). In Table 27.3, the transporters mentioned are listed with gene name and substrate specificity. However, the capacity of amino acid transporters seems to be limited for an effective oral delivery approach. On the other hand, substrate specificity is limited as well. For review, refer to Verrey (2003), Palacin and Kanai (2003), Verrey et al. (2003), Halestrap and Meredith (2004), and Ganapathy et al. (2001).

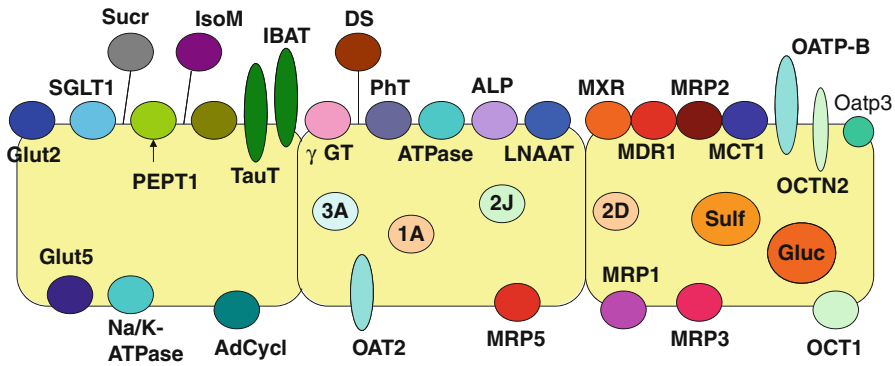

Fig. 27.3 The intestinal barrier. *Sucr* sucrase, *IsoM* isomaltase, *DS* disaccharidase, *TauT* taurocholic acid transporter, *MCT1* monocarboxylic acid transporter, *PhT* phosphate transporter, *LNAAT* amino acid transporter, *3A*, *1A*, *2D*, *2 J* phase I metabolizing enzymes, *Gluc* glucuronidase/phase II, *sulf* sulfatase/phase II, *PEPT1* di/tripeptide transporter, *OCT*, *OCTN2* organic cation transporters, *IBAT* intestinal bile acid transporter, *AdCycl* adenylate cyclase, *MDR*, *MRP* ABC transporters, *ALP* alkaline phosphatase, *γGT* gamma-glutamyl transpeptidase, *OAT* organic anion transporter, *OATP* organic anion transporting polypeptide, *Glut*, *SGLT* glucose transporters

Table 27.3 Amino acid transporters, substrates, and localization. *A* apical side of the membrane, *B* basolateral side of the membrane

Transporter	Gene name	Specificity	Substrates	Cellular localization
LAT1-4F2hc	SLC7A5/SLC3A2	Large NAA	Gabapentin, melphalan, baclophen, L-dopa	A/B
LAT2-4Fhc	SLC7A8/SLC3A2	NAA	Cys	B
B0. + AT	SLC7A9/SLC3A1	CAA	Gabapentin	A
ATB0	U75284	NAA	Pregabalin	A
CAT1	SLC7A1	CAA		B
Y + LAT1-4F2hc	SLC7A7/SLC3A2	CAA		B
Y + LAT2-4F2hc	SLC7A6/SLC3A2	CAA		B
		NAA		
A	SLC5A4	NAA	Glu, amino acids	A/B
ATB0.+	SLC6A14	NAA, CAA	Pregabalin	A
TAUT	SLC6A6	Tau	Tau, Ala	A
TAT1	SLC16A10	AAA	L-dopa	B
EAAC1	SLC1A5	Asp/Glu	Asp-3	B
ASC1-4F2hc	SLC7A10/SLC3A2	Ala, Ser, Cys, Gly, Thr	Aminobutyric acid, β-alanine, D-Ser	B
PAT1	SLC36A1	NAA	D-Ser, D-cycloserine, GABA	A

27.2.6.3 Nucleoside Transporters

At the apical side of intestinal enterocytes, concentrative nucleoside transporters CNT1, CNT2, and CNT3 are identified, whereas at the basolateral membrane, equilibrative nucleoside transporters ENT1 and ENT2 are localized. Table 27.4 gives an overview on nucleosides, nucleobases, and drugs being transported by nucleoside transporters (for a detailed review, see Steffansen et al. 2004; Ritzel et al. 1997, 2001).

The capacity for drug transport by nucleoside transporters is different: the CNTs are discussed as potential drug uptake candidates since they have micromolar affinities. Several antiviral and anticancer drugs are transported by CNTs like 3-azido-3-deoxythymidine (AZT) and gemcitabine by CNT1, 2,3-dideoxyinosine by CNT2 and 5-fluorouridine, zebularine, gemcitabine, cladribine, and fludarabine by CNT3 (Ritzel et al. 2001).

Table 27.4 Nucleoside transporters, substrates, and localization. *A* apical side of the membrane, *B* basolateral side of the membrane

Transporter	Gene name	Specificity	Substrates	Cellular localization
CNT1	SLC28A1	Purine nucleosides	Zaltidabine, cytarabine, cladribine, gemcitabine, 5 deoxy-5-fluorouridine	A
CNT2	SLC28A2	Pyridine nucleosides	Adenosine, cladribine, didanoside	A
CNT3	SLC28A3	Purine and pyridine nucleosides	5-fluorouridine, floxuridine, zebularine, gemcitabine, zalcitabine	A
ENT1	SLC29A1	Purine, pyrimidine nucleosides	Cladribine, cytarabine, fludarabine, gemcitabine, zalcitabine, didanoside	B
ENT2	SLC29A2	Purine, pyrimidine nucleosides	Gemcitabine, didanoside	B

27.2.6.4 Sugar Transporters

Hexose transporters are divided into two families: the sodium-dependent glucose transporters (SGLT, transport of hexoses against a concentration gradient) and the Glut family (transport of hexoses down a concentration gradient). At the apical membrane of enterocytes SGLT1, Glut2 and Glut5, and SGLT6 have been identified. Basolaterally, hexoses are transported by Glut5 and SGLT6. The localization of SGLT3 and SGLT4 is unknown. SGLT1 transports D-glucose, D-galactose, alpha-methyl-D-glycopyranoside, inositol, proline, pantothenate, iodide, urea, myo-inositol, and glucose derivatives. SGLT6 is capable of transporting D-glucose. SGLT3 transports D-glucose and myo-inositol. Glut5 transports D-fructose whereas Glut2 identifies D-glucose, D-fructose, and streptozotocin. For reviews, please refer to Wright et al. (2003), Wright and Turk (2004), Uldry and Thorens (2004), and Stuart and Trayhurn (2003). Examples of drugs being transported by hexose transporters are rare so far.

27.2.6.5 Peptide Transporters

In the intestine, the following peptide transporters have been identified: PEPT1, peptide/histidine transporter PHT1, PHT2, and the peptide transporter PT1 (Herrera-Ruiz et al. 2001, and others). PT1 belongs to the cadherin family; all other peptide transporters belong to the solute carrier family SLC15A.

For PEPT1, an impressive list of compounds (drugs and prodrugs) has been presented during the last years all of which in retrospective have been identified as PEPT1 substrates (e.g., beta-lactam antibiotics, cephaslosporins, ACE and renin inhibitors, thrombin inhibitors, bestatin, and prodrugs of acyclovir and ganciclovir) (Friedman and Amidon 1989; Walter et al. 1995; Bretschneider et al. 1999; Brandsch et al. 2004 and others). A very comprehensive overview is given in Nielsen et al. (2002). Several reviews deal with PEPT1 as a potential drug delivery target and show therapeutic applications and prodrug approaches (Daniel and Kottra 2004; Nielsen et al. 2002; Steffansen et al. 2003, 2004).

27.2.6.6 Monocarboxylate Transporters (MCT)

Eight different monocarboxylate transporters have been identified playing an important role in the cellular metabolism by transporting endogenous substrates like monocarboxylates, pyruvate, L-lactate, propionate, and butyrate (Halestrap and Price 1999). The best studied transporter, MCT1, is localized basolaterally (according to Tamai et al. 1995). Other authors detected MCT1 in apical intestinal membranes (Ritzhaupt et al. 1998). Tamai studied the uptake and kinetics of several organic acids like benzoic acid, nicotinic acid, pravastatin, and salicylic acid (Tamai et al. 1995). Some beta-lactam antibiotics like cefdinir, phenethicillin, and carindacillin have been proposed as MCT1 substrates (Tsuji et al. 1993; Itoh et al. 1998; Li et al. 1999).

27.2.6.7 Fatty Acid Transporters

In the intestine fatty acid translocase (FAT), fatty acid transporting protein (FATP4) and fatty acid binding protein (FABPpm) have been identified (for review, see Stahl 2003). Natural substrates are long chain fatty acids like myristate, oleate, and palmitate.

27.2.6.8 Organic Anion Transporters and Transporting Polypeptides

In the intestine, only few members of the large group of organic anion transporters have been found: OAT2 (SLC22A7) and OATP-B (SLC21A9) responsible

for the transport of many different compounds (Burckhardt and Wolff 2000; Tamai et al. 2000; Kobayashi et al. 2003). Drugs like fexofenadine, methotrexate, pravastatin, and ouabain are substrates of OATPs (Cvetkovic et al. 1999; Abe et al. 2001; Bossuyt et al. 1996; Dresser et al. 2002). Members of organic anion transporters present in the gut are OAT2 (colon, duodenum, ileum, jejunum), OAT3 (colon), URAT1 (ileum, jejunum), OAT10 (colon, jejunum), which all have been detected at mRNA level (see review of Burckhardt and Burckhardt 2011).

27.2.6.9 Organic Cation Transporters

The family of organic cation transporters is represented at the apical membrane of intestinal cells by OCTN2, whereas the basolateral membrane is equipped with OCT1 (Sekine et al. 1998). In the liver, OCT1 plays a crucial role in uptake of positively charged drugs and compounds preparing hepatic metabolism and elimination. In the intestine, this transporter is localized basolaterally, making a drug targeting questionable: compounds once entering the cells would be identified by OCT1 and excreted via sinusoidal membrane directly for uptake into hepatocytes (for review, see Koepsell and Endou 2004; Koepsell et al. 2003).

OCTN2 was identified as a Na+/carnitine cotransporter and is also located in the apical membrane of proximal tubular cells. Substrates of OCTN2 are TEA, verapamil, pyrilamine, choline, and quinidine (Koepsell et al. 2003).

Other organic cation transporters identified at mRNA level were OCT1, OCTN1, and OCTN2 (small and large intestine) and OCT2, OCT3, and OCTN3 (small intestine)—for a review, see Koepsell et al. (2007).

27.2.6.10 Phosphate Transporters

It is believed that the major part of inorganic phosphor absorption in the intestine occurs via Na^+-dependent phosphate cotransporter NaPi-IIb (SLC34A2, Xu et al. 1999). Small drugs like phosphocarbonic acid and foscarnet are transported by this transporter (Swaan et al. 1995; Tsuji and Tamai 1996). However, the substrate range is rather limited including only inorganic phosphate compounds.

27.2.6.11 Bile Acid Transporters

Bile acids are reabsorbed from the intestine by the bile acid transporter ASBT (SLC 10A2). Substrates are cholate, taurocholate, glycoursocholate, and glycodeoxycholate (Craddock et al. 1998; Wong et al. 1996). Conjugation of oligopeptides to bile salts increased their bioavailability substantially (Kramer et al. 1994). However, it is important to ensure that possible ASBT-mediated drug uptake should not interfere with bile salt absorption. It is known that patients with decreased bile salt uptake have higher risk for colorectal carcinomas may be due to increased bile salt concentration in the GI (Wang et al. 2001).

27.2.6.12 Vitamin Transporters

Several vitamin transporters are expressed in the intestine: SVCT1 responsible for ascorbic acid uptake (Wang 2000) and THTR-2 (SLC 19A3) showing high affinity for thiamine (Eudy et al. 2000; Nguyen et al. 1997; Said et al. 1996). Biotin is transported by the SMVT (Balamurugan and Said 2003). The vitamin transporters are discussed for possible drug targeting although their uptake capacities are low.

27.2.6.13 Transports with Unknown Localization

Several transporters have been identified in intestinal tissue without defining the localization: SGLT3 and SGLT4 for glucose, PHT2 (PTR3) for peptide/histidine, MCT8 for monocarboxylates/thyroid hormone, FABpm for fatty acids (oleate, myristate, palmitate, stearate, arachidonate, linoleate), RFC1 for folate, THTR2 for thiamine, and SMVT for biotin (Steffansen et al. 2004).

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EXAMPLES

Examples of transporter uptake studies utilizing CACO-2 cells can be found in D'Souza et al. (2003), Bretschneider et al. (1999), Tamai et al. (1995), Walter et al. (1995), and Balamurugan and Said (2003).

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28.1 General Considerations

The ability to permeate a biological membrane is a prerequisite for orally administered drugs. A number of active transport processes involving many different transporter proteins have been described in the last years. However, the primary absorption pathway for the majority of drugs after oral dosage still seems to be the passive *transcellular diffusion* pathway (for a detailed discussion, see Sugano et al. 2010).

Biological membranes consist of lipid bilayers featuring a polar headgroup region at the interface to the aqueous phases and a nonpolar region in the center of the bilayer. The main constituents of these membranes are amphiphilic phospholipids and cholesterol.

In order to cross a biological lipid bilayer membrane, drugs have to interact with the hydrophilic headgroup region of the membrane and enter it subsequently from the water environment (unstirred water layer (UWL)). This is followed by diffusion through the hydrophobic region of the bilayer. Consequently, the drugs have to exit the hydrophilic headgroup region again into the water environment at the inner side of the membrane. The overall process is driven by the concentration gradient of the compound across the membrane.

The passive *permeability* of a drug is determined by its lipophilicity, which is the physicochemical property that influence all of the steps involved in the transcellular diffusion. Lipophilicity is a mixed parameter reflecting both hydrophobic and polar membrane affinity properties as well as hydrogen bonding and ionization properties of the drug depending on the lipophilicity scale and lipophilicity measurement method used (Liu et al. 2011).

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Lipophilicity is traditionally expressed as the partition coefficient between water and n-octanol which is called logP (see Chap. 44.3). However, a number of alternative assays for estimating lipophilicity (and predicting absorption) expressed as partition coefficients have been proposed (other organic solvents as isotropic partitioning media, reversed phase HPLC using anisotropic media for partitioning). Artificial membrane preparations or real lipid bilayer membranes (liposomes) have also been used for partition experiments. The intention of these assays is to find improved correlation of lipophilicity values to passive transcellular absorption of drugs than using the classical octanol-water partitioning system by taking into account the ionization characteristics of phospholipid membranes.

In other assays, the permeation of a compound is followed directly by estimating the amount of compound on either side of the membrane barrier. The results of these experiments (e.g., parallel artificial membrane permeation assay, PAMPA, or liposome permeation studies) are expressed as permeability values rather than lipophilicity values even if partition coefficients can be calculated from these studies and the PAMPA permeability results themselves might also be regarded as another lipophilicity scale.

A number of review articles and books described recently the methods used in pharmaceutical research to early on estimate the absorption potential of a compound by means of a variety of cell-free techniques:

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28.2 *HPLC* Methods for *Lipophilicity* Determination

PURPOSE/RATIONALE

Lipophilicity is a main parameter influencing a drug's passive intestinal absorption and is traditionally expressed as the *partition coefficient logP* between water and n-octanol (Hansch and Fujita 1964). The partition coefficient logP is used if the partition behavior of the neutral form of an analyte is considered; the *distribution coefficient logD* is used if a mixture of differently charged forms of the same analyte is under investigation. LogD values take the ionization state of a compound into account by measuring the distribution behavior of a given compound at a fixed pH. A wide range of correlations between lipophilicity expressed as logD(P) octanol-water ($\log D(P)_{OW}$) values and biological properties of compounds have been described over the years including biological absorption and membrane partitioning (Kerns 2001b). The partition coefficient $\log P_{OW}$ or the distribution coefficient $\log D_{OW}$ between water and n-octanol is determined traditionally by the shake-flask method (see Chap. 44.3). The shake-flask method is a rather labor-intensive method and not suited for high-throughput analysis needed in pharmaceutical research. An alternative way is the determination of logD(P) values based on chromatographic retention.

The use of hydrophobic stationary phases and aqueous mobile phases in *chromatography* on reversed

phase (RP) materials is widely established. *Reversed phase* materials typically consist of alkyl chains (mainly C18 in length) immobilized on silica material. The chromatographic retention of a solute on such a system directly depends on its partition between the aqueous mobile and the hydrophobic solid phase (Berthod and Carda-Broch 2004). Therefore, lipophilicity (expressed as partition coefficient logP between stationary and mobile phase, Eq. 28.1) can be estimated from the retention behavior of the solute of interest on reversed phase material as reviewed by Nasal et al. (2003).

$$\log P = \log k - \log\left(\frac{V_s}{V_m}\right) \tag{28.1}$$

V_m and V_s are the volumes of the stationary and mobile phase, respectively; k is the *retention factor* that is derived from the retention time (t_R), taking the dead time (t_0) into account.

$$k = \frac{t_R - t_0}{t_0} \tag{28.2}$$

The advantage of using a chromatographic system is much higher throughput compared to shake-flask assays. The retention time of an analyte is concentration independent, and therefore, no quantification has to be done to establish lipophilicity values. Chromatographic measurements are easily automated including peak picking and data evaluation. Another advantage is that disturbing impurities are separated from the compound of interest during the chromatographic experiment and will not interfere with the estimation of lipophilicity. However, the volume of the stationary phase cannot be estimated easily, so that the partition coefficients have to be calibrated using a set of compounds with known partition coefficients in order to achieve comparable results.

PROCEDURE

The lipophilicity of compounds can be measured with any commercially available HPLC equipment traditionally using octadecyl-bonded silica (ODS) columns (but also other column materials like polymer-based materials without residual silanol groups (Donovan and Pescatore 2002) or monolithic materials have been described, discussed in the review by Kaliszan 2007); most commonly, UV absorption is used as detection system for the chromatography but also LC/MS coupling was described (Camurri and Zaramella, 2001; Kerns et al. 2003). The use of mass spectrometry as detection method offers the possibility to determine the lipophilicity of a mixture of analytes in one run. Recently, the use of ultra-high-pressure liquid chromatography (UHPLC) coupled with mass spectrometry was described in order to decrease the time needed for the analysis (Henchoz 2009b).

The retention factor of a compound is the analytical parameter determined to measure lipophilicity (Eq. 28.1). To measure the retention factor, the retention time of the compound under investigation has to be determined reproducibly together with the dead time. The dead time is determined using the retention time of a not retained substance on reversed phase columns like buffer salts. In principle, injecting a solute onto a reversed phase system using a purely aqueous mobile phase would directly determine the partition coefficient. However, to cover a broad range of lipophilicity, a variety of mobile phase mixtures have to be used as most of the drug substances do not elute from reversed phase columns using purely aqueous mobile phase. Organic cosolvents in the mobile phase have a strong influence on the measured partition between lipophilic stationary phase and mobile phase and have to be considered. Generally, a number of different experiments with increasing percentage of organic solvent (not exceeding about 50–70% organic solvent) have to be carried out, and the determined retention factors are plotted against the organic solvent ratio (φ) used in the respective experiment. The retention factor k_w extrapolated to 0% organic solvent is taken from this plot. Using methanol, the retention factors k and k_w are linearly correlated with a constant factor S, depending on the analyte and the organic modifier (Kaliszan 2007).

$$\log k = \log k_w - S * \varphi \tag{28.3}$$

Valko et al. (1997) and Du et al. (1998) showed that instead of using a number of different isocratic experiments at different organic solvent ratios, one fast gradient experiment on short reversed phase columns could be applied. If a fast gradient is used, any retention time defines a specific mixture ratio of organic and aqueous solvent. Therefore, the retention index k_w was determined directly from one experiment just by measuring the retention time. Short reversed phase

columns are recommended in order to keep the organic solvent ratio low at the point of elution from the column.

Different buffer systems at different pH values (mainly 6.8 or 7.4) with organic modifiers are used as mobile phase. Results obtained with methanol as cosolvent were shown by Baczek et al. (2000) to correlate better to octanol-water partitioning data than results using acetonitrile as organic cosolvent. The H-bonding capabilities of the alcohol seem to be the reason for that.

EVALUATION

The variety of stationary phases available commercially and the lack of standardization between different laboratories make it difficult to compare the retention factors *k* directly as comparison of lipophilicity. Therefore, different lipophilicity scales obtained from chromatographic data (isocratic or gradient elution) have been introduced.

The determination of log k_w as the retention factor at 0% organic modifier estimated from isocratic chromatographic analysis was described earlier. Log k_w is one of the widest used chromatographic lipophilicity parameters and is usually correlated to Log P_{OW} values estimated using the traditional shake-flask method. OECD guidelines (1989) suggest a calibration of k_w values with experimental $\log P_{OW}$ values and the expression of reversed phase chromatographically estimated partition coefficients directly as logP values.

The lipophilicity index φ_o was defined by Valko and Slegel (1993). φ_0 is the volume percent of organic modifier in the mobile phase by which the retention time is twice the dead time, which means the retention factor k is equal to 1. It was reported that using the φ_0 scale, the inter-laboratory comparability was improved compared to using log k_w. The correlation with traditionally determined $\log P_{OW}$ values was shown to be better (Valkó 1993) using the φ_0 index. The analytical advantage of the φ_0 value is that it can be estimated from bracketing experimental results and not from extrapolation to 0% organic modifier.

The *CHI* index was introduced by Valko et al. (1997) in order to match the lipophilicity index φ_0 of isocratic experiments with results from gradient elution. The lipophilicity results from gradient elution experiments are calibrated using standard compounds with known φ_0 value estimated using isocratic elution. The CHI scale was used for neutral drugs and also for acids and bases (Fuguet et al. 2007; Pallicer et al. 2011).

The results of chromatographic lipophilicity measurements using gradient elution are often directly expressed as logD values. In that case, estimated retention factors are calibrated with known $\log D_{OW}$ values of a standard set of compounds used as external or internal standards. However, it has to be pointed out that the correlation between estimated logD values from reversed phase chromatography and octanol-water distribution coefficients $\log D_{OW}$ depends on proper selection of calibration standards and maybe low for a diverse set of compounds (Valko et al. 1997). The reason is the different organic partition media used in the experiments.

Several review articles recently summarized the use of reversed phase chromatography (also discussing the most important stationary phases used) to measure partition coefficients (Valkó 2004; Berthod and Carda-Broch 2004; Nasal et al. 2003; Kaliszan 2007; Henchoz et al. 2009).

CRITICAL ASSESSMENT OF THE METHOD

The lipophilicity measurements using reversed phase HPLC are fast and reliable, and the equipment for the analysis is available in almost every laboratory dealing with drug discovery. Using reversed phase chromatography in the gradient mode, the high throughput needed to cope with the high numbers of compounds delivered from combinatorial or parallel synthesis can easily be achieved. As LCMS analysis on fast generic gradients is used nowadays as the standard tool for purity estimation in drug research, the very same method offers a very robust way to estimate lipophilicity of high numbers of compounds (even mixtures) on the run (Kerns et al. 2003). Good correlation between octanol-water partition coefficients and chromatographic retention factors (isocratic or gradient) for neutral molecules ($\log P_{OW}$) can be achieved if a proper calibration set was chosen. For charged analytes ($\log D_{OW}$), the reversed phase HPLC system may be only an approximate model mainly when structurally diverse compound sets were analyzed (Valkó 2004) and even more when gradient mode was applied. However, for comparison measurements and ranking of lipophilic properties of a high number of compounds, the reversed phase chromatography technique using fast gradient elution seems to be the method of choice even if the correlation to $\log D_{OW}$ maybe not perfect under these conditions.

There are obvious differences between the classical octanol-water partition system and the reversed phase chromatographic partition system mainly due to the different nature (isotropic partition vs. anisotropic partition) of the partitioning media. Related to drug absorption, both classical octanol-water partition coefficients and lipophilicity values using the different chromatographic scales have shown to be predictive. In summary, Taillardat-Bertschinger et al. (2003) stated in their review that the best lipophilicity descriptor used to predict membrane permeation differs according to the compounds under investigation. Wang et al. (2009) added that it is not shown yet which lipophilicity descriptor was best suited to predict membrane permeability for a large diverse set of compounds. Therefore, a number of different methods and stationary phases (including biopartitioning columns, see "Modifications of the Method") might be suitable for a given class of drugs, and the use of a number of different scales was recommended in literature as discussed by Valko (2004).

MODIFICATIONS OF THE METHOD

In order to improve the correlation between isocratic $\log k_w$ values and $\log P_{OW}$ values for diverse sets of compounds, Lombardo et al. (2000, 2001) used an octanol-saturated mobile phase and a Supelcosil LC-ABZ (polar amide column) as stationary phase. Isocratic separation is used in their work. The obtained $\log_w$ data are calibrated with known logD/logP values of a training set, and the obtained lipophilicity data from unknown samples are reported as *ElogP* (ElogD) values. These authors pointed out that *ElogD* values correlate very well with traditionally obtained $\log D_{OW}$ values in case of neutral and basic compounds. Benhaim (2008) expanded this concept by using a modified silica column which can be used over a broad pH range (pH 2–12) and allowed to study the lipophilicity of the neutral form of acids and bases.

Apart from the hydrophobic interactions provided by the alkyl part of the molecule, octanol has also hydrogen bond acceptor and donor functions like lipid membranes have. This property of n-octanol made the octanol-water distribution coefficient that is widely used. However, neither n-octanol nor reversed phase materials can mimic the interfacial character of the bilayer structure. The ionic interactions between charged membrane *phospholipids* and ionisable solutes are also not represented in the properties of octanol or reversed phase materials. To overcome this issue, alternative stationary phases have been developed to determine lipophilicity values more related to intestinal absorption than to classical $\log P_{OW}$ values.

Those stationary phases are made of silica material with covalent bond phospholipids mimicking a cell membrane surface as introduced by Pidgeon et al. (1989). These *immobilized artificial membrane* (*IAM*) stationary phases having diacetylphosphatidylcholine (PC) with or without glycerol backbone bound to silica are also commercially available (Regis Technology, Morton Grove, IL). Kararli et al. (1995) showed that PC is relevant to intestinal absorption as PC together with PE (phosphatidylethanolamine) comprises a major portion of the intestinal brush border membranes.

The lipophilicity measured using IAM columns generally is expressed as the retention factor k_{IAM} or k_{IAMw} of a solute following Eqs. 28.2 and 28.3, respectively, using IAM columns. Valkó et al. (2000) also applied gradient elution to IAM columns, and a CHI_{IAM} index was defined similar to the CHI values as described for reversed phase chromatography. Log k_{IAMw} values of neutral solutes often correlate with other lipophilicity scales, but acidic solutes generally result in different values using different lipophilicity scales (Sun 2008). Log k_{IAM} values have been correlated successfully with blood-brain barrier distribution, small intestine absorption, and Caco-2 cell permeation as summarized by Stewart et al. (1998). Modeling of Caco-2 permeability was proven by the use of IAM chromatography together with other descriptors like polar surface area (PSA) (Chan 2005). The respective correlations were shown to be superior to the correlation with octanol-water partition coefficient or HPLC-determined lipophilicity in another review by Yang et al. (1996). Liu (2008) studied the retention behavior of almost 50 diverse drugs on an IAM column and described the influence of the ionization state of the analytes on their retention. Kotecha et al. (2008) used the highest k_{IAMw} value in the pH range from 4.5 to 7.5 ($\log k'_{IAM}{}^{4.5–7.4}$) in combination with PSA to predict oral absorption for divers set of drugs. IAM chromatography has been reviewed by Giaganis and Tsantili-Kakoulidou (2008).

Also, liposomes as real lipid bilayers (compare next chapter) have been used in chromatography-based lipophilicity assays (ILC, immobilized liposome chromatography) as reviewed recently by Cserhati (2010).

Österberg (2001) compared liposome partition with ILC and other lipophilicity scales. An excellent correlation between liposome partitioning data and ILC results was shown. Beigi (1998) immobilized liposomes onto sepharose material by gel-bead swelling and used it as solid phase in chromatography experiments (*immobilized liposome chromatography*, *ILC*). A bell-shaped correlation between the retention behavior on the liposome columns and human fraction absorbed was reported by these authors. However, the immobilization of liposomes using gel-bead swelling is not very stable over an extended time range, and column-to-column reproducibility seems to be an issue. Liu (2002) coupled avidin covalently to sepharose material and used biotin-tagged phospholipids to immobilize liposomes on this stationary phase. They reported improved column stability compared to the gel-bead swelling approach. Good correlation to human fraction absorbed was shown using these results. Even extracted biological membranes have been used in ILC experiments by Engvall and Lundahl (2004); however, the stability of the columns was shown to be limited. Instead of covalent immobilization, also dynamic coating of liposomes onto reversed phase columns has been described, and improved column stability was reported. Techniques and applications have been reviewed recently (Godard 2011).

Lazaro et al. discussed in 2006 the differences between reversed phase materials compared with IAM material and described a theoretical measure to decide which tool might be the best choice depending on the nature of the biological barrier to be predicted (BBB, intestinal absorption, skin absorption, etc.). Lipid membrane mimicking chromatography (biopartitioning chromatography) methods have been reviewed in detail by Sun (2008).

In addition to the described chromatography-based methods, a number of electrically driven systems have been described recently (Ornskov 2005) using different pseudo-stationary phases. The separation of neutral molecules is achieved due to partition into the charged micelles. MEKC (micellar electrokinetic chromatography) using SDS (sodium dodecyl sulfate) as pseudo-stationary phase or LEKC (liposome electrokinetic chromatography) has been described. For details, refer to the review articles by Henchoz et al. (2009b) and Cserhati (2010).

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28.3 Lipophilicity Determination Using *Liposomes*

PURPOSE/RATIONALE

Lipophilicity expressed as logP$_{OW}$ correlates with membrane affinity and other biological properties as summarized in Kern's (2001) review on physicochemical profiling. However, the interfacial (anisotropic) character of bilayer membranes and the ionizable phospholipid headgroups of biological membranes influence the partition properties of drugs. These effects are not reflected using octanol-partitioning experiments. To analyze these influences, the partition of solutes between water and charged bilayer membranes should be measured.

For this purpose, liposomes are used as lipid phase. Unilamellar liposomes are artificial lipid bilayer vesicles. They can be considered as real model bilayer membranes as they ideally consist of a circular bilayer membrane. The hydrophobic acyl chains are assembled in the hydrophobic core of the liposome, whereas the hydrophilic headgroups point to the water in the inner and outer side of the vesicle. Liposomes can be

produced from a variety of lipids and from mixtures of lipids. This possibility allows studying the influence of membrane constituents on the partition of solutes. Krämer et al. (1997) studied the influence of the presence of free fatty acids in membranes on the partition behavior of propranolol. The influence on α-tocopherol in membranes on the partition behavior of desipramine has been reported recently (Marenchino et al. 2004) using a liposome model.

Balon et al. (1999a) reported improved correlation between liposome partition and human intestinal absorption in comparison to the correlation of intestinal absorption to $\log D_{OW}$. Tammela et al. (2004) showed a good agreement between Caco-2 permeation results and liposome data.

The general use of liposomes and the evaluation of the obtained data have been recently reviewed by Plember van Balen et al. (2004) and Krämer (2001).

PROCEDURE

For liposome partition experiments, freshly prepared liposomes of defined size are incubated with an aqueous solution of the analyte. The partition coefficient is calculated from the ratio of compound present in aqueous environment and lipid environment in equilibrium.

In order to prepare liposomes, the lipid preparation is dried at low temperature under an inert gas atmosphere (protect the lipid from oxidation). The lipid film is swollen with water or buffered aqueous solution, and several freeze-thaw cycles are carried out to get optimal rehydration of the lipid. The rehydrated lipid preparation is filtered using membrane filters with defined pore size. After repeated filtration steps (extrusion), a unilamellar liposome preparation with a defined size distribution is obtained. Large unilamellar vesicles (LUVs) are produced in this way. LUVs are about 100 nm in size; the thickness of the lipid bilayer is about 4 nm. Even smaller liposomes can be derived from sonication (sonication probe or ultra-sonication bath). Separation of the prepared liposomes according to size using sepharose chromatography or ultracentrifugation ensures a homogenous size distribution of the liposomes (Krämer 2001). Balon et al. (1999b) used these sonicated small unilamellar vesicles (S-SUV) for partition experiments.

Several methods are used to determine the partition coefficient of solutes between water and liposomes.

Equilibrium dialysis is used in a number of examples to analyze the ratio of lipid-bound to free analyte. Krämer et al. (1998) described the use of equilibrium dialysis by separating the liposome suspension and the water phase by a semipermeable membrane. The analyte is dissolved in the water compartment of the system and diffuses into the liposome compartment. If equilibrium is reached, the remaining concentration of the analyte in the water compartment is determined by means of a quantification method (mainly HPLC or LC/MS, fluorescence techniques) and the partition coefficient is calculated. Krämer et al. (1997) used a radio tracer substance as analyte to quantify the compound in both compartments using liquid scintillation counting.

The partitioning behavior of an ionizable compound can also be followed directly using a two-phase *potentiometric titration* using commercial instrumentation (Sirius Analytical Instruments) as shown by Avdeef et al. (1998).

EVALUATION

The partition experiments are generally evaluated in terms of a partition coefficient that is calculated from the concentration ratio of analyte bound to liposomes or dissolved in the aqueous phase. For calculating the partition coefficient, the amount of lipid in the system has to be known.

Using equilibration dialysis, the distribution coefficient logD is calculated using the following equation (Plemper van Balen et al. 2004):

$$\mathrm{LogD} = \log\left(\frac{V_{LB}(C_{LB} - C_B)}{V_{Lipo} \cdot C_B} + 1\right) \qquad (28.4)$$

C_{LB} and C_B represent the analyte concentration in the liposome-containing compartment and the aqueous compartment, respectively. V_{LB} is the volume of the liposome-containing compartment, and V_{Lipo} defines the total volume of lipid phase (total amount of lipid used).

The distribution coefficient logD expresses the distribution of a solute if a mixture of ionization states of the analyte molecule is present at a defined pH value. The partition coefficient logP is strictly expressed for a specific ionization state of a solute. LogP values can be obtained from liposome partition experiments if experiments are carried out under a number of defined pH conditions.

Using the potentiometric titration approach (the same approach is also used to determine $\log P_{OW}$ values

and described in the paragraph about physicochemical properties), first, the substance in aqueous solution is titrated against standard acid or base in order to obtain the pKa value. In presence of liposomes, the experiment is repeated, and a shift in the observed pKa value (pKa^{app}) might be noted as analyte has disappeared from the aqueous phase into the liposome phase. From the shift in observed pKa^{app} value, the partition coefficient of protonated analyte and neutral analyte can be calculated (Plemper van Balen et al. 2004).

$$pKa^{app} = pKa - \log\left(\frac{1 + r \cdot P^{B}}{1 + r \cdot P^{BH+}}\right) \qquad (28.5)$$

The ratio of organic to aqueous phase is expressed as r; P^{B} is the partition coefficient of the unionized and P^{BH+} is the partition coefficient of the protonated analyte. Using this approach, depending on the nature of the analyte, several titrations (for monoprotic compounds, at least two experiments) using different ratios of lipid to water phase have to be carried out in order to determine the partition coefficients of the solute. The result is a partition coefficient-pH profile for the compound and its distinct ionization states.

CRITICAL ASSESSMENT OF THE METHOD

Using liposomes for membrane affinity studies has the great advantage that liposomes are a nearly one-to-one model of biological bilayer membranes. Liposomes can be generated from a variability of lipids and mixtures of lipids in order to study the influence of the membrane constituents on the partition behavior of drug candidates.

Mainly the electrostatic interactions of liposomes are closer to those of biological membranes than in the case of n-octanol or reversed phase solid phases. The partition coefficients for neutral molecules mainly correlate well with n-octanol-partitioning data or chromatographic lipophilicity scales including IAM, whereas for charged or zwitterionic molecules, strong differences between partitioning in liposomes and octanol were described. Using liposomes, the difference between charged and uncharged molecules concerning their partitioning coefficient is much lower as compared to the $logP_{OW}$ case. Therefore, the use of liposomes might help in understanding the role charged species play in the overall membrane permeation process of drugs. Balon et al. (1999a) showed that the correlation of liposome partition data with human intestinal absorption is superior to n-octanol partition experiments in some cases, whereas Österberg (2001) showed equivalent correlation of different lipophilicity scales including IAM and liposome partitioning compared to human fraction absorbed. Plemper van Balen et al. (2004) pointed out that the mechanism for permeation might be different for different ionized species of the same molecule or members of the same chemical series, which makes correlation of data complicated.

However, the throughput of liposome partition assays is limited as preparation and validation of liposomes are very time consuming. That seems to be the main reason why liposome partitioning is not widely used in the pharmaceutical industry to date. On the other hand, the results obtained using liposome partitioning show that liposomes are unique tools to study the bilayer *membrane affinity* (MA) of drugs and their correlation to intestinal membrane absorption.

MODIFICATIONS OF THE METHOD

The use of *solid-supported lipid membranes* (*SSLMs*) to measure membrane affinity was recently reported by Loidl-Stahlhofen et al. (2001a, b). To produce solid-supported lipid membranes, a single phospholipid bilayer membrane is noncovalently attached to a solid support. In contrast to covalent immobilization of liposomes on solid materials for ILC or the impregnation of lipids on filter materials (PAMPA), the solid-supported lipid membrane is reported to retain the physiological fluidity and its unilamellarity. SSLM material for membrane affinity measurements is commercially available as *TRANSIL*® material by Sovicell (Leipzig, Germany). The compounds under investigation are incubated in buffered solution with TRANSIL® material of known lipid amount (V_{lipid}) provided by the manufacturer in a 96- or 384-well format. After incubation, the concentration in the aqueous phase (N_{water}/V_{water}) and in the solution before incubation (N_{total}) is determined by HPLC, and *membrane affinity* is calculated using the following equation (Loidl-Stahlhofen et al. 2001a):

$$MA = \frac{c_{lipid}}{c_{water}} = \frac{V_{water}}{V_{lipid}} \times \frac{N_{total} - N_{water}}{N_{water}} \qquad (28.6)$$

A good correlation between $logMA_{SSLM}$ and logMA estimated from equilibrium dialyses using liposomes in solution was reported. The TRANSIL®

approach is a unique use of liposome-like real bilayer membranes as high-throughput method for the estimation of membrane affinity. This technology was recently extended to the study of brain tissues binding to predict the permeability over the blood-brain barrier (Longhi et al. 2011).

Liposomes have also been attached to sensor chip surfaces used in *surface plasmon resonance* (SPR) instruments. *SPR biosensors* measure the quantity of a compound bound to an immobilized partner in real time without the need for fluorescent or radioactive labeling. SPR is an optical phenomenon that occurs when total internal reflection of light at metal surfaces is examined. Changes in SPR occur due to changes in the refractive index at the metal surface (up to about 300 nm). Binding of compounds to a metal-immobilized partner will change the refractive index near the metal surface. Therefore, binding of compounds to metal surface–immobilized partners can be determined as changes in the refractive index in SPR. Liposomes are attached to the dextran matrix that covers the gold surface of a SPR chip. In SPR experiments, the compound of interest is injected in buffered solution to a liposome and reference surface at low flow rates. The result of an SPR experiment is called sensogram, which shows the association and dissociation of an interaction partner to the immobilized system in real time. Baird et al. (2002) used blanc and liposome chip surfaces at the same time, and different sensograms between the control surface and a liposome surface were analyzed. The plateau binding response expressed as response units (RU) for the individual analytes is the measure for membrane affinity in these experiments. Membrane affinity expressed in RU has been successfully correlated to fraction absorbed in humans by Danelian et al. (2000). SPR also offers the unique possibility to study the kinetics of the membrane solute interaction if a dilution series of the compound under investigation is injected into the system. Abdiche (2004) clustered compounds with the same apparent partition coefficient K_D according to their *binding kinetics* and differentiated mainly between fast off and slow off rate drugs. The use of SPR in absorption and distribution studies has been recently reviewed by Bertucci et al. (2007).

Instead of the determination of partition coefficients using liposomes, also the permeation of solutes through liposomal membranes can be studied. This is possible in solution using a number of techniques including the use of radio tracers, fluorescence, or by separation techniques as reviewed by Krämer (2006). Liposomes prepared from egg phosphatidylcholine have also been deposited into the pores and onto the surfaces of a filter support in order to use this construct in a sandwich-like permeability study (Flaten et al. 2006) similar as in PAMPA studies (next chapter). This phospholipid vesicle-based barrier system was validated on 21 diverse drugs and was shown to be as predictive as double-sink PAMPA and the Caco-2 model concerning human oral absorption. In contrast to the PAMPA model, in the phospholipid vesicle-based barrier system, no organic solvent is present in the artificial membrane barrier. These barriers can be used in a broad pH range under steady-state conditions which make them an interesting tool to mimic different sections of the intestinal tract. This model was recently used to study the influence of formulations like solid dispersions on the permeability of poorly water-soluble drugs in comparison to Caco-2 cells (Kanzer et al. 2010).

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28.4 *Parallel Artificial Membrane Permeability Assay (PAMPA)*

PURPOSE/RATIONALE

The use of partition coefficients between water and lipophilic media is of wide use in pharmaceutical research. As discussed in the last chapters, different lipophilicity scales depending on the partition media and the method of lipophilicity determination (Giaganis and Tsantili-Kakoulidou 2008) are used to describe the lipophilicity of a compound and relate it to its absorption behavior in vivo. Differences between the $\log P_{OW}$ and the partition coefficient of partitioning between artificial membranes and water (mainly determined using phospholipid liposomes, see Sect. 28.3) for diverse compounds have been described leading to the development of a number of methods using artificial membrane systems including methods using immobilized artificial membranes (e.g., IAM chromatography).

Artursson and Karlsson (1991) described the permeation across biological cell layers (Caco-2 system and other cell systems, see Chap. 27 "Absorption: In Vitro Tests - Cell Based") in order to predict the human intestinal absorption of a solute. However, the throughput of Caco-2 assays is limited due to the long-growing cycles of the cells and because the assay itself is very laboratory intense. Therefore, it is not easily applicable to the high throughput needed in drug research in a combinatorial chemistry and high-throughput-screening environment. The need for a fast, easy, and cheap method to assess permeability in vitro led to the development of the parallel artificial membrane permeability assay (PAMPA) that uses the filter-immobilized membrane concept to analyze membrane permeation. PAMPA is a permeability assay, whereas the assays described until now in this chapter were partition assays. The difference mainly is that partition coefficients tell you that the molecule should go through the membrane, whereas permeability studies tell you that the molecule did go through the membrane (from Box 2006) which makes interpretation of PAMPA results very intuitive. However, the different PAMPA assays can also be viewed in a global sense as lipophilicity scales (Avdeef et al. 2007) which link PAMPA to the assays using artificial membranes described in the section before.

The development of PAMPA was possible because of the former work by Thompson et al. (1982) and

others about filter-supported lipid bilayers (black lipid membranes, BLM) that showed that stable lipid layers can be formed on filter supports and that the lipid layer is able to close the filter pores enabling measuring the permeation across this lipid layer barrier. Permeation across a biological membrane is the sum of several partition events between the aqueous environment and the membrane plus the diffusion event within the biological membrane and the passage through the unstirred water layer at both sides of the membrane. Using octanol as barrier, partition between octanol and water and permeation through octanol immobilized on filters were correlated by Camenisch et al. (1997), and a sigmoidal relationship was encountered similar to the correlation between logD and Caco-2 permeability, indicating the importance of directly measuring permeation of solutes through *artificial membranes* instead of solely correlating partitioning data.

Kansy et al. (1998) first proposed a high-throughput permeation assay called parallel artificial membrane permeation assay (PAMPA) using an artificial phospholipid membrane brought onto a supporting hydrophobic filter. They presented a promising hyperbolic correlation between human absorption and PAMPA permeability however with a steep initial slope. The correlation allowed classifying compounds in classes of low, intermediate, and high permeability. Today, PAMPA is used in a variety of labs worldwide in drug research and development labs, and a commercial instrument and software are available (pION Inc.). A number of review articles summarized the different uses of the PAMPA assay (Faller 2008; Avdeef 2005). A critical article and rebuttal commentary was published in 2007 (Galinis-Luciani et al. 2007 answered by Avdeef et al. 2007).

PROCEDURE

PAMPA is generally carried out in the 96-well format using a PAMPA sandwich construction. The sandwich consists of a standard 96-well plate and a filter plate put on top (Microtiter plate filter plates that are commercially available). The bottom standard 96-well plate is filled with buffer so that the liquid surface will be in contact with the filter material of the filter plate put later on top. The filter material is impregnated with a lipid solution (e.g., 1–20% egg lecithin) in an organic solvent (e.g., dodecane) and put carefully on top of the donor plate in order to avoid any air bubbles between the liquid surface and the filter plate (Kansy et al. 1998). Then the filter plate is filled with buffer, and the permeation experiment is started by the addition of the analyte to the donor plate on top of the sandwich. The concentration of the analytes in the donor and the acceptor wells after incubation is determined. The analyte concentration in the acceptor well after permeation is compared to the analyte concentration in the donor solution before incubation to estimate permeation of the analyte under investigation.

As the focus of PAMPA is on high throughput, the concentration of the analytes in the acceptor wells after incubation is determined classically by fast UV measurements at various wavelengths using a UV plate reader. However, for up to 40% of the tested compounds in the pharmaceutical industry, no permeability coefficient could be determined mainly due to low solubility of the compounds or low UV response (Mensch et al. 2007; Faller 2008). HPLC-UV (Zhu et al. 2002) and HPLC-MS (Liu et al. 2003) have been used as alternative for the quantification in PAMPA studies. HPLC MS/MS and UHPLC MS/MS which are extensively used as quantification methods in other ADME studies are also widely used in the quantification of PAMPA studies. The high specificity and sensitivity of UHPLC MS/MS offers the possibility to pool compounds (cassette approach) to further increase the throughput (Mensch et al. 2007). SPE-MSMS as described by Lim et al. (2010) can also be used for PAMPA studies and can be integrated with a number of various ADME methods (Luippold et al. 2011). A number of alternative mass spectrometry methods as reviewed by Shou and Zhang (2010) might be used as detection systems for PAMPA studies as well.

PAMPA is often used at various pH values in order to measure *permeability pH profiles* as the permeability of ionizable compounds depends heavily on the pH of the buffer. As the pH range of the intestinal tract varies between pH 6 and pH 8, this is the range of pH values that mostly is used. Kerns et al. (2004) recommended to measure from pH 4 to pH 7.4 in order to predict both bases and acids correctly. Ruell et al. (2003) used permeation pH profiles from pH 4 to pH 9 together with the pKa values of the compounds under investigation to establish the optimum pH value for a single pH PAMPA measurement. The double-sink PAMPA method (Bermejo et al. 2004) applies a pH gradient between donor and acceptor plate together with an ionic surfactant in the acceptor plate to minimize membrane retention.

EVALUATION

The permeation in PAMPA experiments can be followed directly as the percentage of compound permeated into the acceptor compartment after a given time (%T) as described in the original work by Kansy et al. (1998) which reflects an equilibrium analysis at a given time point.

$$\%T = 100 \times \frac{C_A{}^{end}}{C_D{}^{start}} \tag{28.7}$$

$C_A{}^{end}$ is the concentration of the solute under investigation in the acceptor well after incubation, and $C_D{}^{start}$ is the concentration of the analyte in the donor well at the beginning of the experiment.

Permeation in PAMPA experiments is also expressed as a flux rate P_{app} with the unit [cm/s] under the assumption that transport equilibrium is not reached and back transport can be neglected in course of the experiment. P_{app} values represent kinetic information and describe the flux of compounds over the membrane.

$$Papp = \frac{V_A}{A \times (C_D - C_A)} \times \frac{dC_A}{dt} \tag{28.8}$$

V_A is the volume in the acceptor well, A is the filter area, C_D is the concentration of analyte in the donor compartment at the beginning of the experiment, C_A is the concentration of analyte in the acceptor compartment at the beginning of the experiment and (dC_A/dt) is the increase of drug concentration in the acceptor compartment during the course of the experiment (Camenisch et al. 1997).

P_{app} values are often calculated from the ratio of analyte signal in the acceptor compartment at a single time point divided by the analyte signal in equilibrium without membrane barrier. Also %T and P_{app} values are related to each other following the equation taken from Zhu's et al. (2002) work:

$$P_{app} = \frac{V_D \times V_A}{(V_D + V_A) \times A \times t} \times \ln\left[\frac{100 \times V_A}{100 \times V_D - \%T(V_D + V_A)}\right] \tag{28.9}$$

Both P_{app} and %T values have been correlated to fraction absorbed in human, and hyperbolic curves were encountered. This correlation is calibrated using a set of compounds with known human absorption properties, and unknown solutes are classified according to their position on the hyperbolic correlation curve into groups of high-/low-/medium-absorbed compounds.

Avdeef et al. (2001) described the influence of membrane retention of solutes observed in PAMPA experiments on the data evaluation. *Membrane retention* is analyzed using a mass balance between compound left in donor compartment after incubation and compound determined in acceptor compartment after incubation. For calculating P_{app} under consideration of membrane-bound fraction, Avdeef et al. (2001) used an equation different from Eq. 28.8, where the total amount of solute is substituted by the nonmembrane-bound fraction of solute in the equation.

CRITICAL ASSESSMENT OF THE METHOD

The PAMPA technology started to evolve with Kansy's et al. (1998) work published in 1998, and since then, the number of published work and the use of the technique in industry and academia grew tremendously. The development of a commercially available instrument/software (pION Inc.) shows the impact the development of PAMPA had on the drug research. Nowadays, PAMPA is the method of choice for high-throughput in vitro cell-free permeability assays in the discovery phase in pharmaceutical industry. The high-throughput possibilities offered by PAMPA are superior to the other in vitro techniques described in this chapter (including logP (D) measurements using RP-HPLC) at least if UV absorbance is used as analytical tool.

Also, the fact that only passive transcellular permeation is regarded in PAMPA experiments (even if a paracellular component can be added in silico, Sugano et al. 2002) is valuable as it allows comparing PAMPA results to cellular permeation experiments that feature all possible permeation mechanism including paracellular permeation and active transport by carrier proteins (active transport and active efflux). Kerns et al. (2004) recommended this comparison to get an insight into the permeation mechanism applied by a compound under investigation. The possibility to obtain permeability pH profiles is helpful to identify the relevant permeability value of a compound. The full pH profile cannot be determined by cellular assays due to the limited pH range usable with living cells.

PAMPA is relatively insensitive to DMSO percentage or other solubility mediating formulations in donor or acceptor compartment which gives the possibility to study the permeability of compound from different formulations which is not easily possible using living Caco-2 cells. This approach, in conjunction with LCMS analyses, was used to study the permeability of poorly soluble compounds in different recipients (Liu et al. 2003; Avdeef et al. 2008).

On the other hand, PAMPA is a purely artificial method, and PAMPA membranes do not perfectly reassemble real lipid bilayer structures. The thickness and material of the supporting PVDF filters also influence artificially the permeation of compounds depending on the lipophilicity of the compounds more than the thin polycarbonate filter does in Caco-2 experiments. Also, the best choice of membrane constituents for PAMPA experiments is still under investigation. One has to take into account that PAMPA today is a summary term on a lot of different methods applied in different laboratories using different membrane constituents, *sink conditions*, permeation times, etc., which makes inter-laboratory comparison difficult.

Therefore, the knowledge of the lipophilicity expressed as $\log P_{OW}$ will still be of high interest in drug research. Pampa will not be able to substitute Caco-2 or other cell types for the study of permeation, but it will be able to deliver reproducible and predictive data about passive transcellular permeation of a high number of analytes. More time can then be used for the cell-based assays to investigate in depth the mechanism of permeation or efflux of compounds of interest.

MODIFICATIONS OF THE METHOD

The PAMPA permeability depends heavily on the artificial membrane used in the experiment. Today, the lipid choice varies between the labs. Kansy et al. (1998) used a solution of 1–20% lecithin in dodecane or hexadecane for their work. Sugano et al. (2001) presented improvements on the used membrane composition in terms of better correlation of the permeability data to the % absorption data in human and higher overall permeability. These authors used a so-called biomimetic lipid made from phosphatidylcholine (0,8%), phosphatidylethanolamine (0,8%), phosphatidylserine (0,2%), phosphatidylinositol (0,2%) and cholesterol (1%) dissolved in 1,7 octadien. The commercially available membrane system used in pION systems (*double-sink* PAMPA™) consists of 20% phospholipid mixtures in dodecane featuring 16% negative charge on the surface (Bermejo et al. 2004). Recently, a three-lipid-component PAMPA system was described consisting of 2.6% 1,2 dioleoyl-sn-glycero-3-phosphocholine, 0.9% 1,2 dioleoyl-sn-glycero-3-[phospho-*L*-serine] and 1.5% cholesterol in n-dodecane (Teksin 20101) which showed good agreement between PAMPA permeability coefficients and Caco-2 permeability coefficients. A so-called tri-layer artificial membrane was proposed by Chen et al. (2008). This artificial membrane consists of a hexadecane layer in between two lipid layers in a PVDF filter. The hexadecane layer stabilizes the bilayer-type structure. The lipid layers were prepared in volatile solvents which evaporate after coating, removing all excess solvent. Using this membrane preparation which is commercially available as precoated plates from BD Biosciences, an improved predictability of human absorption and decreased membrane retention compared to lipid solution–based strategies were reported (Chen et al. 2008).

Whereas, generally, PAMPA is used with artificial membranes composed of mixtures of phospholipids with organic solvents, Wohnsland and Faller (2001) used a permeation screen using pure hexadecane, Ottaviani et al. (2006) used isopropyl myristate with silicone oil as barriers. The use of a pure alkane as barrier for permeation allows to measure the permeation through the alkane barrier and partitioning between the alkane phase and water in the same experiment. The permeation data using hexadecane were correlated to % absorption in human, and a good correlation was reported showing the predictive character of this assay (Wohnsland and Faller 2001). The partition coefficient $\log P_{ow}$ can be estimated from PAMPA assays directly as described by Faller (2001 and 2005) and discussed by Avdeef et al. (2007).

Also, the filter support was proven to have influence of the permeability determined by PAMPA experiments. Zhu et al. (2002) used a hydrophilic PVDF membrane as support and obtained permeability results yet after 2 h of incubation in contrast to 15 h using the hydrophobic filter materials used by other authors. Wohnsland and Faller (2001) used polycarbonate material instead of PVDF because of the reduced thickness of the polycarbonate material and more uniform surface structure as compared to the PVDF filters. Ruell et al. (2003), Wohnsland and Faller (2001), and others discussed the influence of the

unstirred water layer on both sides of the filter plate on the results from PAMPA assays. Avdeef et al. (2004) introduced individual stirring in each well of the 96-well plate using stirring disks rotating parallel to the membrane surface for PAMPA experiments. PAMPA experiments as short as 15 min due to the intense stirring were reported.

In intestinal absorption, the concentration gradient over the lipid membrane is the driving force for permeation of compounds. Low compound concentration is generally found at the basolateral side. This low local concentration at the inner side of the membrane is achieved by removal of compound with the blood flow or binding of the compound to plasma proteins, bile acids or lipids (Youdim et al. 2003). These sink conditions are mimicked in some PAMPA protocols by trapping the analytes in the acceptor compartment using a pH gradient between donor and acceptor well. Zhu et al. (2002) presented a method where acids and bases have to be analyzed under different sink conditions aiming in trapping an ionized species in the acceptor compartment. Double-sink™ conditions are offered commercially by pION. This method combines pH gradients between donor and acceptor compartments depending on the acid/base properties of the analytes with the use of a surfactant in the acceptor compartment mimicking serum albumin and trapping mainly neutral compounds.

PAMPA assays were also used for the prediction of other systems than intestinal absorption which involve membrane permeation as a key limiting factor, e.g., blood-brain barrier permeation and skin permeation. These barrier systems differ to the intestinal absorption system by the order of magnitude of the concentration sink in the acceptor compartment as well as in the lipid composition of their lipid barrier and the thickness of the unstirred water layer.

Li et al. (2003) used porcine brain lipids as artificial membrane to investigate blood-brain permeation and showed correlation between in vivo assays for brain permeation and PAMPA assay. Recently, Tsinman (2011) expanded the use of porcine brain lipid extracts and reported the use of an in-combo (PAMPA results combined with calculation of H-bond descriptors) approach for passive BBB permeability prediction. A number of different PAMPA systems have been compared by Mensch (2010) concerning BBB permeability prediction, and the recommendation was to combine PAMPA results with efflux data from cell-based adsorption assays. Skin permeability was studied using PAMPA by Ottaviani et al. (2006) and Alvarez-Figueroa (2011). However, for the prediction of skin permeability, the solubility of the solutes should be taken into consideration in addition to the permeability results (Alvarez-Figueroa 2011).

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Volker Krone

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29.1 Introduction

The use of radiolabeled molecules allows a drug and its labeled metabolites to be followed throughout the body and excreta over time. The radioactivity concentration can be tracked in blood and plasma as well as in tissues. Whether the drug with its specific radioactivity administered to the body is completely captured can be proven by calculating the so-called mass balance.

Whereas the radioactivity measurement alone does not allow distinguishing between drug and metabolite(s), samples obtained from the described studies should be also used for standard determination of the drug and its known metabolites, receiving information about the drug and the known metabolite kinetics directly. The gap between radioactivity concentrations and the concentrations determined by direct bioanalytical methods defines the contribution of unknown metabolites.[1]

Using one and the same set of studies with equally withdrawn samples for determination of all radiokinetic and pharmacokinetic data possible is not only economically favorable, but allows illuminating comparisons with a minimum of assumptions and therefore high confidence to interpretation.

Radiokinetic studies deliver the key data of absorption and elimination to which all data and statements of other investigations have to fit—or in case of conflict need a plausible explanation at least.

As examples in the following chapter, the frequently used radiokinetic studies in dogs and rats are described, including placental transfer and milk transfer in rats. Investigations in other species can often be performed similarly. The final choice of species should be done in consensus with toxicology (at a later stage of development including cancerogenicity and segment II studies), regarding the metabolic patterns of in vitro cross-species comparisons and regarding the pharmacological in vivo model.

A suggestion of necessary types of radiokinetic studies and general hints for conducting those animal ADME studies can be found in Campbell and Jochemsen (1994). More insight and aspects regarding mass balance studies are given by Beumer. For general introduction in the mechanisms and physiology of drug absorption, refer to Bermejo or Granero.

29.2 Choice of the Radiolabel

^{14}C is the label of choice for most drugs since it is stable biologically (when the right labeling position is chosen), the detection is comfortable,[2] and in case of combustion of samples, the produced $^{14}CO_2$ can be nicely absorbed quantitatively. ^{3}H labeled drugs are easier to synthesize sometimes, but they are often less stable biologically or at least worse to predict in their biological stability. Because of its much higher specific activity, the ^{3}H-label is favorable in the case of high molecular weight drugs and/or very low doses. ^{35}S, ^{33}P, ^{125}J are used comparatively seldom.

The position of the label should be away from sites chemically unstable or from sites of metabolic attack to ensure that the label is kept in the main metabolic fragments. Of course, this is difficult for complex molecules, especially when the metabolic attack takes part in the center of the drug molecule. Then it can become necessary to introduce a second label and to repeat the set of radiokinetic studies. A double-labeling strategy to minimize the number of radiokinetic studies has normally to be refused due to complexity of interpretation of the data.

A quality control including stability of the radiolabeled drug is mandatory. The chemical degradation and also the radiolysis can be responsible for a short expiry date. The higher the purity and the content of the labeled drug is, the better the results of the radiokinetic studies can be interpreted.[3]

[1]The estimation of the drug absorption (not to be mixed up with bioavailability) using non-radiolabeled drugs and not using the mass balance approach would be much less reliable, since the entity of metabolites cannot be captured in the matrices necessary to be followed, normally.

[2]Half-life of 5,730 year for ^{14}C, making an half-life correction unnecessary. As a weak β – radiator (up to 156 keV) the risk of handling is an acceptable compromise in the laboratory (protection area!).

[3]Imagine the case of a projected nonabsorbable drug, for instance, for topical application, and the situation of having detected 3% absorption with a radioanalytical purity of the labeled drug of 97%. Or imagine the case of a minor part of radioactivity with a long terminal half-life suggesting a metabolite with an accumulation potency. The radioactivity represents the sum of the original compound and/or radioactive-labeled metabolites and not to forget possible synthetic side-products which can be present in traces (depending on the purity and content of the synthetic material). Discussing traces of

The stability and homogeneity of the radiolabeled compound should be tested also in the galenic formulation intended to be used.

29.3 Determination of the Radioactivity Concentration

Radioactivity measurements radioactivity measurements determination of the radioactivity concentration are carried out by the liquid scintillation counting procedure in β-spectrometers using an external standard device which permitted the counting efficiency to be determined by the channel ratio method (explained, for instance, by Dyer (1980)).

Samples are either measured directly after adding a commercial scintillation cocktail[4] or after dissolution and discoloration[5] or after combustion in suitable combustion machines.[6]

A thorough calibration of the β-spectrometer regarding different sample matrices and sizes, the scintillator used, different dissolutions, different colorations of the samples, different amounts of CO_2 after combustion and of course different radioactivity concentrations should be ensured. Calibration and control samples can be set up by using internal standard kits (for instance, ^{14}C-ORG Standard capsules from PerkinElmer).

Blank samples (of every type of matrix) have to be measured in the batches with the study samples. Their mean value can be used as background value to estimate the limit of quantitation.

The definition of the limit of quantitation (LOQ) is handled quite differently. For example, an easy, often used approach is the definition of using the double of the mean blank value as LOQ. This definition sounds simple and a scientific theoretical justification seems not to be available. However, the thus defined LOQ is then a reasonable value when standard deviation (SD) of the blank values is low (<10%, according to own experience). More sophisticated is the following definition: The mean blank value plus 3 times the standard deviation is required for a limit of detection (LOD) and the mean blank value plus 10 times the standard deviation is required for LOQ (see, for instance, Krull (1998)). Outliers can be identified, for instance, by the Grubbs test (for instance, explained in www.graphpad.com).

29.4 Control of Applied Dose

For small animals or dosing of a large number of animals, individual doses are determined by weighing the syringes containing the galenic formulation prior to and after each administration; stability and homogeneity of radioactive formulation is determined on aliquots before and after each series of administration. (The choice of the dose used is matter of opinion and has often to be decided between the (expected or already determined) pharmacodynamically active dose and the NOAEL of the subchronic or chronic safety studies. In the latter case and the oral route, a suspension formulation will be most often applied (also for the purpose of resulting a similar exposure like in safety studies) which has to be characterized chemically and physically (solid state properties) to guarantee reproducibility and comparability to other studies with the drug substance. A second group of animals are often dosed intravenously in parallel. Limited by the necessary dissolution in the i.v. formulation, the doses are lower, normally.)

For individual dosing of larger animals/few animals, the radiolabeled drug is directly weighed in separate devices regarding the individual weight of the animal. All used devices for administration are collected and washed with solvent. The collected solvents are used for back-measurement (dose loss) of radioactivity. The radioactivity found is subtracted from the originally calculated dose.

radioactivity, for instance, traces crossing the placenta, keep in mind that these traces may be due to synthetic side-products. Thus, whenever possible try to use radiolabeled compound as clean as possible.

[4]For instance, in case of urine samples when the quench is in the range of the calibration curve or bile samples.

[5]For instance, with Solvable® from Perkin Elmer for dissolution and H_2O_2 for discoloration.

[6]For instance, with a Tri-Carb® 307 combuster which can be equipped with a robot unit for automatic sample handling from Perkin Elmer. Since carry-over effects are not negligible in case a low radioactivity sample follows a high radioactivity sample, a reasonable arrangement of samples in a sequence is essential.

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29.5 Radiokinetics and Mass Balance in Dogs

PURPOSE AND RATIONALE

To evaluate the pattern and the rate of excretion and to investigate the time course of radioactivity concentrations in blood and plasma with the aim of getting information about the absorption process, the AUC, C_{max}, and the elimination half-life of radioactivity in blood and plasma. Possibly, indices for enterohepatic cycle or for metabolites with much different volume of distribution than the original compound may be found. An estimate of the absorption rate comparing dose-normalized AUCs or the renal excretion after i.v. and oral (or any other) administration can be done and a major binding to formed blood elements can be noticeable by comparison of blood and plasma concentrations. Samples for metabolic profiling and metabolite structure elucidation can be collected during this study ideally.

The route of administration projected to be used therapeutically should be applied. If possible—and the solubility of the drug allows—the study should also be performed after intravenous administration. The results of both routes should be compared. Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration.

Normally, the radiokinetic study is performed administering a single dose per administration route.

PROCEDURE

Three healthy male beagles receive an intravenous dose into a jugular vein or into the vena cephalica antebrachii. After a washout period dependent on the duration of excretion of the drug/metabolites (typically 4 weeks at minimum), the same animals are dosed orally by using a stomach tube. Normally, 5–10 MBq/animal is a sufficient radioactive dose for a ^{14}C-label, in case of ^{3}H about 50 MBq/animal could be used. Up to 168 h after administration, blood, urine, and feces are collected. At each collection time, about 3.5–5 mL of blood are taken for radioanalysis (2–3 aliquots) and for processing plasma for subsequent radio- and bioanalysis and metabolite investigation. The plasma is aliquoted immediately after centrifugation without warm-up.

29.5.1 Proposal for Standard Collection Times

Blood: 0 h (before administration), 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 24 h, 48 h, 72 h, 144 h, 168 h after oral administration and 0 h (before administration), 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 24 h, 48 h, 72 h, 144 h, 168 h after intravenous administration.

Urine: 0 h (before administration), 0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Feces: 0 h (before administration), 0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Cage washing: 0–24 h, 24–48 h, 48–168 h after administration.

Radioanalysis is performed by the liquid scintillation procedure either directly after addition of a scintillator (urine, cage washing) or following combustion and addition of a scintillator (blood, plasma, feces).

29.5.2 Details of the Oral Administration

29.5.2.1 Material

- Bulge tube (stomach tube) for oral dosing of dogs (soft, transparent plastic tube with a diameter of about 1 and 60 cm of length)
- Nonreturn valve
- Disposable gloves
- Highly absorbent paper
- Glass funnel
- Bottle with tap water

29.5.2.2 Procedure

The dog is placed on an undersheet and its head is secured by an assistant. The upper and lower jaws are

separated by exerting pressure on the lips with both hands. The stomach tube moistened with mains water is introduced into the open throat. Caution: Do not introduce the tube in the trachea. Pour mains water into the glass funnel fitted on the stomach tube over the nonreturn valve to check that the tube is correctly positioned. If outflow difficulties occur, adjust the position of the tube by moving it slightly backward and forward. Then introduce the test substance to be administered. Then rinse the tube and remove.

29.5.3 Details of the Intravenous Administration into the Vena Cephalica Antebrachii or the Vena Saphena Parva

29.5.3.1 Material

- Curved scissors
- Disposable gloves, Kleenex
- Disposable syringe with formulation for administration with fixed cannula
- Electric hair clippers

29.5.3.2 Procedure

An assistant fixes the dog. The vena saphena parva (hindlimb vein) or the vena cephalica antebrachii is excised from hairs. The vein is compressed using a rubber tubing tourniquet or by firmly encircling the limb with the hand above the vein. Insert the needle of the disposable syringe containing the study substance preparation to be administered into the congested vein, release the tourniquet, and check that the needle is in the correct position by aspirating a small amount of blood into the syringe. Then carefully administer the substance and remove the needle and syringe. Prevent secondary bleeding by exerting pressure on the skin injection site with a Kleenex swab.

29.5.4 Details of Sample Collection of Excreta (Urine, Feces, Cage Washing)

29.5.4.1 Material

- Metabolism cage
- Two liter urine collection container (cooled)
- Dash bottles containing warm mains water
- Polyethylene bottles in various sizes
- Labels, plastic feces scraper, large glass beaker or polyethylene bag, disposable gloves, Kleenex, scrubbing brush with handle, cooling device for the urine tube
- Mixer

29.5.4.2 Procedure

The urine is collected according to the preinstructed collection intervals directly from the urine collection container of the metabolism cage. The samples are weight and directly after stirring and homogenizing the urine, aliquots are taken for radioanalysis. The remainder is bottled and frozen for additional investigations planned (metabolite profile and identification and/or bioanalysis).

The feces are collected from the sitting device and the ground plate using the plastic scraper. In case of direct processing of the feces sample, the feces are collected in a weight glass beaker. In case a direct processing is not possible, the feces are collected in a weight plastic sac and are frozen.

The weights are determined and registered as raw data. To homogenize the feces each sample is diluted with demineralized water—according to the consistence with the two- to four-fold of the feces weight. The weight of the filled bottle is determined and the data stored until evaluation.

The sample with the mixture of feces and distilled water is homogenized with a mixer which should be cleaned rigorously before using it again and which is proven to be without contamination.

For preparation of samples for combustion, preweighted combustion cones were filled with an appropriate amount of homogenate (typically about 250 mg). The combustion cones and covers including the homogenate are weight again and the data stored until evaluation.

The cage washing is done after removal of feces and urine with the help of warm tap water dash bottle (about 2 L). Cleaning of the cage is done with the scrubbing brush starting with the walls, the sitting device, and followed by the ground plate. The fluid is weight and after thorough mixing, aliquots are filled in scintillation cups for radioactivity measurement.

29.5.5 Details of Sample Collection (Blood, Plasma)

29.5.5.1 Material

- Monovets with sine use cannulas (butterfly)
- Tourniquet loop

- Disposable gloves, Kleenex, hair clipper
- Needle, needle holder, surgical suture material, scissors, protective collar for dogs, antibiotic powder

29.5.5.2 Procedure

The site for blood collection has to be different from the site of intravenous administration. Thus, the vena cephalica antebrachii can be used for collection and the vena saphena parva for administration (or vice versa). The dog is kept by one person and the hairs at the selected veins at the forelimbs or hindlimbs are removed by gently clipping. The vein on the dog's limb is compressed by the assistant by encircling the limb with the hand or applying a rubber tourniquet loop. The puncture procedure is then performed. Blood should be withdrawn slowly by aspirating gently.

When the monovet is sufficiently filled, remove the compression and withdraw the needle. Secondary bleeding is prevented by immediately exerting compression on the puncture site with a Kleenex swab.

29.5.6 Alternative Route for Blood Sample Collection

A favorable, safe method for multiple blood collection is a permanent catheter in the vena jugularis. The dog is fixed in a frame with a hammock. An assistant fixed the head upward, slightly stretched to the opposite site to which the vein puncture should be done. The animal's neck is shaved with clippers over the jugular vein and wiped with a commercial skin disinfectant solution. After applying the tourniquet loop at the base of the neck, the jugular vein is clearly prominent. The jugular vein is then fixed between two fingers and punctured toward the heart with a split needle. The catheter is located inside the split needle and, as soon as blood is visible, is inserted into the vein. The split needle is then carefully withdrawn and opened. The base of the catheter is secured to the skin with surgical suture material. After the syringe has been inserted, aspirate until blood is visible in the syringe. Then rinse out the catheter with sterile physiological sodium chloride solution (about 2 mL). The correct position of the catheter can be checked by repeating this operation if required. Then seal the base of the catheter with a closure cap. To prevent obstruction when pronounced coagulation occurs, a drop of anticoagulant can be added to the NaCl solution.

For restless animals, the catheter can be prevented from being removed by the dog by fitting with a protective collar of the type commonly used in veterinary practice. Dusting the puncture site and fixation suture with antibiotic powder prevents inflammation.

Catheter insertion should be carried out under the most aseptic conditions possible.

Before blood sampling, the physiological saline solution has to be removed from the catheter using a syringe. This is done by withdrawing 0.5 mL from the catheter before collecting the actual sample with the prepared monovet. After blood collection, the catheter is rinsed and filled again with about 2 mL of physiological sodium chloride solution.

For generating plasma, part of the collected blood is transferred into a centrifuge tube and treated in the centrifuge with 1,500 G.

29.5.7 Animal Maintenance

During the study period, the animals are maintained on metabolism cages, one in each cage with a separating device for urine and feces, allowing to collect the excreta separately. Conventionally, these cages consist of several parts which have to be cleaned carefully before assembling (box, device for sitting, food, water, urine collection). It has shown to be favorable to control the cleanness by rinsing the cage with an alcohol-water mixture and subsequent determination of radioactivity of the rinsing solution.

Before study start, the animals should have time for acclimatization, depending on the extent of changes in the surroundings and the type and duration of transport. In case of a maintenance in a lab in the neighborhood under similar conditions as in the study, an acclimatization time of at least 1–2 days might be sufficient.

The feed (500 g commercial pelleted diet about noontime, such as ALLCO Vollkost Menü or sniff® Spezialdiäten GmbH; D-59494 Soest, Deutschland) and tap water ad libitum is given about 20 h before administration.

Following conditions should be controlled throughout the study duration:

- Room temperature
- Lighting time

Proposed values for these conditions (according to the guideline 2010/63/EU of the European Parliament

and the Council, Sept 22 2010 and the recommendation 2007/526/EG of the Commission, June 18 2007):

- Room temperature: 15–21°C
- Lighting time: 6°am–6°pm (light), 6°pm–6°am (dark)

EVALUATION

The concentrations of radioactivity expressed as μg equivalents of drug per g of matrix (μg equiv./g) and alternatively as part of dose eliminated (% administered dose) are determined in each matrix and collection interval.[7] The temporal course of the excretion and the radioactivity concentration in blood and plasma are represented by graphs[8] and tables using either Windows Excel function, a specific pharmacokinetic software program (like WinNONLIN; www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). The software also allows calculation of the half-lives of radioactivity elimination via urine and feces (when appropriate),[9] to generate cumulation plots or general survey plots like bar diagrams (see "Example").

In case that an essential part of dose is excreted via urine, an estimation of absorption is often done giving the ratio of urine excretion (oral/intravenous) as absorption rate. It should be compared with the ratio of the dose-normalized plasma AUCs (oral/intravenous).

All these data are finally drawn together for detailed discussion and evaluation. Special attention has to be taken on major differences of data following intravenous and oral administration.

An essential requirement for the quality of the study is a balance value near to 100%. A value >95% and <105% is reasonable. Balance values of 90–95% or 105–110% are often accepted too, but they should indicate the investigator that something might run out of control or the process/handling could be optimized. Balances much worse than those should be explained.

In case an essential concentration is available in blood and plasma at early time points after oral administration (may be C_{max} is reached soon also), a rapid onset of absorption can be stated.

When there is no continuous decline of concentrations after intravenous administration, this might be an indication for:

- An inappropriate administration or a precipitation of the drug from the formulation at the application side or just behind the side in the blood stream. This might be the case when there is a plateau concentration or even an increase at early collection times.
- An enterohepatic circulation when a side maximum exists (time depending on absorption and elimination rate, often seen 4–8 h after administration).
- One or more of the generating metabolites have a much smaller volume of distribution than the original drug.
- Other (often more complex) explanations.

In case of a drug concentration ratio (in plasma/blood) distinctly lower than expected from hematocrit, a binding of the drug or its metabolites to formed blood elements is indicated.[10]

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study.

[7]It might be necessary to use a normalized apparent collection interval for graphical reasons in case of dissimilar collection intervals.

[8]Instead of the collection interval, the mean time of the collection period is often used in the graphic presentations.

[9]Via determination of the rate constant which is calculated by linear regression of ln concentration on time using a least three data points which appeared to be randomly distributed about a single straight line. Half-lives were calculated as ln2/rate constant.

[10]The following equation describes the percent of total radioactivity in plasma relative to total radioactivity in blood

$$\text{Plasma/Blood}\,(\%) = \frac{(V_B) \times (P \times [1 - HCT])}{(V_B \times B)} \times 100 = \frac{P \times (1 - HCT)}{B} \times 100$$

where
V_B = volume of blood
P = drug concentration in plasma
B = drug concentration in blood
HCT = hematocrit

The comparison of both, the radioactivity concentrations and the sum of concentrations determined by specific methods, allows estimation of the gap (of unknown metabolites) which often develops time-dependently: For instance, at the beginning of the study (after intravenous administration), radioactivity concentrations in plasma should fit to the drug concentration. Usually, the gap (as a percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption via the ratio of dose-normalized plasma AUCs (of radioactivity) after oral and intravenous administration is based on the assumption that:

1. A first pass effect including an instant excretion via bile does not exist.
2. The metabolic pattern is comparable independent of the route of administration.[11]
3. The distribution (drug and metabolites) and the metabolism in special tissue are not effected by the bolus i.v. administration with high initial concentrations (see, e.g., Chiou (1989)).
4. Dose-exposure linearity exists (under a more stringent view even a first order kinetic is demanded).

When these criteria are not fulfilled, then either the absorption can be underestimated (for instance, when precondition (1) is not true (2) or distinctly overestimated up to a calculated "super-absorption" for more than 100% (possible, for instance, when precondition (3) or (4) are not true)).

Only AUCs obtained from the same "type of blood" samples should be used. For instance, do not compare central arterial blood with venous peripheral blood (Chiou 1989) as long as one is not sure that both are virtually comparable.

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, microemulsions, or suspensions can be responsible for distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

MODIFICATIONS OF THE METHOD

The radiokinetic and mass balance study can also be applied to other non-rodent species.[12] The framework is similar to the study described above. Often monkeys, mini-pigs, and rabbits are used. Instead of or additional to the oral administration, the method can be adopted to other routes of administration.

Similar basic procedures including evaluation are described by Webber et al. (2004); additionally, the investigation of biliary excretion and tissue distribution was integrated within the study.

Blood collection from the femoral artery is mentioned by Davis et al. (1994) after surgically preparing vascular access ports to the femoral artery. Using dogs with surgically instrumented indwelling venous access ports into the femoral vein and using a 6-min infusion instead of an intravenous bolus injection is mentioned by Krishna et al. (2002). Occasionally, such a short-term infusion is recommended instead of a bolus intravenous administration (Chiou 1989, p. 283). The advantage is to avoid high blood concentrations shortly after intravenous administration which may be in conflict with the assumed linear dose-exposure relationship (validity of linear relation at border concentration?). The advantage of the intravenous bolus injection is the lower/shorter stress situation for the animals (and may be also for the experimenter) which might influence the reproducibility of the study results.

Sometimes several dose levels are investigated with the described method: a low dose for which a pharmacological response is expected or a possible much higher dose at the NOAEL (for concomitant metabolic profiling and estimation of metabolite/drug concentration ratios at this relevant level of exposure). In the case of evaluation of altered routes of elimination at toxic doses, studies with doses and retrieving toxic metabolites higher than NOAEL may considered as an exception.

Determination of blood, plasma, and feces samples not using combustion but either direct measurement (plasma) or solubilizing and de-coloration, the samples is mentioned, for instance, by Okuyama et al. (1997).

[11] An example where this assumption is not fulfilled is described by Okuyama et al. (1997); consequently the authors only mention the ratio of AUCs after oral and intravenous administration and do not correlate this ratio to absorption.

[12] Mass balance results in rats, mice, dogs, monkeys, and humans after administration of the same drug, including bile excretion results from rat, dog, monkey, and human (!), are described by Donglu Zhang.

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EXAMPLE

AVE 3559 was developed for the treatment of obesitas. It was ^{14}C-labeled and investigated in a dog radiokinetic study to determine the time course of radioactivity concentrations in blood/plasma and to determine the pattern and rate of excretion, the residual concentrations at the end of the study, 168 h after administration, and the mass balance following oral and intravenous route of administration.

Three healthy male Harlan beagles weighing 15–16 kg received an approximate dose of 10 mg/kg ^{14}C-AVE 3559 orally by using a stomach tube (exact weights and doses see table). The identical animals received approximately 2 mg/kg ^{14}C-AVE 3559 into a jugular vein 5 weeks following the oral administration. The pharmaceutical preparation used for both the oral and intravenous route was a solution in transcutol/water (25/75 w/w). The drug concentration in the formulation was about 2 mg/g. Thus, the animals received about 75-g formulation orally and 15 g intravenously. The radiolabeled drug was blended with non-labeled drug in such a way that each dog received about 7.5 MBq radioactivity.

Blood, urine, and feces were collected as described above. The remaining parts of samples were used for bioanalysis and metabolism studies.

Three aliquots from each (processed) sample were used for radioactivity determination. The aliquots from the urine samples were measured directly in the liquid scintillation counting procedure after addition of the scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany). The other matrix aliquots taken up on Combusto Cones (Perkin Elmer), weighed, dried at room temperature, were combusted in a Tricarb® combuster (Perkin Elmer, Model 307) and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb® (Perkin Elmer). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Perkin Elmer2500 TR) after addition of the scintillator Permafluor E+® (Perkin Elmer).

29.5.8 Evaluation and Discussion

As an example for the numerous generated graphs, the time course of the mean radioactivity blood and plasma concentration values (including standard deviation) after intravenous and oral administration is given in the summary graph (Fig. 29.1). Additionally, the mean amounts of dose excreted in urine and feces depending on time after administration is given in Fig. 29.2.

The pharmacokinetic values were calculated by the pharmacokinetics software WinNonlin, and were summarized in Table 29.1.

Already 15 min after oral administration mean radioactivity concentrations of 5.60 μg equiv./g (range: 3.9–7.4 μg equiv./g) and 8.47 μg equiv./g (range 5.8–11.1 μg equiv./g), respectively, were present in blood and plasma indicating a rapid onset of absorption. C_{max} was interindividually different (see table) and reached 0.5–4 h after administration. The mean maximum concentrations amounted 8.4 μg equiv./g in blood and 11.7 μg equiv./g in plasma. The AUCs (until the last concentration point) amounted to mean values of 178 μg equiv./g × h for blood and 254 μg equiv./g × h for plasma.

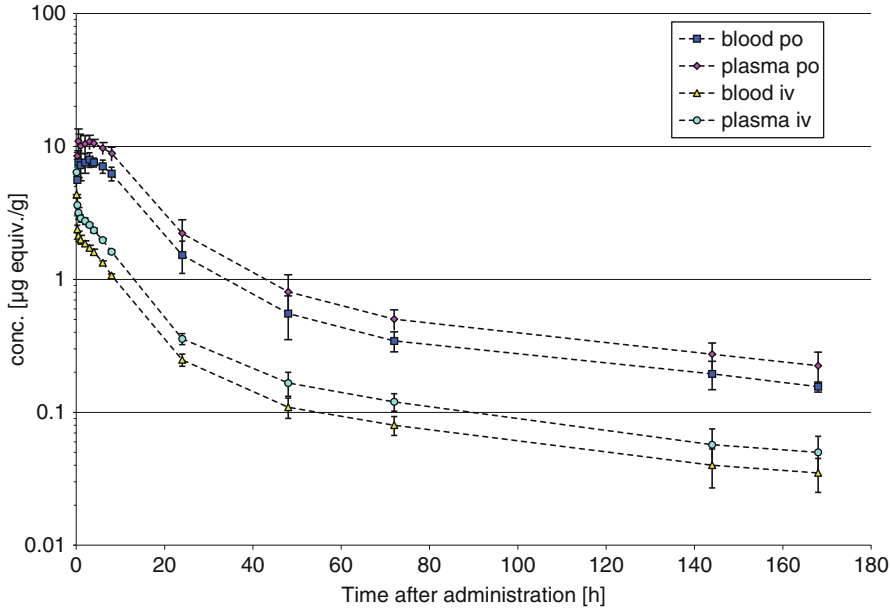

Fig. 29.1 ^{14}C-AVE 3559: mean concentration in blood and plasma after administration p.o. (10 mg/kg) and i.v. (2 mg/kg)

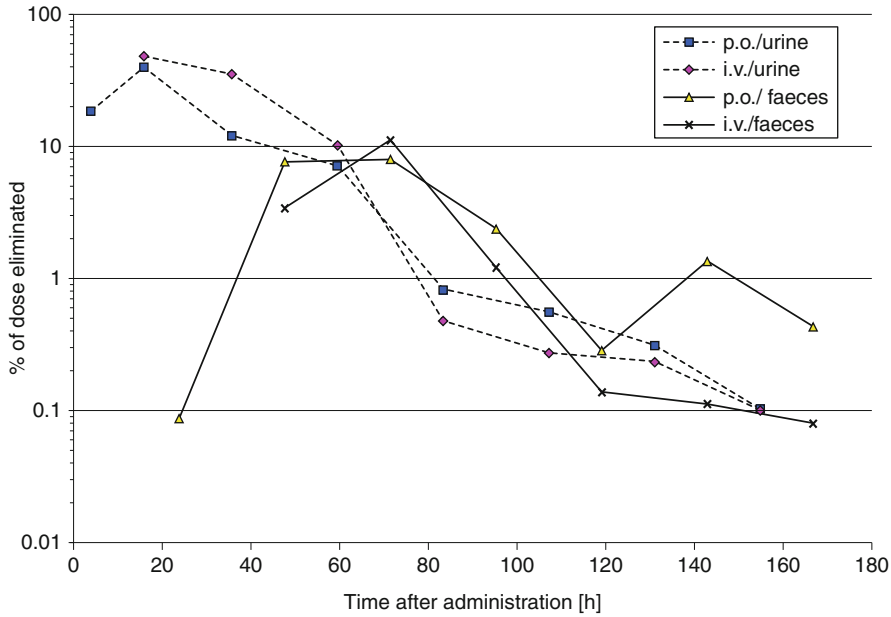

Fig. 29.2 ^{14}C-AVE 3559: mean% of dose eliminated via urine and feces after administration p.o. (10 mg/kg) and i.v. (2 mg/kg)

After reaching peak concentrations, the radioactivity was eliminated in a first elimination phase with mean half-lives of 8.1 h from blood and 8.4 h from plasma and with mean half-lives of about 84 h from blood and plasma in a terminal elimination phase.

These half-lives correspond to the elimination processes and half-lives found after intravenous administration with mean half-lives of 7.3 and 7.5 h in blood and plasma for the first elimination process and 71 and 74 h for the terminal elimination phase. Mean C_{max} values 5 min after administration were 4.3 and 6.4 µg equiv./g. The mean AUCs (until last concentration point) amounted 34.8 and 52.7 µg equiv./g × h.

Table 29.1

Route of administration	Oral				Intravenous			
Dog no.[a]	1	2	3	MW	4	5	6	MW
Body weights (kg)	14.7	16.0	15.3	15.3	15.4	15.9	15.0	15.4
Doses (mg/kg)	10.24	10.03	10.05	10.11	2.041	1.915	1.928	1.961
Blood values								
C_{max} (µg equiv./g)[b]	9.5	7.3	8.4	8.4	4.5	4.2	4.3	4.3
					(5.9)	(5.4)	(6.0)	(5.8)
t_{max}(h)	0.5	3	3	2.2	0.08	0.08	0.08	0.08
$t_{1/2}$ [c](h)	8.0	9.4	7.0	8.1	7.1	7.1	7.7	7.3
$t_{1/2}$ z [d](h)	71.4	81.5	101	84.6	66.6	66.6	79.7	71.0
AUC[e](µg equiv./g × h)	176	176	183	178	34.0	34.0	36.5	34.8
	(187)	(196)	(211)	(198)	(36.4)	(36.3)	(41.0)	(37.9)
Plasma values								
C_{max} (µg equiv./g)[2]	13.6	10.0	11.4	11.7	6.6	6.8	5.8	6.4
					(8.6)	(8.8)	(7.6)	(8.3)
t_{max}(h)	0.5	3	4	2.5	0.08	0.08	0.08	0.08
$t_{1/2}$ [c](h)	8.3	9.7	7.1	8.4	7.0	7.9	7.6	7.5
$t_{1/2}$ z [d](h)	68.7	78.4	102.7	83.3	61.4	92.8	67.8	74.0
AUC[e](µg equiv./g × h)	245	253	264	254	50.0	54.6	53.6	52.7
	(262)	(279)	(305)	(282)	(53.1)	(62.8)	(58.7)	(58.2)
Urine								
Total amount (%)	85.85	72.83	79.30	79.3	84.41	79.03	79.48	81.0
$t_{1/2}$(h)	22.6	21.4	15.2	19.7	16.0	14.8		
Feces								
Total amount (%)	13.45	22.80	15.64	17.3	10.76	18.04	16.07	15.0
$t_{1/2}$(h)	28.5	13.5	27.9	23.3	12.3	12.0	17.0	13.8
Cage washing								
Total amount (%)	1.35	2.20	3.57	2.4	2.85	2.97	1.38	2.4
Recovery								
Total amount (%)	100.7	97.8	98.5	99.0	98.0	100.0	96.9	98.3
Absorption rate (%)								
cal. via urine excretion (%)	102	92	100	98				
cal. via plasma AUC (%)	97	88	94	93				

[a]Dog 1 = dog 4; dog 2 = dog 5; dog 3 = dog 6
[b]Dog 4–6: number in brackets give C_{max} extrapolated to $t_{max} = 0$
[c]Half-lives calculated using noncompartmental models (200.201)
[d]Terminal half-lives calculated using noncompartmental models (200.201)
[e]AUC all calculated using the trapezoidal rule (AUC information is given in brackets)

The higher plasma than blood concentrations at all measuring points (especially at the last measuring time points) after oral and intravenous administration make a strong binding of a radioactive component (drug or metabolite) to blood cells in dog unlikely.

After both routes of administration, the main part of the administered radioactivity was excreted renally with means of 79% and 81% (oral and intravenous route). In the feces, an average of 17% after oral and 15% after intravenous administration were found.

The recovery rate after oral administration amounted to a mean of 99%. Following intravenous administration the mean balance was 98%.

An absorption of radioactivity between 88% and 97% based on plasma AUC could be calculated. Based on urinary excretion data a mean absorption rate of 98% could be computed.

29.6 Mass Balance Study in Rats

PURPOSE AND RATIONALE

To evaluate the pattern and the rate of excretion, the residual concentration of the drug in the body 1 week after administration, and the estimation of the absorption in case of a relevant renal excretion. The kind of study is obligatory for the registration as a drug when the rat is chosen as the rodent model (in toxicology or as a relevant pharmacological model[13]).

The route of administration projected to be used therapeutically should be applied also in the mass balance study. If possible and solubility allows, the study should also be performed after intravenous administration and the results of both routes should be compared. Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration.

Normally, the mass balance study is performed administering a single dose per administration route. In case of extraordinary circumstances, for instance, when there is a suspicion of an accumulation of any metabolite, a mass balance with repeated dosing can be considered.

The study is performed separately from the radiokinetics blood/plasma study in rats not to influence the results by collecting blood during the study course.

PROCEDURE

29.6.1 Animal Study Part

Out of eight healthy male Sprague Dawley rats weighing about 200 g corresponding to an age of 6–10 weeks, four rats receive an oral dose of the radiolabeled drug by using a stomach tube (typically 10 mg/kg body weight; 2 mg/g formulation; total radioactivity of about 1–2 MBq/animal in case of ^{14}C-label). Four other animals receive an intravenous dose into a tail vein (typically 2–5 mg/kg body weight).

[13]For an example of a mass balance study in mice (besides rat and dog) refer to Miraglia L.

In this description the i.v. and oral administration is described in more detail.

29.6.1.1 Material for Oral Administration

- Bulge tube (stomach tube) for oral dosing of rats
- Syringes
- Undersheet and disposable gloves
- Drug formulation

29.6.1.2 Procedure of Oral Administration

- The drug formulation is transferred into a syringe with the bulge tube put on the top of the syringe.
- The rat is held tight on its neck above the undersheet.
- Slightly turning, the bulge tube is introduced into the throat of the animal.
- Syringe content is administered intra-gastrally.
- If appropriate a rinsing solution might follow the drug administration.

29.6.1.3 Material for Intravenous Administration into the Tail Vein

- Fixation tube for rats adjusted to the size of the rat
- Electrically warmed and controlled water bath (42°C)
- Kleenex, cellulose, disposable gloves
- Syringe containing the formulation (solution!) for administration
- Syringe with pyrogen-free, physiological saline solution
- Vein-dwell-cannula consisting of Teflon (about 0.8-mm diameter)

29.6.1.4 Procedure for Intravenous Administration

- The rat is placed into the fixation tube. The tail of the animal is fixed outside the tube.
- The tail is put into the water bath with a constant temperature between 40°C and 42°C.
- This procedure causes the appearance of the tail vein without burdening the animals.
- The vein is punctured with the dwell-cannula so that blood at the end of the leading-cannula appears. During extracting, the leading-cannula blood should also arrive in the dwell-cannula.
- For additional control of the correct position of the dwell-cannula, 0.2–0.3 mL of physiological saline solution is administered. The administration has to occur easily, the aqueous saline solution has to flow into the vein immediately.

- The syringe is changed cautiously by the syringe containing the drug formulation. The content is administered.
- Also this syringe is changed cautiously against a second syringe containing physiological aqueous saline solution. Again 0.2–0.3 mL is administered to transfer the formulation which rested in the cannula completely into the body.

Note: To avoid an injury of the tail vein, all the administrations should be conducted with caution.

29.6.2 Animal Maintenance

During the study period, the animals are kept in metabolism cages, one in each cage with a separating device for urine and feces, allowing collection of the excreta separately. Conventionally, those cages consist of several parts which have to be cleaned carefully before assembling (cage cylinder, device for sitting, funnel and separation device, cover, drinking bottle, tubing to the food vessel, and food vessel). It has shown to be favorable to control the cleanliness by rinsing the cage with an alcohol-water mixture and subsequent determination of radioactivity of the rinsing solution.

Before study start, the animals should have time for acclimatization (default 7 days), depending on the extent of changes in the surroundings and the type and duration of transport. In case of a maintenance in a lab in the neighborhood under similar conditions as in the study, an acclimatization time of at least 1–2 days might be sufficient. In case of longer transport or from a different environment, acclimatization times up to 14 days can become necessary. One animal is housed per metabolism cage.

Before administration of the drug, the animals should have an empty stomach but not go hungry, so that the food is withdrawn 3–4 h before the administration in the morning (for instance, achieved by a timer-controlled unit withdrawing the food). The feed (commercial pelleted diet, for instance, sniff® Spezialdiäten GmbH; D-59494 Soest, Deutschland) and tap water is provided ad libitum.

Following conditions should be controlled throughout the study duration: room temperature, relative humidity, lighting time.

Proposed values for these conditions (according to the guideline 2010/63/EU of the European Parliament and the Council, Sept 22, 2010 and the recommendation 2007/526/EG of the Commission, June 18 2007):

- Room temperature: 20–24°C
- Relative humidity: 45–65%
- Lighting time: 6 am–6 pm light, 6 pm–6 am (dark)

Animal identification has to be ensured, e.g., by permanent marking on the tail root with the animal number. Prior to the start of the study, the animals have to be assessed and have to show no clinical abnormalities.

29.6.3 Collection and Sample Preparation of Excreta

29.6.3.1 Material for Collection of Urine, Cage Washing, and Feces

- Clear collection of excreta plastic bottles (100 mL) with covers, with appropriate labeling and registered tare weight (e.g., entered in LIMS)
- Bottles for spraying deionized water
- Precision balance
- Kleenex, disposable gloves
- If necessary: cooling device and light protection box
- Ultra-Turrax® or mixer

29.6.3.2 Urine Collection

Typical collection intervals are: 0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

During each collection interval, the excreted urine is collected from the separation device in a labeled plastic bottle. To collect also the dried adherent parts of the urine on the surface, all contaminated devices are rinsed with deionized water until the clear plastic bottles are filled to about three-quarters. The weight of the filled bottle is determined and the data stored until evaluation.

29.6.3.3 Cage Washing

Typical collection intervals are: 0–24 h, 24–48 h, 48–168 h after administration.

At predetermined occasions, additional washings of the cage are performed. The collection is done during the change of the bottles containing the urine. When necessary (in case of very hydrophobic drugs or metabolites) the rinsing fluid may contain organic, water-miscible fluids. The cage washing samples are treated in the same way like the urine samples.

29.6.3.4 Feces Collection and Sample Preparation

Typical collection intervals are: 0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

During each collection interval, the excreted feces pellets are collected from the separation device in a labeled plastic bottle. The weights are determined and registered as raw data. To homogenize the feces each sample is diluted with demineralized water, according to the consistency with the two- to four-fold of the feces weight. The weight of the filled bottle is determined and the data stored until evaluation.

The sample with the mixture of feces and distilled water is homogenized with the Ultra-Turrax® intensively. The Ultra-Turrax® should be cleaned rigorously before using it again.

For preparation of samples for combustion, pre-weighted combustion cones were filled with an appropriate amount of homogenate (typically about 250 mg). The combustion cones and covers including the homogenate are weighed again and the data stored until evaluation.

29.6.4 Preparation of the Carcass

29.6.4.1 Material

- Standard commercial small meat mincer
- Ultra-Turrax® (e.g., from Ika, Staufen, Germany)
- Precision balance
- Evaporation basins, scissors, pincer, spatula, Kleenex, disposable gloves, plastic bottles, or glass bottles

29.6.4.2 Procedure

After exsanguination/killing any organs and tissues of each rat planned to be investigated separately are removed and stored in a pre-weighed small evaporation basin. The rest of the body is ground with the meat mincer. In order to minimize the residues in the mincer, three Kleenex wipes are ground in the mincer afterward and are added to/in evaporation basin. The mincer is disassembled and residuals are removed with pincer and spatula. The residuals are added also to the evaporation basin. To homogenize the residues, each sample is diluted with deionized water, according to the consistency with the two- to four-fold of the carcass weight and mixed intensively with the Ultra-Turrax®. The Ultra-Turrax® should be cleaned rigorously before using it again. All weights have to be registered thoroughly. The weights of the removed organs and the blood of exsanguination also have to be collected.

For preparation of samples/aliquots for combustion, pre-weighted combustion cones (as from Perkin Elmer) are filled with an appropriate amount of homogenate (typically about 250 mg) or blood/plasma of exsanguination. The combustion cones and covers including the homogenate are weight again and the data stored until radioanalytical evaluation.

Radioanalysis is performed by the liquid scintillation procedure either directly after addition of a scintillator (urine, cage washing) or following combustion and addition of a scintillator (carcass, blood, plasma, feces).

EVALUATION

The concentrations of radioactivity expressed as µg equivalents of drug per gram of matrix and alternatively as part of dose eliminated (in percentage of the administered dose) are determined in each matrix and collection interval.[14] The temporal course of the eliminated parts of excretion is represented by graphs[15] and tables using either Windows Excel function, a specific software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). They also allow calculation of the half-lives of radioactivity elimination via urine and feces (when appropriate),[16] to generate cumulation plots or general survey plots like bar diagrams (see "Example").

These data and the radioactivity in carcass, blood and plasma of exsanguination, special organs/tissues, cage washing, and the balance are finally drawn together for detailed discussion and evaluation.

[14]It might be necessary to use a normalized apparent collection interval for graphical reasons in case of dissimilar collection intervals.

[15]Instead of the collection interval, the mean time of the collection period is often used in the graphic presentations.

[16]Via determination of the rate constant which is calculated by linear regression of ln concentration on time using a least three data points which appeared to be randomly distributed about a single straight line. Half-lives were calculated as ln2/rate constant.

Special attention has to be taken on major differences of data following intravenous and oral administration.

In the case that an essential part of dose is excreted via urine, an estimation of absorption is often done giving the ratio of urine excretion (oral/intravenous) as absorption rate.

An essential requirement for the quality of the study is a balance value near to 100%. A value >95% and <105% is reasonable. Balance values of 90–95% or 105–110% are often accepted as well, but they should indicate the investigator that something might run out of control or the process/handling could be optimized. Balances much worse than these should be explained.

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study. The comparison of both the radioactivity concentrations and the sum of concentrations determined by specific methods allows the estimation of the gap which often develops time-dependently: For instance, in the beginning of the study, radioactivity concentrations in plasma should fit to the drug concentration. Usually, the gap (as percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption based on the urine excretion ratio (oral/intravenous) should be compared with the ratio of dose-normalized plasma AUCs after i.v. and oral administration from the rat radiokinetic study. In case of major differences between both methods of absorption determination, a thorough investigation/discussion of a route of administration-dependent metabolism and/or volume of distribution might help to explain the apparent discrepancy.

Both methods of absorption calculation mentioned are based on the fact that after absorption the drug/metabolites reach the central circulation. In case of oral administered drugs targeting the liver and being eliminated biliary instantly, it is obvious that both methods mentioned fail. In such a special case, an absorption estimation might be possible via a biliary excretion study.

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, microemulsions, or suspensions can be responsible for very distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

MODIFICATIONS OF THE METHOD

Instead of or additional to the oral administration the method can be adopted to subcutaneous, inhalation, intratracheal or dermal route (as, for instance, described by Dix et al. (2001), Simonsen et al. (2002), Mathews (1998), Koyama (2010), Hoffmann (2010), Gledhill (2005)), or any other route of administration.

Sometimes several dose levels are investigated with the method described: a low dose for which a pharmacological response is expected or a possible much higher dose at the NOAEL (for concomitant metabolic profiling and estimation of metabolite/drug concentration ratios at this relevant level of exposure). In case of evaluation of altered routes of elimination at toxic doses and retrieving toxic metabolites, studies with doses higher than NOAEL may be considered as exception. Determination of blood, plasma and feces samples not using combustion but either direct measurement (plasma) or solubilizing and de-coloration of the samples is mentioned, for instance, by Okuyama et al. (1997) or Mathews et al. (1998).

The in situ absorption from different segments of the gastrointestinal tract by dosing the radiolabeled drug directly into "digestive tract loops" is explained by Mano et al. (2004) to get information about the ability for absorption along the different parts of the gut.

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EXAMPLE

HMR 1556 was a drug candidate developed for treatment of hypertension. It was ^{14}C-labeled and investigated in a rat mass balance study to determine the pattern and rate of excretion, the residual concentrations at the end of the study, 168 h after administration, and the mass balance following oral and intravenous route of administration.

29.6.5 Choice of Formulation

Because of low solubility of the compound in pure water, for the oral administration, a suspension in an aqueous solution of hydroxyethylcellulose (0.5%) was used. The solid state physical parameters (particle size, surface area, amorphicity, and crystal modification) and the suspension properties (agglomeration tendency, homogenicity, and dissolved part of the drug) had to be investigated. For the intravenous administration, an aqueous solution containing saline, DMSO, and PEG 400 was used as formulation.

29.6.6 Animal and Radioanalytic/Kinetic Study Part

Of 8 healthy male Sprague Dawley rats weighing between 187.4 and 197.6 g, four animals received an approximate dose of 10 mg ^{14}C-HMR 1,556/kg body weight orally by using a stomach tube. Four other animals received a dose of approximately 5 mg/kg intravenously into a tail vein. For further details, see Table 29.2. Urine samples (0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration), feces samples (0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration), cage washings (0–24 h, 24–48 h, 48–168 h after administration), blood and plasma samples after exsanguination and carcass were collected and weighed as described above. Three aliquots from each (processed) sample were used. The aliquots from the urine samples were measured directly in the liquid scintillation counting procedure after addition of the scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany). The other matrix aliquots were combusted in a Tri-Carb® 307 combuster (Perkin Elmer, Model 307) and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb® (Perkin Elmer). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Perkin Elmer 2500 TR) after addition of the scintillator Permafluor E+® (Perkin Elmer).

29.6.7 Evaluation and Discussion

As an example for the numerous generated graphs, the linear cumulation plot (% of dose amount excreted) and the semilogarithmic plot of drug amount (given as dose amount excreted during a 24-h interval) excreted in urine depending on time after intravenous administration is given in Fig. 29.3a, b.

After the oral administration of approximately 10 mg ^{14}C-HMR 1,556/kg body weight, between 42.8% and 50.8% (mean 46.05%) of the administered radioactivity was excreted via the urine (including cage washings). In the feces, between 46.9% and 58.3% (mean 51.2%) was found. After the intravenous administration of approximately 5 mg ^{14}C HMR 1,556/kg body weight, between 52.3% and 63.9% (mean 56.9%) of the administered radioactivity was excreted via the urine (including cage washings). In the feces, between 33.4% and 43.2% (mean 38.6%) was found.

Table 29.2 HMR 1556 dosage

Animal-No	1	2	3	4	5	6	7	8
Body weight (g)	195.8	195.1	190.1	197.4	197.6	191.5	187.4	194.4
Route of administration	oral				intravenous			
Dose (mg/kg)	9.535	9.694	9.949	9.543	5.794	5.591	5.658	5.444
Time of killing (h aft. adm.)	168							
Preparation	Suspension with HEC				Solution in water with DMSO/PEG 400/Saline			
Conc. in prep. (mg/g)	1.884				2.063			
Amount prep. adm. (g)	0.991	1.004	1.004	1.000	0.555	0.519	0.514	0.513
Batch	Z 29075-2				Z 29075-2			
Spec. radioact. (MBq/g)	1990.00				1990.00			
Adm. rad. (MBq/animal)	3.72	3.76	3.76	3.75	2.28	2.13	2.11	2.11
Study objectives	Mass Balance Excretion via urine and faeces Residues in carcass							

The calculated absorption rate based on the comparison of the urine excretion after oral and intravenous administration amounted to approximately 80%.

The calculated half-lives for the renal elimination amounted to a mean of 12.2 h (oral dose) and 9.0 h (intravenous dose). The fecal elimination took place with mean half-lives of 10.3 h and 8.8 (oral and intravenous dosing).

After both routes of administration, only very low amounts of the total radioactivity administered were to be found in the carcasses and blood of exsanguination (0.2%).

The recovery rate after oral administration of approximately 10 mg/kg body weight amounted to a mean of 97.4%. Following intravenous administration of approximately 5 mg/kg, the mean balance was 95.7%.

The main results are summarized in Fig. 29.4 and Table 29.3.

29.7 Blood/Plasma Radiokinetics in Rats

PURPOSE AND RATIONALE

Investigate the time course of radioactivity concentrations in blood and plasma with the aim to getting information about the absorption process, the AUC, C_{max}, and the elimination half-life of radioactivity in blood and plasma. Possibly indices for enterohepatic cycle or for metabolites with very different volume of distribution from the original compound may be found. An estimate of the absorption rate comparing dose-normalized AUCs after i.v. and oral (or any other) administration can be done and a major binding to formed blood elements can be noticeable by comparison of blood and plasma concentrations.

This type of study is obligatory for the registration as a drug when the rat is chosen as rodent model (in toxicology).

Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration. If possible, and solubility allows, the study should also be performed after intravenous administration and the results of both routes should be compared.

Normally, the radiokinetic study is performed administering a single dose per administration route.

The study is performed separately from the mass balance study in rats, in order not to influence the balance by collecting blood during the study course.

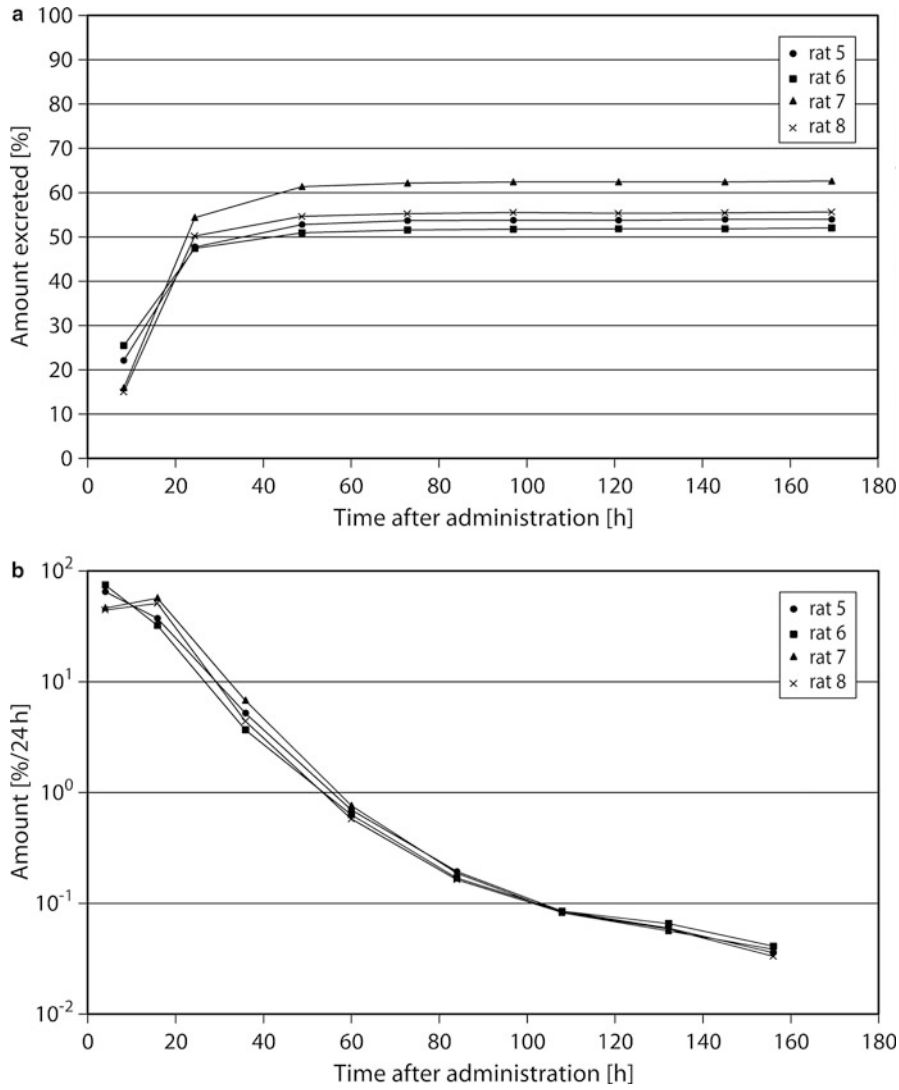

Fig. 29.3 ^{14}C-HMR 1556: excretion via urine following intravenous administration of approx. (**a**) cumulative plot (**b**) amount (%/24 h)

PROCEDURE

29.7.1 Animal Study Part

The radiolabeled drug is administered to 54 healthy Sprague Dawley rats per gender (weighing about 200 g corresponding to an age of 6–10 weeks); 27 animals receive an oral dose by stomach tube (for instance, 10 mg/kg bw) and 27 animals receive an intravenous dose into a tail vein (for instance, 1–5 mg/kg; total radioactivity of about 2 MBq/animal in case of ^{14}C-label).

The administration and the animal maintenance can/may follow the procedure and description given for oral and intravenous administration of the mass balance study.

29.7.2 Collection and Sample Preparation

29.7.2.1 Material

- Box for anesthesia with Isofluran
- Scalpel
- Disposable gloves
- Small evaporating basins

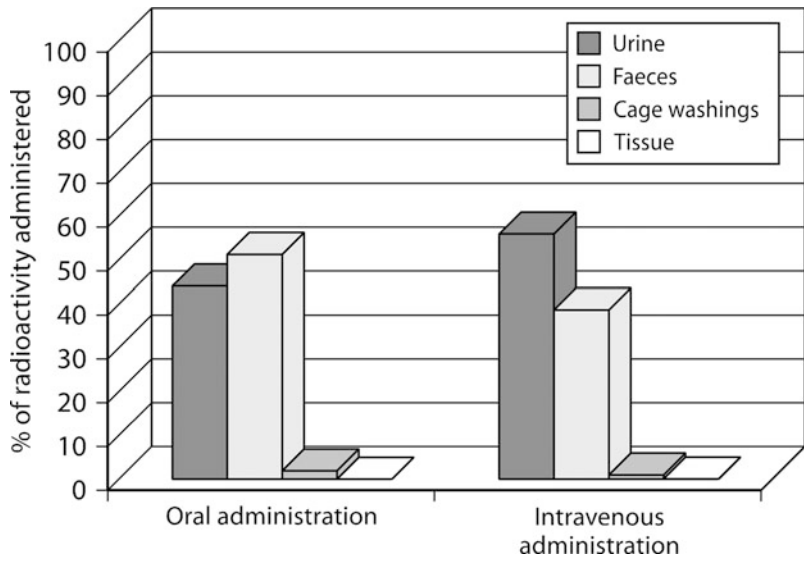

Fig. 29.4 ^{14}C-HMR 1556: Balance after oral administration of approximately 10 mg/kg body weight and intravenous administration of approximately 5 mg/kg body weight to male rats

Table 29.3 ^{14}C-HMR 1556. Excretion in urine and feces, cage washings, and amount of radioactivity in blood of exsanguination and carcass after oral administration of approximately 10 mg/kg and intravenous administration of approximately 5 mg/kg body weight to male rats (% of administered radioactivity)

	Oral						Intravenous					
	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD	Rat 5	Rat 6	Rat 7	Rat 8	Mean	SD
Body weight (g)	195.8	195.1	190.1	197.4	**194.6**	3.2	197.6	191.5	187.4	194.4	**192.7**	4.3
Dose (mg/kg)	9.535	9.694	9.949	9.543	**9.68**	0.193	5.794	5.591	5.658	5.444	**5.622**	0.146
Urine												
Total amount (%)	41.81	42.95	49.31	42.01	**44.02**	3.56	53.82	51.79	62.35	55.37	**55.83**	4.58
$t_{1/2}$ (h)	9.3	11.3	12.4	15.7	**12.2**	2.7	9	8.8	9.1	8.9	**9**	0.1
Feces												
Total amount (%)	58.3	47.75	46.88	51.8	**51.19**	5.21	38.19	43.23	33.37	39.6	**38.6**	4.08
$t_{1/2}$ (h)	7.7	9.3	11.6	12.6	**10.3**	2.2	8.7	8.7	9.4	8.3	**8.8**	0.5
Cage washings												
Total amount (%)	1	2.93	1.45	2.74	**2.03**	0.95	1.35	0.49	1.58	0.77	**1.05**	0.5
Carcass												
Total amount (%)	0.16	0.2	0.13	0.16	**0.16**	0.03	0.17	0.19	0.24	0.21	**0.2**	0.03
Recovery												
Total amount (%)	101.3	93.8	97.8	96.7	**97.4**	3.1	93.5	95.7	97.5	96	**95.7**	1.6

- Commercially available aggregation inhibitor like heparin-sodium or sodium citrate
- Small centrifuge tubes
- Centrifuge

At each observation time point three rats are put in the box for anesthesia separately (especially critical at early time points; a staggered approach makes sense), which is permanently flushed with narcosis gas. The cover is closed and the rat is left there until complete apnea. Taking the rat outside the box again the reflex cornealis is proven. When the reflex is extinguished the vessels lateral below the throat are cut with the scalpel. The blood pouring out is collected in the small evaporating basins which already contain small amounts of heparin-sodium in cases where plasma preparation is intended (instead of serum).

Alternatively, blood can be collected from the abdominal aorta after anesthesia and cerebral

dislocation directly into syringes/containers containing a small amount of lithium or sodium heparin by abdominal incision at linea alba, and putting abdominal viscera aside. For generating plasma, part of the collected blood is transferred into centrifuge tubes and treated in the centrifuge with 1,500 G.

For typical sample collection time points in a 48-h study see example below.

Blood and plasma samples are taken up prepared for combustion (e.g., aliquots are transferred on cones for combustion), weighed, dried at room temperature, combusted, and the $^{14}CO_2$ formed is absorbed by Carbo-Sorb (Perkin Elmer). The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

Remaining blood and plasma are suitable for concomitant metabolism and bioanalytical studies.

EVALUATION

The concentrations of radioactivity expressed as μg equivalents of drug per g of matrix at all collection times and in blood and plasma are determined. The temporal course of the concentrations is represented by graphs and tables using either Windows Excel functions, a specific pharmacokinetic software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). They also allow calculation of the half-lives of radioactivity and the AUCs (see "Example"). The ratio of the dose-normalized AUCs (oral/intravenous) is used as the extent of absorption.

All these data are finally drawn together for detailed discussion and evaluation. Special attention has to be taken to major differences of data following intravenous and oral administration.

In the case that an essential concentration is available in blood and plasma at early time points after oral administration (e.g., C_{max} is reached soon) a rapid onset of absorption can be stated.

When there is no continuous decline of concentrations after intravenous administration, this might be an indication for:

- An inappropriate administration or a precipitation of the drug from the formulation at the application side or just behind the side in the blood stream. This might be the case when there is a plateau concentration or even an increase at early collection times.
- An enterohepatic circulation when a side maximum exists (time depending on absorption and elimination rate—often seen 4–8 h after administration).
- One or more of the generating metabolites show a much smaller volume of distribution than the drug itself.
- Other (often more complex) explanations.

In case of a drug concentration ratio (in plasma/blood) distinctly lower than expected from hematocrit, a binding of the drug or its metabolites to formed blood elements is indicated.[17]

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study. The comparison of both the radioactivity concentrations and the sum of concentrations determined by specific methods allows the estimation of the gap which often develops time-dependently: For instance, in the beginning of the study after intravenous administration, radioactivity concentrations in plasma should fit to the drug concentration. Usually the gap (as a percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption via the ratio of dose-normalized plasma AUCs after oral and intravenous administration based on the assumption that:

1. A first pass effect including an instant elimination via bile does not exist.
2. The metabolic pattern is comparable independently of the route of administration.

[17]The following equation describes the percent of total radioactivity in plasma relative to total radioactivity in blood:

$$\text{Plasma/Blood}\,(\%) = \frac{(V_B) \times (P \times [1 - HCT])}{(V_B \times B)} \times 100 = \frac{P \times (1 - HCT)}{B} \times 100$$

where:
V_B = Volume of blood
P = Drug concentration in plasma
B = Drug concentration in blood
HCT = Hematocrit

3. The volumes of distribution (drug and metabolites) and the metabolism in special tissue are not effected by the bolus i.v. administration with high initial concentrations (Chiou 1989).
4. Dose-exposure linearity exists (under a more stringent view even a first order kinetic is required).

When those criteria are not fulfilled, then either the absorption can be underestimated (for instance, when precondition (1) is not true (2) or distinctly overestimated up to a calculated "super-absorption" for more than 100% (possible, for instance, when precondition (3) or (4) is not true)).

Only AUCs obtained from the same "type of blood" samples should be used. For instance, do not compare central arterial blood with venous peripheral blood (Chiou (1989)).

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, microemulsions, or suspensions can be responsible for very distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

When establishing the method, one should cautiously practice the collection of blood by the cut lateral below the throat. In any circumstances injuring the esophagus has to be avoided, since small parts of formulation remaining in the esophagus (for instance, because of reflux or a sticky formulation) can contaminate the blood of exsanguination.

MODIFICATIONS OF THE METHOD

There are several methods of collecting blood from other sites.[18] Each method has advantages and disadvantages. Whereas central blood of exsanguination enables additional bioanalytics and metabolism from the same original sample (5–7 mL of blood), the collection of tail vein blood[19] (sample sizes of about 100–150 µl) enables intraindividual radiokinetics to be obtained.

In case of using preoperated animals (such as animals with jugulars and/or carotis catheter, see, for instance, Davis et al. (1994), Krishna et al. (2002) or Mun (2009) using an automated blood sampling system also for a radiokinetic study), the physical suitability has to be assessed critically. The reproducibility of these studies is much more critical and excellently prepared and recovered animals are constantly necessary. The comparison of results from central arterial blood (carotis), for instance, with peripheral venous blood, is questionable (Chiou (1989)).

Sometimes it is recommended to use a short-term infusion instead of a bolus intravenous administration. The advantage is to avoid high blood concentrations shortly after intravenous administration which may be in conflict with the assumed linear dose-exposure relationship (validity of linear relation at border concentration?). The advantage of the intravenous bolus injection is the lower/shorter stress situation for the animals (and may be also for the experimenter) which again has consequences for the reproducibility of the study results.

The determination of the volatile radioactivity in urine, feces, and expired air can be performed within the framework of the radiokinetic study (for instance, with the animals provided for the 24 h sample) (About 60% of exhaled radioactivity is mentioned by Xu et al. (2009) after administering radiolabeled dimethyl fumarate to rats and human. An example with a distinct difference of exhalation between mice and rats is described by Chen et al. (2007)). The air of the metabolism cage is continuously extracted, therefore, (for instance, with 3.5 L/min) and measured using a gas flow counter (Exhalameter, Raytest, Straubenhardt, Germany).

[18]For instance, blood of exsanguination collected after heart puncture; jugularis puncture, jugularis or carotis catheter, retroorbital blood, blood from the vena femoralis, sublingual blood after short narcosis or blood from the tail vein.

[19]Caution: The administration should not be done at the site of sample collection to avoid contamination. For instance, an i.v. administration into the vena femoralis after a short transient anesthesia can be recommended.

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EXAMPLE

HMR 1766 is a guanylate-cyclase activator and is being developed for treatment of hypertension. It was ^{14}C-labeled and investigated in a rat radiokinetic study to determine the time course of radioactivity concentrations in blood and plasma.

29.7.3 Animal and Radioanalytic/Kinetic Study Part

Of 54 healthy male Sprague Dawley rats weighing between 165 and 216 g, 27 animals received an approximate dose of 12 mg ^{14}C-HMR 1,766/kg body weight orally by using a stomach tube; 27 other animals received a dose of approximately 2 mg/kg intravenously into a tail vein. In both cases, an aqueous solution containing PEG 400 was used as formulation. As an example for which details of data are collected during administration see Table 29.4 for the data for intravenous administration (analogously the data from the p.o. administration part are recorded).

Blood samples (0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h after oral administration and 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h after intravenous administration) were collected. Parts of the blood samples were processed to obtain plasma samples. Of each sample and matrix 3 aliquots were generated, taken up on Combusto Cones (Perkin Elmer), weighed, dried at room temperature, combusted in a Tricarb® combuster (Perkin Elmer, Model 307), and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb® (Perkin Elmer). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Perkin Elmer 2500 TR) after addition of the scintillator Permafluor E+® (Perkin Elmer).

29.7.4 Evaluation and Discussion

The time course of the mean radioactivity blood and plasma concentration values of three animals is given in the summary graph Fig. 29.5.

Half-lives were calculated by the pharmacokinetics' software WinNonlin. In case of the intravenous administration, a two compartmental model was chosen; in case of oral administration, a non-compartmental model. The AUCs were calculated using the linear trapezoidal rule. The results are summarized in Table 29.5.

Already 15 min after oral administration, radioactivity concentrations in blood and plasma (5.90 and 10.67 µg equiv./g, resp.) were found indicating a fast absorption. The maximum concentrations (C_{max}) occurred 0.5 h after administration and amounted to a mean of 9.34 µg equiv./g (blood) and 16.95 µg equiv./g (plasma). The elimination of radioactivity from blood and plasma was biphasic. The half-lives for the first phase amounted to 1.1 h (blood) and 0.9 h (plasma), for the second phase half-lives of 7.5 and 6.3 h were calculated. In all animals, radioactivity concentrations were detectable up to the last measuring time 48 h after dosing and showed averages of 0.025 and 0.024 µg equiv./g (blood and plasma, respectively).

After intravenous administration, the maximum radioactivity concentrations were measured at the first measuring time 5 min after administration, exhibiting mean concentrations of 4.79 and 7.64 µg equiv./g (blood and plasma). Following the peak values, the radioactivity decreased in a biphasic process with mean half-lives of 0.4 h for phase I and 10 h (blood and plasma, respectively) for phase II. In all animals, blood and plasma levels were observed up to 48 h after administration (0.006 and 0.007 µg equiv./g in blood and plasma, respectively)

The extent of absorption after oral dosing was determined by using the dose corrected comparison of the mean areas under the blood (and plasma)—radioactivity concentration curves after oral and intravenous administration (AUC values). This comparison revealed an absorption rate of more than 90% depending on AUC values used for calculation (blood or plasma). This value was very similar to the result in a mass balance study where the absorption was calculated by a comparison of the renally excreted radioactivity following oral and intravenous administration.

Since all plasma radioactivity concentrations are distinctly higher than the corresponding blood values,

Table 29.4 Data for intravenous administration

Animal-No	113	114	115	116	117	118	128	129	130
Body weight (kg)	0.188	0.212	0.196	0.179	0.189	0.186	0.210	0.202	0.206
Route of admin.	Intravenously								
Dose (mg/kg)	1.962	1.763	2.058	2.213	2.084	2.054	1.952	2.010	1.999
Time of killing (h after adm.)	0.083			0.25			0.5		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.788						0.951		
Amount prep. adm. (g)	0.469	0.473	0.511	0.503	0.499	0.484	0.431	0.427	0.433
Batch	Z 29023-0-10						Z 29023-0-11		
Spec. radioact. (MBq/g)	1512.78						1324.47		
Radioact./animal (MBq)	0.559	0.564	0.609	0.600	0.595	0.577	0.543	0.538	0.545
Study objectives	Drug levels in blood and plasma								
Animal-No	**131**	**132**	**133**	**119**	**120**	**121**	**122**	**123**	**124**
Body weight (kg)	0.201	0.215	0.208	0.194	0.189	0.187	0.203	0.197	0.181
Route of admin.	Intravenously								
Dose (mg/kg)	2.110	1.920	2.003	1.925	2.019	1.980	2.012	1.977	2.060
Time of killing (h after adm.)	1			2			4		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.951			0.788					
Amount prep. adm. (g)	0.446	0.434	0.438	0.474	0.483	0.470	0.517	0.495	0.473
Batch	Z 29023-0-11			Z 29023-0-10					
Spec. radioact. (MBq/g)	1324.47			1512.78					
Radioact./animal (MBq)	0.562	0.547	0.552	0.565	0.576	0.560	0.616	0.590	0.564
Study objectives	Drug levels in blood and plasma								
Animal-No	**125**	**126**	**127**	**134**	**135**	**136**	**137**	**138**	**139**
Body weight (kg)	0.189	0.196	0.186	0.202	0.216	0.210	0.213	0.206	0.207
Route of admin.	Intravenously								
Dose (mg/kg)	2.053	1.984	1.941	1.883	1.818	1.789	2.009	2.017	2.049
Time of killing (h after adm.)	8			24			48		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.788			0.951					
Amount prep. adm. (g)	0.492	0.493	0.458	0.400	0.413	0.395	0.450	0.437	0.446
Batch	Z 29023-0-10			Z 29023-0-11					
Spec. radioact. (MBq/g)	1512.78			1324.47					
Radioact./animal (MBq)	0.587	0.588	0.546	0.504	0.520	0.498	0.567	0.550	0.562
Study objectives	Drug levels in blood and plasma								

there is no indication for a major binding of radioactivity to formed blood elements in this study.

Since the intravenous administration ensures the maximum exposure especially in case of anesthetized or preoperated animals, this route is often favored.

29.8 Bile Fistula Study in Rats

PURPOSE AND RATIONALE

To investigate the rate and the extent of the excretion of the drug/metabolites with the bile. If required, the investigation can be supplemented to study the enterohepatic circle. The bile fistula study becomes necessary when relevant parts of the administered radioactivity are eliminated fecally suggesting a considerable biliary excretion during the mass balance study in rats.

PROCEDURE

29.8.1 Part 1 (Collecting and Investigating the Bile)

Four healthy male anesthetized Sprague Dawley (or Wistar) rats weighing about 300 g (10–12 weeks old) and provided with a permanent bile fistula receive an intravenous dose (such as 5 mg/kg; for

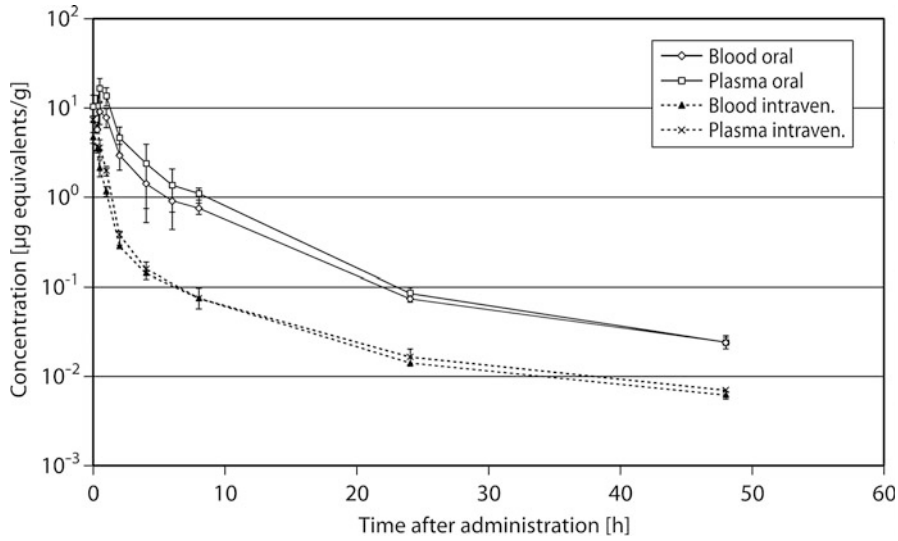

Fig. 29.5 ^{14}C-HMR 1766: Blood and plasma radioactivity concentrations after oral administration of approximately 12 mg/kg and after intravenous administration of approximately 2 mg/kg body weight to male rats. The variability (SD) of the animal values (n = 3) is given as bars

Table 29.5

Route of administration	Oral	Intravenous
Rat-nos.	59–85	113–139
Body weights (kg)	0.165–0.189	0.179–0.216
Doses (mg/kg)[a]	10.602–13.092	1.763–2.213
	(11.88)	(1.99)
Blood		
C_{max} (µg equiv./g)	9.34	4.79
t_{max} (h)	0.5	0.083
$t_{1/2}$I (h)	1.1	0.4
$t_{1/2}$II (h)	7.5	9.6
AUC (µg equiv./g × h)	28.96	5.29
Plasma		
C_{max} (µg equiv./g)	16.95	7.64
t_{max} (h)	0.5	0.083
$t_{1/2}$I (h)	0.9	0.4
$t_{1/2}$II (h)	6.3	10.4
AUC (µg equiv./g × h)	46.39	7.78

[a]In brackets: mean value of 27 animals each

instance, into the tail vein, procedure see Sect. 29.6 without the necessity to use the described tube for the animals).

Details of installing the permanent bile fistula:

29.8.1.1 Material

- Ketamin (for instance, Hostaket® from Intervet)
- Midazolam (for instance, Dormicum® V from Roche)
- Disposable syringes, disposable gloves, disposable cannulas
- Small Braunülen (= indwelling venous cannulas)
- Skin disinfectant
- Heatable operating table for small animals
- Surgical microscope
- Electric clipper with 1/2 mm cutter head (for instance, from Aesculap Favorita)
- Surgical instruments: scalpel, surgical shears of various sizes, bent forceps, iridectomy scissors, surgical skin staples, stapling forceps, surgical gloves, swabs
- Catheter consisting of polythene tubing ID 0.28 mm, OD 0.61 mm (for instance, from Portex, Hythe, Kent, GB) or similar material (like flexible silicon catheter)

The rats are fasted for about 4 h and are anesthetized with 80 mg/kg bw Ketamin plus 5 mg/kg Midazolam administered intravenously into the tail vein via an indwelling venous catheter. The amount corresponds to a volume of about 0.3 mL. Anesthesia is monitored by checking the reflexes and is maintained by repeat dosing of ketamine/midazolam.

The abdomen of the anesthetized rat is shaved, the rat is then fixed in dorsal position on the heated operating table (thermostat set to 37°C), and the abdomen is disinfected with a commercial skin disinfectant.

Laparotomy is carried out at the linea alba, the catheter is introduced into the common bile duct

and fixed at such a distance from the hilus that the flow of pancreatic juice is not hindered. The animals are kept on heating pads to maintain body temperature throughout the experiment.

The test compound is injected into a tail vein. The study period is 8 h as from the time of dosing. At the end of the study period, the animals are killed painlessly by an overdose of the anesthetic.

The bile removed leads to fluid depletion of the organism. The corresponding volume was thus substituted for by intravenous infusion of isotonic sodium chloride solution (about 1 mL/h).

The bile of each animal is collected separately under cooled conditions during, for instance, four intervals (0–2 h, 2–4 h, 4–6 h, 6–8 h) via the permanent bile fistula. The samples are weighed and aliquots thereof measured directly after the addition of a commercial scintillation cocktail and water. In addition, the amounts of radioactivity in urine (0–8 h) are determined.

The remainder of the bile is used for metabolic profiling and structure elucidation of metabolites and, if necessary, for the investigation of the enterohepatic circulation as described in the following.

29.8.2 Part 2 (Enterohepatic Circulation)

Enterohepatic circulation of the non-diluted bile obtained in parts are pooled in order to receive a representative mixture of metabolites on one hand (that is from different collection intervals) and on the other hand a radioactivity concentration as high as possible.

The pooled bile is intensively homogenized, the homogenicity and the exact concentration proven by radioanalysis of several aliquots. The amount for application (about 1 g/animal) is withdrawn. The receiver animals are prepared like the animals in the first part of the study. However, the pooled bile is administered intra-duodenally via a flexible tubing. This tube is advanced from the mouth through the stomach as far as the beginning of the duodenum. During the administration, the tube is fixed with the fingers inside the duodenum to prevent reflux of the administered bile into the stomach.

Bile samples are collected and processed as described in part 1 of the study.

EVALUATION

29.8.3 Part 1

With the knowledge of the amount of bile, the concentrations determined are converted into percentages of administered radioactivity. The results can be displayed graphically (versus time). In case of portions of administered radioactivity of at least three time points being distributed about a single straight line in the semilogarithmic plot, the rate constant can be calculated by linear regression and subsequently the half-lives.

The data are finally drawn together for detailed discussion and evaluation with special attention to known results from other radiokinetic studies: such as the mass balance study and the obtained fecal elimination or the quantitative whole body autoradiography.

29.8.4 Part 2

The part of dose found in the bile of the receiver animals dosed with a part of the bile withdrawn from the donor animals gives an estimate about the magnitude of the enterohepatic circulation.

CRITICAL ASSESSMENT OF THE METHOD

The anesthesia slows down processes of the liver. It has to be assumed that liver clearance and elimination are faster when animals are awake. Therefore, models with woken animals are sometimes favored (see "Modifications of the Method").

A rough estimation whether anesthesia might relevantly misrepresent the situation of an awake animal should be performed comparing the bile excretion with the excretion via feces from the mass balance study (see "Example"), the urine excretion or the radioactivity concentration in blood. (So the bile fistula study should be extended to collect also body fluids such as terminal blood or the urine during the anesthesia.)

The estimation of enterohepatic circulation can be valid only when biliary excretion is virtually complete in the observed time interval.

MODIFICATIONS OF THE METHOD

The intravenous administration ensures a "complete" absorption and is therefore often the favored route of

dosing in this type of study. However, metabolism, distribution, and excretion may change with the route of administration and might impact the results of a bile fistula study. Thus, it has to be considered which route of administration or even two routes have[20] to be chosen. The comparison of the metabolic pattern in plasma or even in feces after different routes of administration may support this decision.

It may be worthwhile to dissect and measure additional tissues/organs/excreta at the end of the study, for instance, to investigate whether a direct secretion into the bowels took place (might be seen after i.v. administration), whether the liver retained radioactivity or to balance the radioactivity over urine, bile, and carcass (without bowels) to estimate the absorption more precisely.

Alternative models with woken animals during bile collection (Johnson et al. (1978) or for instance: Tse FLS et al. (1983) or as used in Bruin (2008)) are sometimes favored excluding the influence of anesthesia. However, those models often also cannot represent the situation of a "normal" rat, since operation and catheterization may also impact the study result.[21]

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EXAMPLE

Hoe 642 is an inhibitor of cellular Na+/H+ exchange and thus a drug with cardioprotective activity.

^{14}C-HOE 642 was intravenously injected into a tail vein (5 mg/kg; 1 MBq/animal) to 4 healthy male, anesthetized Wistar rats (approx. 320 g) with bile fistula. For administration, the test compound was used as aqueous solution with 0.9% saline (3.5 mg/g formulation; approximately 0.5 g formulation/animal) adjusted to pH 7 with sodium hydroxide.

Bile samples were collected as described above, weighed, diluted with water, weighed again, and aliquots were measured after addition of scintillator.

The radioactivity measurements were carried out by the liquid scintillation procedure, using a β-spectrometer of type BF 5000 (Berthold, Wildbad, Germany). As scintillator Rotiszint eco plus (Roth, Karlsruhe, Germany) was used. Blank values were concurrently measured in the studies and deducted from those measured. These blank values originated from bile fluid of the animals taken before dosing.

The amounts of radioactivity that were excreted with the bile are illustrated in the graph in Fig. 29.6.

During the study period between 6.9% and 7.9% (mean = 7.6%) of the radioactivity administered were excreted with the bile. The major part thereof was eliminated within the first 2 h after injection. The portion of radioactivity excreted with the bile decreased at later measuring intervals. In the last collection interval, i.e., 6–8 h after dosing, an average of 0.09% of the radioactivity administered was still present in the bile.

Half-lives were calculated. They were based on a monophasic process with values between 0.7 and 1 h. A previous mass balance study with ^{14}C-HOE 642 revealed a fecal elimination of about 17% (2% up to 8 h, 14% between 8 and 24 h, and 1% at later time points) following intravenous injection (comparable dose). Since in the bile fistula study the portion still present in the bile in the last collection period was only about 0.1%, it was assumed that biliary secretion is virtually complete. The difference to the excreted fecal elimination obtained in the mass balance study may be due to the general anesthesia of the rats in the bile study or radioactivity may reach the gastrointestinal tract by other ways (direct secretion into the intestine or the stomach, secretion by the salivary glands with subsequent swallowing of the saliva. The salivary glands are known to exhibit high radioactivity concentrations after intravenous injection from radioluminography studies with ^{14}C-HOE 642).

[20]Instead of an oral an intra-duodenal administration should be chosen, when using anesthetized animals.

[21]The procedure as described in the main part still has supporters. See Hoehle et al. (2009).

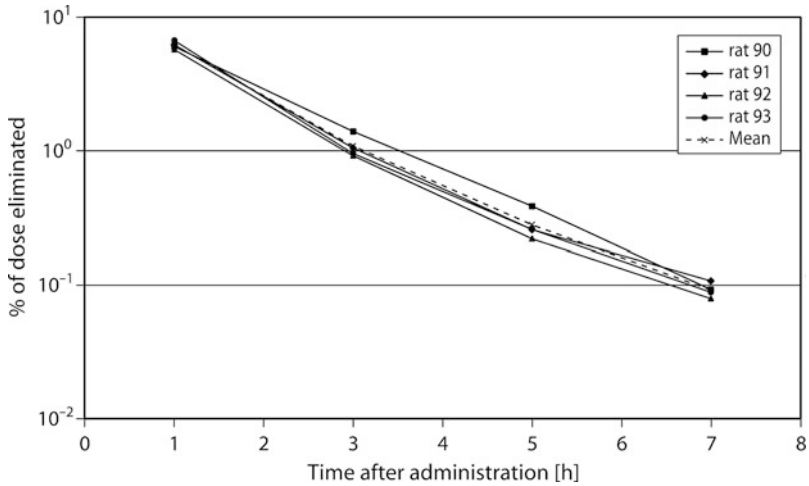

Fig. 29.6 ^{14}C-HOE 642: amount of dose excreted via bile after i.v. (5 mg/kg) administration to rats (the lines between the data of the measuring points should be understood as trend lines)

The presence of a lower radioactivity portion in the bile than in the feces and the virtually monophasic elimination process makes the existence of an enterohepatic circulation improbable.

29.9 Diaplacental Transfer Study in Rats

PURPOSE AND RATIONALE

To obtain information about the distribution of a drug and/or its biotransformation products in dams and fetuses in relation to time.

PROCEDURE

Nine healthy female, 18 days pregnant Sprague Dawley rats weighing about 300 g (approximately 12 weeks old) receive the radiolabeled compound through the route of administration projected to be used therapeutically (for oral or intravenous administration to rats and animal maintenance, see the procedure described in the rat mass balance study).

29.9.1 Material

- Box for anesthesia with CO_2 or with Isofluran
- Scalpel, scissors straight and curved
- Bone shears, pincers
- Gloves for single use
- Small evaporating basins
- Plastic tubes and commercially available aggregation inhibitor like heparin-sodium
- Centrifuge tubes and centrifuge
- Disposable syringes and cannulas
- Dash bottle with ethanol/water 1:1

Three animals/time point are killed painlessly (CO_2 anesthesia, exsanguination as described in the "blood/plasma radiokinetic study in rats") and are each immediately dissected.

The rat is placed in a dorsal position on an undersheet and the fur is moistened with ethanol/water. The abdominal cavity is opened and the abdominal wall folded back. The uterus is exposed and the amniotic fluid is withdrawn by puncturing the amniotic sac with a syringe. The uterus is then placed on a plastic film and opened. The placentas with the fetuses are detached from the uterus. The placentas are then detached from the fetuses. After removing the fetal membranes the fetuses are removed and immediately killed under CO_2 atm. The amniotic fluid is aspirated with a disposable syringe.

The organs and tissues listed below are removed. The number of fetuses is determined and documented. The fetuses per dam and their organs, respectively, are pooled. The order of removal is prescribed in the following dissection schedule.

- Blood/plasma (from exsanguination, see study "blood/plasma radiokinetics in rat")
  Opening of the abdominal cavity
- Liver
- Extraction of uterus
- Amniotic fluid
- Remove/kill fetuses immediately with CO_2-gas

- Fetal blood/plasma
- Fetal liver
- Fetal carcass
- Placenta

Examinations are performed concerning the radioactivity concentrations and portions.

29.9.2 Processing of Samples

Blood and plasma samples are taken on cones for combustion, weighed, dried at room temperature, combusted, and the $^{14}CO_2$ formed is directed into an absorption fluid. The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

After removal, larger organs and tissues are homogenized with Ultra-Turrax® appliances (for instance, from Ika, Staufen, Germany) after addition of deionized water, the amount of which depends on the consistency of the tissue. Smaller tissues are finely cut. The specimens are dissolved in volumetric flasks at 60–70°C in Solvable® (Perkin Elmer) and water. Ethanol is added if required to prevent foam formation. Addition of approximately 0.2 mL Perhydrol® (Riedel de Haën, Seelze, Germany) or hydrogen peroxide, respectively, is sufficient to remove discolorations. Measurements are then performed after addition of the scintillator.

EVALUATION

The radioactivity concentrations in the collected samples are determined. They are expressed in µg equivalents of drug/g and in % of administered radioactivity. The temporal course of the concentrations and the portions of radioactivity found can be represented by graph and tables. In cases where mean portions of administered radioactivity of a tissue/body fluid of all three time points are distributed about a single straight line in a semilogarithmic plot (ln concentrations on time), the rate constant can be calculated by linear regression and subsequently the half-life (ln2/rate constant).

The data are finally drawn together for detailed discussion and evaluation with special attention on the placenta border: Do significant amounts of radioactivity cross the placenta and are the fetuses exposed to drug/metabolites? Does the radioactivity remain in the fetus/organs/body fluids longer than in the dams?

CRITICAL ASSESSMENT OF THE METHOD

The radioactivity represents the sum of the original compound and/or radioactive-labeled metabolites and not to forget possible synthetic side-products which can be present in traces (depending on the purity and content of the synthetic material). Discussing traces of radioactivity, for instance, traces crossing the placenta, keep in mind that these traces may be due to synthetic side-products. Thus, whenever possible, try to use radiolabeled compound as clean as possible.

MODIFICATIONS OF THE METHOD

The described procedure may be seen as a minimum for investigation of the placental transfer. Of course, whenever necessary or useful, the procedure may be enlarged by additional time points and organs/tissues/body fluids (such as ovary, kidney of dams) to be investigated.

The radioactivity of the organs can also be determined after combustion alternatively. Normally the method of digestion, discoloration, and direct determination of radioactivity is a little bit more sensitive, in case more material can be used than in the combustion process.

Instead of the aforementioned procedure of a common quantitative distribution study, the placental transfer can be investigated well also by whole body autoradiography (see the section on autoradiography and, for instance, Endo et al. (1992), or Umehara (2008). The quantitative whole body autoradiography was extended also for the use of pregnant rabbits (Herman 1998).

A bidirectional placental (maternal to fetal and fetal to maternal) transfer was described, for instance, by Thomas (1995).

Younger rats (8–10 weeks) and performing the investigation already on day 12 of gestation is mentioned, for instance, by Pohland (1991).

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Table 29.6 HWA 456 dose and stydy design

Animal-No	11	12	13	20	21	22	17	18	19
Body weight (kg)	0.288	0.318	0.284	0.276	0.303	0.270	0.297	0.313	0.280
Route of administration	oral								
Dose (mg/kg)	19.161	19.014	19.405	19.470	19.347	20.005	18.631	19.318	20.903
Time of killing (h after administration)	6			24			48		
Preparation	Suspension in starch mucilage								
Conc. in prep. (mg/g)	5.618			5.551			5.618		
Amount prep. adm. (g)	0.982	1.076	0.980	0.968	1.056	0.973	0.984	1.075	1.040
Batch	Z 29030-2								
Spec. radioact. (MBq/g)	849.00								
Adm.rad.(MBq/animal)	4.68	5.13	6.67	4.56	4.98	4.59	4.69	5.13	4.96
Study objectives	Determination of concentrations in maternal and fetal tissues								

Pohland RC, Vavrek MT (1991) Ameltolide. II: placental transfer of radiocarbon following the oral administration of a novel anticonvulsant in rats. Teratology 44:45–49

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Umehara K-I, Seya K, Iwatsubo T, Noguchi K, Usui T, Kamimura H (2008) Tissue distribution of YM758, a novel If channel inhibitor, in pregnant and lactating rats. Xenobiotica 38(10):1274–1288

EXAMPLE

HWA 486 (Leflunimide, Arava) is a compound against rheumatoid arthritis.

Nine healthy female 18 days pregnant Sprague Dawley rats weighing between 270.0 and 317.9 g received ^{14}C-HWA 486 in an approximate dose of 20 mg/kg body weight. The compound was administered orally by a stomach tube. As formulation a suspension in starch mucilage was selected. Examinations were performed concerning the radioactivity concentrations and portions in blood, plasma, and liver of the dams as well as in amniotic fluid, placenta, and in blood, plasma, liver, and carcass of the fetuses.

Details of the dose and study design are given in Table 29.6.

Samples were processed, the radioactivity determined, and data evaluated as described above.

The radioactivity concentrations found in the different tissues/organs/body fluids are summarized in the graph (Fig. 29.7).

Taking the weight of the investigated tissues/organs/body fluids into account and expressing the observed radioactivity as part of the dose administered (in %), the graph (Fig. 29.8) shows the amount of drug/metabolites reaching the tissues/organs/body fluids:

At the first measuring time (6 h after dosing), the highest levels for dams were detected in the plasma (mean 58.99 µg equiv./g), followed by the liver (43.50 µg equiv./g) and the blood (38.16 µg equiv./g) indicating a considerable extent of absorption. In the placenta, a mean of 16.10 µg equiv./g was found, in the amniotic fluid 3.06 µg equiv./g (0.30%). Concentrations in fetal plasma (13.96 µg equiv./g), fetal blood (11.19 µg equiv./g), fetal carcass (7.39 µg equiv./g), and fetal liver (6.31 µg equiv./g) were lower than the concentration in the maternal blood. Thus, a close barrier function of the placenta with complete retention of radioactivity in this organ was not present.

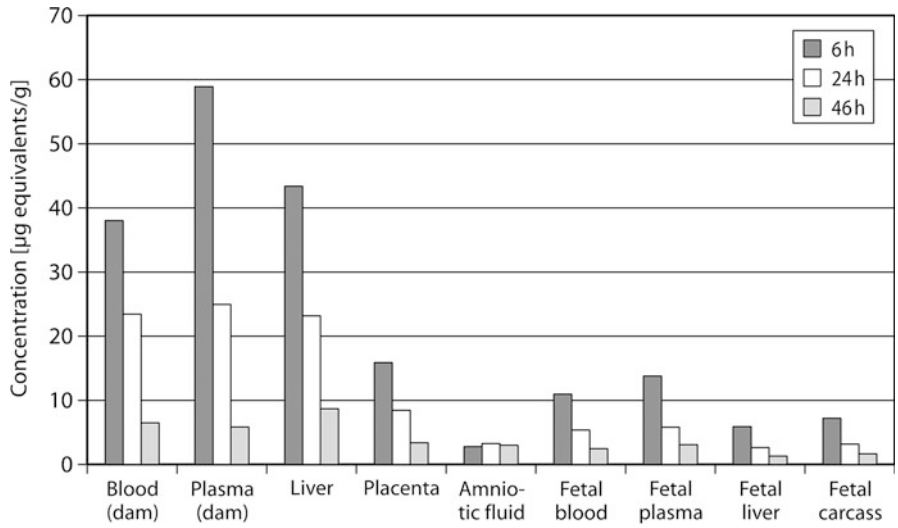

Fig. 29.7 ^{14}C-HWA 486: radioactivity concentrations in tissues/organs/body fluids from pregnant rats

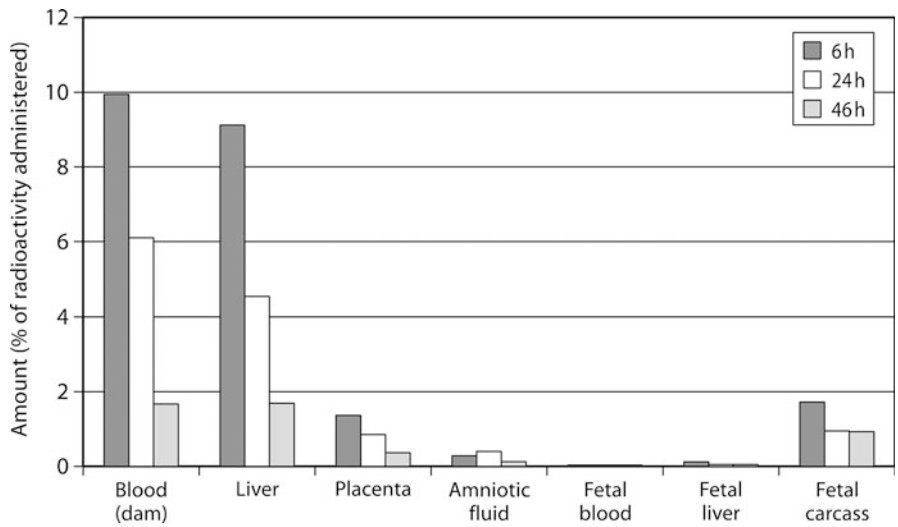

Fig. 29.8 ^{14}C-HWA 486: amount of dose in tissues/organs/body fluids from pregnant rats

Twenty-four hours after administration, the concentrations in all examined organs of dams had decreased as compared to the first measuring time. The concentrations in plasma (25.26 µg equiv./g) were slightly higher than in blood (23.84 µg equiv./g). The liver was in a similar range (23.22 µg equiv./g) and the placenta showed 8.71 µg equiv./g. Fetal liver, fetal blood and plasma, and fetal carcass also showed lower concentrations than at the first measuring time. All concentrations were below the concentration in maternal blood.

At the last measuring time, 48 h after dosing, the concentrations in maternal organs had decreased further: The highest concentrations were detected in the liver (8.83 µg equiv./g, corresponding to 1.7% of radioactivity administered), followed by the blood (6.63 µg equiv./g; 1.7%), and the plasma (5.97 µg equiv./g). In the placenta, 3.45 µg equiv./g (0.4%) were found. Concentrations in fetuses had dropped further. All measured concentrations were considerably below the concentrations in the maternal blood: Fetal blood (2.57 µg equiv./g), fetal plasma (3.28 µg equiv./g), fetal liver (1.36 µg equiv./g), and fetal carcass (1.63 µg equiv./g).

Since the concentrations showed a virtual linear decrease of radioactivity with time in the semilog scale (see Fig. 29.9) the estimation of half-lives made sense (with the exception of the amniotic fluid). The half-lives ranged from 13 h (plasma, dam) to 20 h (plasma, fetus).

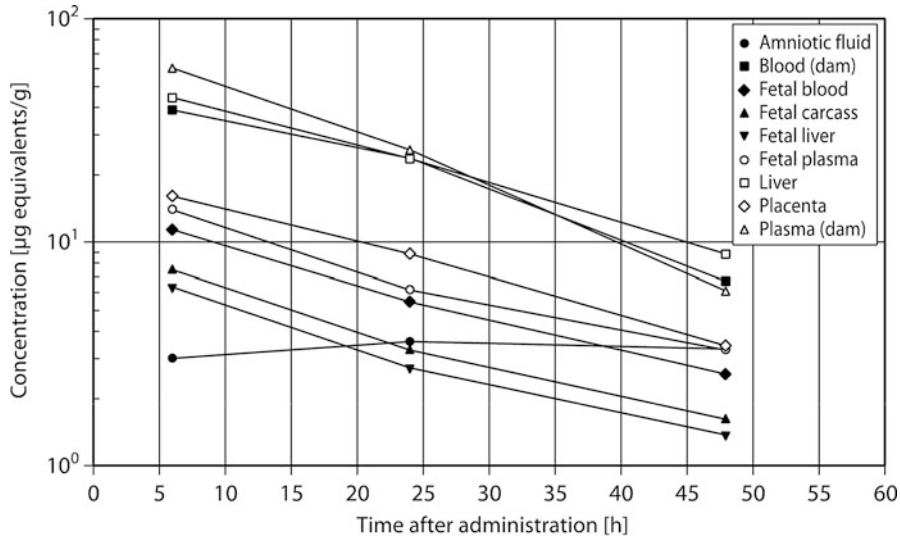

Fig. 29.9 ^{14}C-HWA 486: concentration-time profile of tissues/organs/body fluids from pregnant rats

In summary, it may be stated that after oral administration of ^{14}C-HWA 486 to the dams the compound was well absorbed. The absorbed radioactivity was able to penetrate into the fetuses. The concentrations in the maternal tissues were considerably higher than in the fetus at all measuring times. The radioactivity departs from the maternal organism parallel to the fetal organism. Only in the amniotic fluid, no clear decreasing tendency could be observed.

29.10 Milk Transfer Study in Rats

PURPOSE AND RATIONALE

For registration of a drug, data are required on excretion with the milk as well as data about the correlation between the blood and milk level.

PROCEDURE

Ten lactating healthy female Sprague Dawley rats weighing about 300 g (approx. 13 weeks old; day 11 post partum) receive the radiolabeled compound through the route of administration projected to be used therapeutically. For oral or intravenous administration to rats as well as details concerning animal maintenance see Sect. 29.6.

About 5 min before the selected milking times, each dam receives about 0.03 mL oxytocin (for instance, Oxytocin Injektionslsg.10 IE/mL; Vetoquinol GmbH; Germany, www.vetoquinol.de Ravensburg) subcutaneously, because the amounts are otherwise insufficient for analysis. At the examination times (such as given in the example), milk was first taken by manual milking, and blood then gained from the tail tip. The animals not being accustomed to the milking procedure, milk could not be obtained at each examination time.

For milking, the animals were held by the nape. A sucking pump[22] was put alternately onto the individual teats to collect the withdrawn milk into an Eppendorf® tube. When the vacuum was interrupted at regular intervals with a finger, the device operated like a milking machine. The appropriate partial vacuum and rhythm had to be established for each animal. Higher milk flow was obtained by massaging and thus stimulating the teats with the fingers prior to actually beginning milking.

In cases where the milking and blood sampling are very narrow at first, it is recommended to use two alternative groups of animals.

[22]Can be easily constructed connecting a water-jet pump with a microwash bottle; an Eppendorf vessel is placed under the inlet tube; at the other end the inlet tube is fitted with a polyethylene tube and a microfunnel.

The "microwash bottle" can be assembled from a 15-mL scintillation vessel with two openings in the lid and polyethylene tubing.

29.10.1 Blood and Milk Samples

Blood and milk samples were taken on cones for combustion, weighed, dried at room temperature, combusted in a combuster, and the $^{14}CO_2$ formed is directed into an absorption fluid. The subsequent radioactivity measurements are carried out after addition of scintillator to the samples. Blank values are concurrently measured in the studies and deducted from those measured. These blank values originated from identical material from untreated animals.

EVALUATION

The radioactivity concentrations of the collected samples are determined. They are expressed in µg equivalents of drug/g matrix. The temporal course of the concentrations can be represented by graph and tables. With a specific pharmacokinetic software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com), the half-lives and the AUCs of the radioactivity in blood and milk can be calculated.

To calculate the portion of administered radioactivity excreted with the milk, it is necessary to know the quantitative milk secretion of each particular rat. However, this is not possible within the present study due to the stress inflicted on the animals by the unusual milking procedure which leads to milk retention. Assuming values known from literature (Mepham 1983), the portion excreted with the milk per day can be estimated at 23 mL.

CRITICAL ASSESSMENT OF THE METHOD

Keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself.

MODIFICATIONS OF THE METHOD

Milking rats under anesthesia in milk transfer studies is mentioned, for instance, by Endo (1992), Tanayama (1974), Saillenfait (1997), or Wang (2011). Even milking of mice seems possible (Oskarsson 2004). Milk from mice or rat pups' stomach is used, for instance, by Gonzalez (2011) and Sumner (2010). Additionally, the distribution in the pups can be investigated by quantitative whole body autoradiography as mentioned, for instance, by Bruin (2008).

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Wang L, He K, Maxwell B, Grossman SC, Tremaine LM, Humphreys WG, Zhang D (2011) Tissue distribution and elimination of [14 C]apixaban in rats. Drug Metab Dispos 39(2):256–264

EXAMPLE

HWA 486 (Leflunimide, Arava®) is a compound against rheumatoid arthritis. The oral route of administration was selected for this study because this was the route planned in humans.

Ten lactating healthy female Sprague Dawley rats weighing between 265.0 and 351.2 g received HWA 486-[^{14}C] in an approximate dose of 16 mg/kg body weight. The compound was administered orally by a stomach tube in form of a suspension in starch mucilage. For details of the study conducted, see Table 29.7.

The concentrations of radioactivity in blood and milk were determined at different times up to 24 h after dosing.

Table 29.7 HWA 486 study conduct

Animal-No	1	2	3	4	5	6	7	8	9	10
Body weight (kg)	0.311	0.305	0.273	0.302	0.265	0.318	0.351	0.328	0.330	0.349
Route of administration	oral									
Dose (mg/kg)	15.85	16.11	18.79	16.29	19.63	15.54	15.66	16.60	16.40	15.72
Time of blood sampling (h after adm.)	0.5, 1, 2, 3, 4, 5, 6, 8, 24									
Time of milk sampling (h after adm.)	0.5, 1, 2, 3, 4, 5, 6, 8, 24									
Preparation	Suspension in starch mucilage									
Conc. in prep. (mg/g)	4.927					4.974				
Amount prep. adm. (g)	1.000	0.996	1.039	1.000	1.056	0.995	1.106	1.095	1.088	1.104
Batch	Z 29030-2									
Spec. radioact. (MBq/g)	849.00									
Adm. rad. (MBq/animal)	4.18	4.17	4.34	4.18	4.42	4.20	4.67	4.62	4.59	4.66
Study objectives	Blood and milk levels									

29.10.2 Blood and Milk Samples

Blood and milk samples were taken on Combusto Cones® (Perkin Elmer), weighed, dried at room temperature, combusted in a Tri-carb® combuster (Perkin Elmer, Model 307,.), and the $^{14}CO_2$ formed was absorbed with Carbo-Sorb® (Perkin Elmer). The subsequent radioactivity measurements were carried out after addition of scintillator to the samples.

29.10.3 Radioactivity Measurements

Radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Perkin Elmer TRI-CARB 2500 TR and T 2700), using an external standard device which permitted the counting efficiency to be determined by the channel ratio method.

The scintillator Permafluor E+ recommended by Perkin Elmer for their automatic combustors was used in the case of combusted samples. The scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany) was used for the samples measured directly.

Blank values were concurrently measured in the studies and deducted from those measured. These blank values originated from identical material from untreated animals.

The resulting mean concentrations (bars indicate SD) are given in Fig. 29.10.

The pharmacokinetic values in the blood and milk given in Table 29.8 were calculated by the pharmacokinetics' software WinNonlin, using the mean concentrations, a noncompartmental model, and the linear trapezoidal rule.

Already at the first measuring time (30 min after dosing), the concentrations in milk were considerably higher than the corresponding levels in blood in all

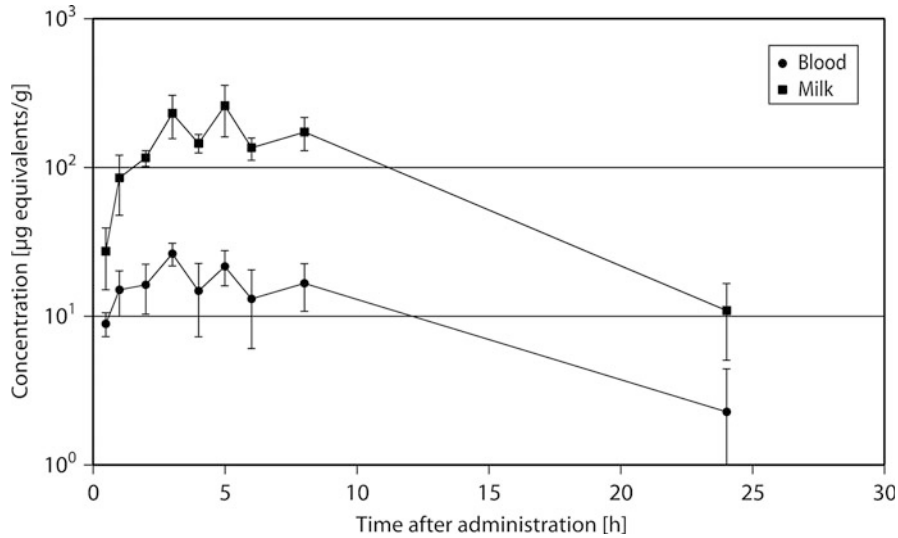

Fig. 29.10 ^{14}C-HWA 486: concentration-time profile of blood and milk from lactating rats

Table 29.8

Parameters	Blood	Milk
C_{max} (µg equiv./g)	26.3	252.9
t_{max} (h)	3	5
$t_{1/2}$(h)	6.4	4.4
$Conc._{24\ h}$ (µg equiv./g)	2.28	10.8
AUC (µg equiv./g × h)	282.4	2,635.5
AUC_{inf} (µg equiv./g × h)	303.5	2,704.2

animals examined. The mean values amounted to 27.01 µg equiv./g in milk and 8.85 µg equiv./g in blood. This indicates a rapid penetration of the compound into the mammary gland and a rapid onset of the elimination via the milk.

The highest mean concentrations in the blood were observed at 3 h after dosing amounting to 26.3 µg equiv./g. In the milk, the detected mean C_{max} concentrations were approximately nine times higher and amounted to 252.9 µg equiv./g. They were present at 5 h after dosing. At all measuring times, the concentrations in blood were considerably lower than in the milk, indicating an accumulation of the compound and/or its metabolites in milk.

The concentrations in the milk and in the blood decreased after reaching of the C_{max} concentrations in a similar way and speed. The calculated half-lives (from C_{max} to 24 h after dosing) amounted to 4.4 h for the elimination from the milk and 6.4 h for the elimination from the blood, indicating a continuous providing of new radioactivity from the blood to the milk. There was no retention in the mammary gland longer than in the blood. A rough estimation of the amount of radioactivity administered excreted via the milk amounted to >25%.

Analogous to the C_{max} concentrations, the calculated AUC values in the milk were approximately 9–10 times higher than those in the blood.

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Bioanalytical Assays: Gas Chromatography (GC)

30

Dietmar Schmidt

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30.1 Introduction

The quantitative determination of analytes in biological matrices such as blood and urine is called bioanalysis. Bioanalysis regulatory authorities and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance requires that the concentration-time profiles of drugs and/or metabolites in man or in animals are studied so their respective pharmacokinetics can be calculated and used as a basis for the evaluation of preclinical (especially toxicological) and clinical studies.

Gas chromatography (GC) is a chromatographic technique that is used to separate volatile organic compounds. A gas chromatograph consists of a mobile (gas) phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary phase in the column. Mobile phases are generally inert gases such as hydrogen, helium, argon/methane, or nitrogen. The upper part of the injection port is sealed with a rubber septum through which a syringe needle is inserted to inject the sample. The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture. Since the partitioning behavior is dependent on temperature, the separation column is positioned in a programmable, thermostat-controlled oven. Starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point, components accomplish separation of analytes showing a wide range of boiling points.

Gas chromatography columns are of two different designs: either packed or capillary. Packed columns are typically glass or stainless steel coils (typically 1–5 m total length and about 5 mm inner diameter) that are filled with the stationary phase. Capillary columns are thin fused-silica (purified silicate glass) capillaries (typically 5–50 m in length and 250 μm inner diameter) that have the stationary phase coated on the inner surface. Capillary columns provide much higher separation efficiency (a typical 25-m wall-coated open tubular GC column gives about 50,000 theoretical plates[1]) than packed columns (an average packed column of 5 m has only 5,000 theoretical plates), but are more easily overloaded by too much sample.

After the components of a mixture are separated using gas chromatography, they must be detected as they exit the GC column. There exist a number of detectors, which can be used in gas chromatography. Different detectors give different types of selectivity: *Nonselective* detectors like the thermal-conductivity (TCD) detector or the flame-ionization (FID) detector, which are the two most common detectors in gas chromatography, respond to all (organic) compounds except the carrier gas. On the contrary, a *selective detector* responds to a range of compounds with a common physical or chemical property. Representatives of the latter group of detectors are the nitrogen–phosphorus detector (NPD), the electron-capture detector (ECD), the mass-selective detector (MSD), and—last, but not least—the tandem mass spectrometer (MS/MS).

However, gas chromatography is not usually the method of choice in the bioanalytical field because it requires the vaporization of the analytes, and—unfortunately—most analytes in bioanalysis are not very volatile. This low volatility may result from the sheer size of the molecules and thus from large dispersion forces holding the molecules together (Knapp 1979). For smaller molecules (as for the many drug candidates), the observed low volatility may result from strong intermolecular attraction between polar groups (e.g., N − H, O − H, and S − H groups that can undergo hydrogen bonding).

It is beyond the scope of this chapter to describe the theory of GC; for a detailed description, the reader is referred to one of the many textbooks devoted to this topic, but the textbook *Analytical Gas Chromatography* (Jennings 1987) with its detailed discussion of capillary GC is strongly recommended.

As mentioned earlier, given the limitations of GC, alternative methods such as high-performance liquid chromatography (HPLC) with ultraviolet (UV) or with fluorescence (FL) detection and—particularly in the past few years —high-pressure liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) have evolved as the major bioanalytical techniques for the bioanalysis of analytes in biological

[1]The number of theoretical plates is the number of discrete distillations that would have to be performed to obtain an equivalent separation. This number is commonly used as a measure of separation efficiency and is a valuable number to use when comparing the performance of various chromatographic columns.

matrices. Nevertheless, due to the inherent advantages of modern fused-silica capillary columns in terms of resolution, inertness, and detection limits, in combination with different detectors like the flame-ionization detector (FID), the nitrogen–phosphorus detector (NPD), the electron-capture detector (ECD), the mass-selective detector (MSD), or even the tandem mass spectrometer (MS/MS), GC continues to play a role in modern bioanalytics (Venn 2000, p. 131). An example of the implementation of each of the above-mentioned GC detectors will be discussed in this chapter but necessarily not in the referred order.

To overcome the already mentioned low volatility of a number of analytes, one approach has been to generate more volatile derivatives before the actual bioanalysis. Although derivatization cannot increase the volatility of large molecules, for smaller molecules, masking of polar groups by derivatization can yield dramatic increases in volatility. Polar N − H, O − H, and S − H groups that can undergo hydrogen bonding contribute significantly to intermolecular attraction and thus low volatility. Replacement of hydrogen in these groups by alkylation, acylation, or silylation significantly increases volatility, especially in compounds with multiple polar groups. The monosaccharides are a prime example of a group of relatively low molecular weight compounds that exhibit low volatility even up to temperatures at which they begin to decompose. Replacement of the active hydrogens with trimethylsilyl groups yields volatile products that readily undergo GC analysis (Knapp 1979). Carboxylic acids are another molecule class, which can easily be derivatized using diazomethane (CH_2N_2) to yield the respective methyl esters, which often are volatile enough to allow the use of GC. In 1984, Hajdù et al. introduced a specific GC assay, which allowed the rapid analysis of ramipril, an angiotensin-converting enzyme (ACE) inhibitor in human urine. To our knowledge, this was the first time that an ACE-inhibitor bioanalysis was performed directly using GC and not by either radioimmunoassay (RIA) (Hitchens et al. 1981; Ribeiro et al. 1996) or a discontinuous enzyme assay (Horiuchi et al. 1982; Tocco et al. 1982).

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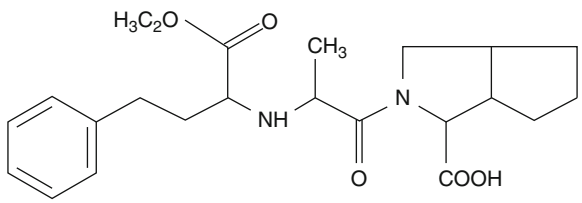
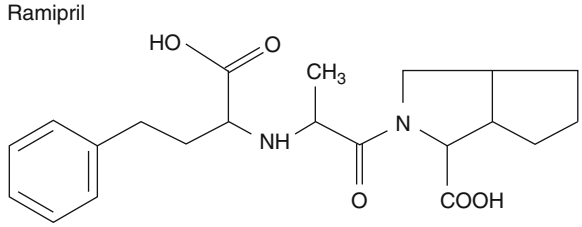
30.2 Urinary Analysis of Ramipril Using Gas Chromatography with Nitrogen–Phosphorus Detection (GC-NPD)

PURPOSE AND RATIONALE

This assay is used for the quantitative determination of the angiotensin-converting enzyme (ACE) inhibitor ramipril, (2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2-azabicyclo [3.3.0]octane-3-carboxylic acid), and its pharmacologically active metabolite, ramiprilat, in human urine using capillary gas chromatography with nitrogen-specific detection (NPD).



Ramipril



Ramiprilat

Note: The nitrogen–phosphorus detector responds to nitrogen–phosphorus compounds about 100,000 times more strongly than normal hydrocarbons. Due to this high degree of selectivity, the NPD is commonly used to detect pesticides, herbicides, and drugs.

The NPD is similar in design to the FID (flame-ionization detector), except that the hydrogen flow rate is reduced to about 3 mL/min, and an electrically heated thermionic bead (NPD bead) is positioned near the column orifice. Nitrogen or phosphorus containing molecules exiting the column collides with the hot bead and undergo a catalytic surface chemistry reaction. The resulting ions are attracted to a collector electrode, amplified, and output to the data system. The NPD is 10–100 times more sensitive than FID.

30.2.1 Reagents

Ramipril, ramiprilat, and the internal standard were supplied by Hoechst AG[2] (Frankfurt, Germany). Analytical grade reagents were used at all times; methanol and chloroform were redistilled before use. Vac Elut and Bond Elut C18 and Si extraction columns are proprietary products manufactured by Varian Incorporated (Harbor City, CA, USA) and obtained from ICT (Frankfurt/Main, Germany). Dimethyldichlorosilane (DMCS) and trifluoroacetic anhydride (TFAA) were purchased from Macherey-Nagel & Co. (Düren, FR Germany) and used without further purification. N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald), for diazomethane generation, was supplied by Aldrich (Milwaukee, WI, USA). As internal standard, 2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-spiro-[5,6]-2-azadecane-3-carboxylic acid was used.

- Buffer solution pH = 1.0: 3.73 g KCl and 134 mL 0.1 mol/L HCl were filled up to 1 L with redistilled water.
- Water pH = 3.3: Redistilled water was adjusted to pH = 3.3 with buffer solution 1.0.
- Elution mixture: Redistilled chloroform and methanol were mixed in the ratio 2:1 (v/v).
- n-Hexane/toluene: n-Hexane and toluene were mixed in the ratio 1:3 (v/v).
- Diazomethane: Ethereal diazomethane solution was prepared by the diazomethane generator kit.

[2]Successor company: Aventis Pharma Deutschland GmbH.

30.2.2 Assay

The need to isolate the analytes from the biological matrix in a form suitable for GC bioanalysis constitutes the most labor-intensive step. The isolation procedure is largely independent of the chromatographic technique, i.e., is not specific to the employed GC method. The only GC-specific requirement is that the final extract is obtained in a relatively volatile solvent and, because of injection volume constraints, in the smallest practical volume (about 50 µL). For the extraction of ramipril and of ramiprilat from urine, disposable C18 columns were used. After derivatization with diazomethane (Albro and Fishbein 1969), the sample was purified by the use of a Si column, to eliminate a number of endogenous impurities from urine, which are not removed during purification over the C18 column. After a second derivatization step with trifluoroacetic anhydride (Walle et al. 1972), both compounds were analyzed in form of their methyl ester-, trifluoroacetyl derivatives by capillary gas chromatography using a nitrogen-specific detector.

PROCEDURE

1. To prevent adsorption of the compounds at the glass surface, the centrifuge tubes are treated with 2 mL n-hexane/DMCS (5%) for at least 10 min prior to use, rinsed with methanol, and subsequently dried.
2. To 1 mL urine in a centrifuge tube, 2 µg internal standard dissolved in 50 µL redistilled water are added and then adjusted to pH = 1.4–1.5 with 0.1 mol/L HCl.
3. The sample thus prepared is applied onto a C18 column—which has been preconditioned with methanol (6 mL) followed by redistilled water (6 mL) and 0.1 mol/L HCl (3 mL)—and slowly sucked through under vacuum (using the Vac Elut System at about 20-kPa pressure drop).
4. Subsequently, the column is washed with 4 × 3 mL redistilled water at the same reduced pressure.
5. The pressure drop is increased to 50–60 kPa, and the column is sucked dry for 10 min.
6. The column is placed into a centrifuge tube, and the sample is eluted with 2 × 0.75 mL elution mixture.
7. The column is dried by centrifuging at 4,000 rpm for 1 min, whereby the entire eluate is collected in the tube.

8. The eluate is methylated by adding 0.5 mL ethereal diazomethane solution at room temperature for about 10 min and subsequently evaporated to dryness at 40°C under N_2 gas.
9. The residue is taken up in 100 µL methanol followed by adding 1 mL n-hexane/toluene.
10. The sample is applied onto a Si column—which has been preconditioned with methanol (5 mL) followed by chloroform (5 mL) and n-hexane (5 mL)—and slowly sucked through under vacuum (20-kPa pressure drop).
11. The pressure drop is increased to 50–60 kPa, and the column is sucked dry.
12. The column is placed into a conically tapered centrifuge tube, and the sample is eluted with 2 × 2 mL chloroform.
13. The eluate is evaporated to dryness at 60°C under N_2 gas.
14. The residue is taken up in 250 µL ethyl acetate, treated with 1 mL n-hexane/TFAA (5%).
15. For derivatization, the stoppered tube is placed into a heating block of 60°C for about 30 min.
16. Subsequently, the sample is evaporated to dryness at 60°C under N_2 gas and taken up in 50 µL toluene, 2 µL of which is injected.

30.2.3 GC System

Hewlett–Packard 5880A gas chromatograph with nitrogen-specific detector (NPD), (Hewlett–Packard, Palo Alto, CA, USA)

Column: Cross-linked fused-silica capillary column, Hewlett–Packard OV 101, 12 m × 0.21 mm inner diameter (ID)

Injection: Split mode
Gases: Carrier gas (helium), inlet pressure 10 psi
Split ratio: 1:20 mL/min
Septum rinsing (helium): 3 mL/min
Detector makeup (helium): 20 mL/min
Detector (hydrogen): 3 mL/min
Detector (air): 60 mL/min
Temperatures: Injection port 250°C
Detector: 300°C
Oven program:
Initial temperature: 160°C
Heating rate: 16°C/min
Final temperature: 260°C (4 min)
Detection: NPD detector
Sample size: 2 µL
Analysis time: About 12 min per sample

EVALUATION

Ramipril and ramiprilat were admixed with human urine in concentrations of 0.050–10,000 µg/mL. The urines were divided into five aliquots and analyzed.

Precision: The standard deviation (SD) is used as a measure of precision; the respective data are as follows:

For values less than 1,000 µg/mL, the precision was 2.4% of the measured value for ramipril and constant 0.003 µg/mL for ramiprilat.

From 1,000 µg/mL upward, the precision can be formally divided into an absolute and a relative error term:

Ramipril: 0.01 ± 2.1% of the measured value
Ramiprilat: 0.033 ± 1.8% of the measured value

Accuracy: The bias served as measure. The largest deviation was for ramipril, 8.0% relatively; for ramiprilat, 13.2% relatively.

Linearity: In the measuring range given, the results obtained by this method were linear.

Limit of detection: About 0.020 µg/mL was found as limit of detection. This corresponds to a signal-to-noise ratio of 3:1.

Specificity: The method is specific and permits simultaneous determination of both compounds. Even a tenfold excess of one compound does not impair the results.

CRITICAL ASSESSMENT OF THE METHOD

The NPD can detect compounds like drugs that contain nitrogen or phosphorus in the ppb range. However, the NPD requires regular maintenance; reduced sensitivity often indicated the depletion of the active element on the thermoionic bead.

The NPD is a destructive detector that can be used in series only after nondestructive detectors (e.g., ECD). The NPD is sensitive to water that affects the condition of the thermoionic bead. The active element of the bead eventually will become depleted (especially when using halogenated solvents like dichloromethane, chloroform, etc.) and requires replacement.

MODIFICATION OF THE METHOD

Due to the discovery of two further urinary metabolites of ramipril (the respective diketopiperazine derivatives of ramipril and ramiprilat), the above-described method was slightly modified (Schmidt and Keller 1985). Instead of the rather time-consuming second

extraction step by means of a disposable Si column, the sample is cleaned by a liquid/liquid extraction step. After methylation of the compounds with diazomethane, the eluate is evaporated to dryness at 40°C under N_2 gas. Subsequently, the residue is dissolved in n-pentane/diethyl ether (3:2. v/v) and washed with 5% hydrogen carbonate solution. After separation of the upper organic layer, this is evaporated to dryness at 40°C under N_2 gas and then treated with 1 mL n-hexane/TFAA (5%) as described before (Hajdù et al. 1984). This method allows the selective determination of ramipril and its three metabolites in human urine; the limit of quantification amounted to 0.020 µg/mL for each of all four analytes. Using this assay, thousands of urine samples originating from phase I–III clinical studies were analyzed.

In the following assay, the application of capillary gas chromatography for the determination of another ACE inhibitor, but this time in human plasma and with mass-selective detection, is described.

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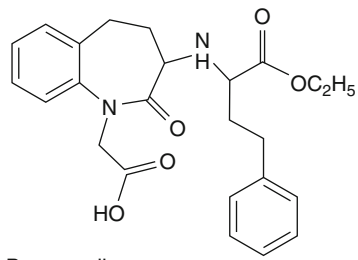
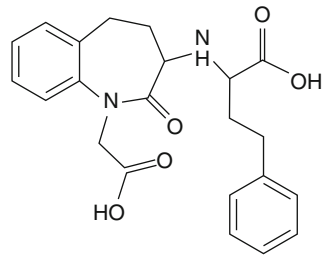
30.3 Plasma Analysis of Benazepril Using Gas Chromatography with Mass-Selective Detection (GC-MSD)

PURPOSE AND RATIONALE

This assay is used for the quantitative determination of benazepril and its active metabolite, benazeprilat, in human plasma by capillary gas chromatography–mass-selective detection (Pommier et al. 2003). Benazepril hydrochloride, 3-{[1-ethoxycarbonyl-3-phenyl-(1S)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3S)-benzazepine-1-acetic acid hydrochloride, is a prodrug-type angiotensin-converting enzyme (ACE) inhibitor which, on absorption, is hydrolyzed to a pharmacologically active metabolite, the dicarboxylic acid (benazeprilat).



Benazepril



Benazeprilat

Note: Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized atoms or molecules to separate them from each other. Mass spectrometry (MS) (or mass-selective detection) is therefore useful for quantitation of atoms or molecules and also for determining chemical and structural information about molecules. Molecules have distinctive fragmentation patterns that provide structural information to identify structural components.

The general operation of a mass spectrometer is:

- Create gas-phase ions
- Separate the ions in space or time-based on their mass-to-charge ratio
- Measure the quantity of ions of each mass-to-charge ratio

30.3.1 Reagents

Benazepril, benazeprilat, and the corresponding deuterium-labeled internal standards (IS) were supplied by Novartis (Basle, Switzerland). All the chemicals were of analytical grade. Hexane and toluene (Pestipur SDS) were obtained from Solvants Documentation Synthèse (Pépin, France). Methyl

tert-butyl ether and 2 mol/L trimethylsilyldiazomethane solution in hexane were purchased from Fluka (Saint-Quentin Fallavier, France). Hydrochloric acid (0.1 mol/L), 0.5 mol/L sulfuric acid, and sodium carbonate were from Merck (Darmstadt, Germany). 3M Empore C18-SD 96-well disk plates containing 12 mg C18 were obtained from Varian (Les Ulis, France).

30.3.2 Assay

An analytical method for the determination of benazepril and its active metabolite, benazeprilat, in human plasma by capillary GC with mass-selective detection was developed and validated according to international regulatory requirements (Guidance for Industry 2001; Shah et al. 2000). After addition of the internal standards, the compounds were extracted from plasma by solid-phase extraction using automated 96-well plate technology. Unlike earlier published gas chromatography–mass spectrometry (GC-MS) methods (Kaiser et al. 1987; Sioufi et al. 1988), the present method halved the volume of plasma (0.5 mL instead of 1 mL formerly required) without diminishing the lower limit of quantification (LLOQ) and reduced considerably the volume of solvent for conditioning, washing, and eluting. After elution, the compounds were converted into their methyl ester derivatives by means of a safe and stable diazomethane derivative (Blau and Halket 1993; Rimmer et al. 1996), trimethylsilyldiazomethane solution. The methyl ester derivatives were determined by mass-selective detection at m/z 365 for benazepril and benazeprilat and m/z 370 for D_5-benazepril and D_5-benazeprilat.

PROCEDURE

1. To 0.5 mL plasma in a polypropylene tube, successively 100 µL of the internal standard solution (150 ng) and 100 µL of 0.1 mol/L hydrochloric acid are added.
2. All tubes are then placed on the platform of the Packard MultiPROBE II, and a 96-well disk plate is placed on top of a vacuum manifold.
3. The 96-well disk plate is conditioned automatically with 100 µL of methanol, and 300 and 80 µL of 0.1 mol/L HCl.
4. Then, 500 µL of the prepared samples is loaded onto the 96-well disk plate.
5. The loaded samples are washed with 100 µL of water, and then, the analytes and the internal standards are eluted twice with 500 µL of methanol.
6. The eluates are transferred into extraction tubes, and 300 µL of trimethylsilyldiazomethane solution are added.
7. The reaction runs at room temperature in a dry bath for 30 min, and the reaction mixture is evaporated to dryness under N_2 gas at 40°C.
8. To the dry derivatized plasma sample, add 0.5 mL of 0.5 mol/L sulfuric acid and 1 mL of hexane.
9. The mixture is shaken mechanically for 5 min at 240 rpm and centrifuged at 1,600*g* for 2 min.
10. The upper organic phase is discarded, and the aqueous phase is alkalized with 1 mL of 2 mol/L sodium carbonate and shaken with 2 mL methyl *tert*-butyl ether for 5 min at 240 rpm.
11. After centrifugation at 1,600 g for 5 min, the organic phase is separated and evaporated to dryness under nitrogen at 40°C.
12. The residue is dissolved in 100 µL toluene, 2 µL of which is injected onto the gas chromatograph.

30.3.3 Robotic System

For sample preparation, a Packard MultiPROBE II liquid handling robotic system was used (Packard Instruments, Meriden, CT, USA) using the WinPREP software.

30.3.4 GC System

Hewlett–Packard 5890 Series II gas chromatograph equipped with a capillary inlet system and an HP 7673 automatic sampler was used (Hewlett–Packard, Palo Alto, CA, USA).

Column: Fused-silica capillary column, Restek Rtx-1, 15 m × 0.25 mm inner diameter (ID); (Restek, Evry, France)

Injection: Splitless mode, 30 s splitless period

Gases: Carrier gas (helium); inlet pressure 8 psi

Split flow: 1:50 mL/min

Septum rinsing (helium): 3 mL/min

Temperatures: Injection port 280°C

Detector: 300°C

Oven program:

Initial temperature: 190°C (0.5 min)

Heating rate: 30°C/min

Final temperature: 290°C

Detection: Hewlett–Packard 5970B mass-selective detector (MSD), the MSD was maintained at 280°C

The detector was turned on from 3.5 to 5 min after injection. The selected ions monitored for the methyl ester derivatives were m/z 365 for benazepril and benazeprilat and m/z 370 for D_5-benazepril and D_5-benazeprilat. These fragments are obtained by cleavage of the carboxyethyl [M–73] or carboxymethyl group [M–59] in the side chain.

Sample size: 2 μL

Analysis time: About 6 min per sample

EVALUATION

Daily calibration standards were prepared at six different concentrations, in duplicate, in the range of 2.5–1,000 ng/mL for benazepril and benazeprilat. Calibration curves ($y = ax + b$) were presented by plots of the peak area ratios (y) of the methyl ester derivative of benazepril or benazeprilat to the methyl ester derivative of the I.S. versus the concentration (x) of the calibration standards and were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. Concentrations in quality control (QC) samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve. Inter-day repeatability was determined on three different days. Good agreement between the nominal and the back-calculated concentration for calibration samples was observed. The precision ranged from 2.1% to 7.4%, and mean accuracies were within 8% of the nominal value for the two compounds.

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest (2.50 ng/mL), near the lowest (7.50 ng/mL), near the middle (200 ng/mL), and the highest (900 ng/mL) concentrations of the calibration range. Accuracy was determined by calculating the mean recovery for the determined concentrations in standard samples. Precision was assessed from the relative standard deviation (RSD) as a percentage of the mean recovery. The following validation criteria for accuracy and precision were used to assess method suitability: mean recoveries should be within 85–115%, except at the lower limit of quantification (LLOQ), where it should not exceed 20% (Guidance for Industry 2001). Series of five quality control samples were prepared at four different concentrations in the range of 2.50–900 ng/mL (LLOQ) for benazepril and benazeprilat, by spiking drug-free plasma with the corresponding working solutions. It could be shown that the results met the acceptance criteria.

CRITICAL ASSESSMENT OF THE METHOD

The latter assay has several advantages in comparison to the first (which certainly is somewhat older):

The mass-selective detector is more specific and allows a lower limit of quantification than the nitrogen–phosphorus detector (NPD).

The use of trimethylsilyldiazomethane with methanol provides a less hazardous method for preparing methyl esters under mild conditions than ethereal diazomethane solution. Trimethylsilyldiazomethane is commercially available, obviating the need to synthesize diazomethane daily. It is a stable and safe substitute for either hazardous diazomethane or corrosive reagents containing boron trifluoride.

The procedure has a good throughput, with a combination of automated sample extraction with a 96-well disk plate using a Packard MultiPROBE II roboter.

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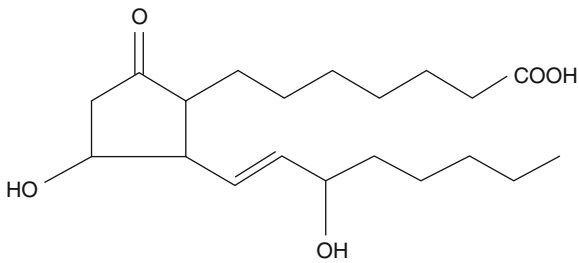
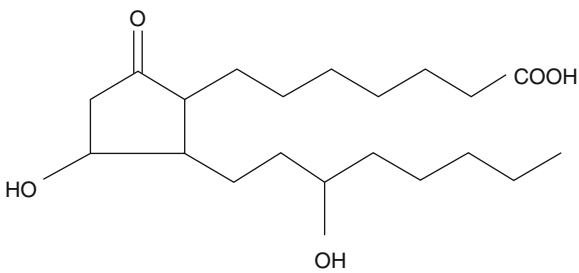
30.4 Simultaneous Determination of Different Prostaglandins in Human Plasma Using Gas Chromatography/Negative-Ion Chemical-Ionization/Tandem Mass Spectrometry (GC-NICI-MS/MS)

PURPOSE AND RATIONALE

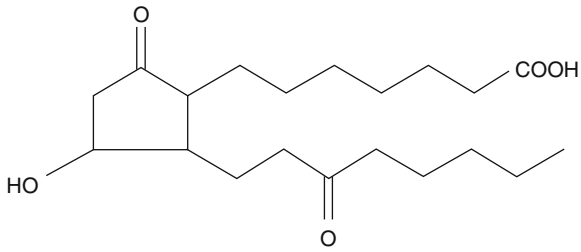
Prostaglandin E_1 (PGE_1) has been used therapeutically for the intravenous treatment of peripheral arterial occlusive disease (Hirai and Nakayama 1986) and of erectile dysfunction as an intracavernous (i.c.) injection (Porst 1996). PGE_1 is rapidly converted to 15-keto-prostaglandin E_0 (15-keto-PGE_0), the major circulating metabolite, and prostaglandin E_0 (PGE_0), a metabolite with activity comparable with PGE_1. The following assay is used for the sensitive (in the low pg range) and selective simultaneous routine determination of prostaglandin E_1, prostaglandin E_0 and 15-keto-prostaglandin E_0, in human plasma by capillary gas chromatography/negative-ion chemical-ionization/tandem mass spectrometry (GC-NICI-MS/MS) (Hammes et al. 1999).



Prostaglandin E_1 (PGE_1)



Prostaglandin E_0 (PGE_0)



15-keto-Prostaglandin E_0 (15-keto-PGE_0)

Note: Tandem mass spectroscopy has become to mean the mass spectrum of a mass spectrum, hence, *MS/MS*. Essentially in MS/MS, mass analyzers are coupled together via an interface known as a collision cell. Ions of a selected mass (precursor ions) are transmitted by the first mass analyzer into the collision cell. Here, they collide with the neutral atoms of an inert gas; generally, argon is used. In this process, a fraction of the translational energy inherent in the precursor ion is converted into internal energy. The internal energy is lost by the precursor ion decomposing or fragmenting into product ions. The product ions are transmitted through a second mass analyzer and subsequently determined quantitatively (Venn 2000, p. 256).

30.4.1 Reagents

PGE_1 was purchased from Acros Organics (St. Augustin, Germany), PGE_0 and 15-keto-PGE_0 from Cascade Biochem (Reading, UK). The deuterated internal standards D_6-PGE_1, D_4-PGE_0 and D_6-15-keto-PGE_0 were synthesized by the Chemistry Department (Schwarz Pharma, Monheim/Rhein, Germany). Ethyl acetate, acetonitrile, dichloromethane, hexane, and methanol were obtained from Promochem (Wesel, Germany); ethanol and formic acid from Merck (Darmstadt, Germany); and pentafluorobenzyl bromide (PFBBr), N, N-diisopropylethylamine (DIPEA), MOX reagent (2% methoxyamine hydrochloride in pyridine), and N, O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) from Pierce (Oud Beijerland, Netherlands). Indomethacin was from Sigma (Deisenhofen, Germany) and Biostabil from Biotrans (Dreieich, Germany). All chemicals were of the highest grade available and used without further purification. Bond Elut C18/Si cartridges were obtained from ICT (Frankfurt/Main, Germany). Water was purified with the NANOpure system delivered by Werner (B.-Gladbach, Germany).

30.4.2 Assay

An analytical method for the determination of PGE_1, PGE_0 and 15-keto-PGE_0 in human plasma by capillary GC with tandem mass spectrometry was developed and validated. After addition of the deuterated internal standards, the compounds were extracted from plasma by solid-phase extraction using C18 cartridges, followed by derivatization to the pentafluorobenzyl (PFB) ester methoxime. After evaporation of the derivatization reagents to dryness, the samples were purified on Bond Elut Si cartridges and converted to their trimethylsilyl (TMS) ethers. Quantitation was achieved by gas chromatography/negative-ion chemical-ionization/tandem mass spectrometry. The lower limit of quantification (LLOQ) was 2 pg/mL for PGE_1 and PGE_0, and 10 pg/mL for 15-keto-PGE_0, extracted from 2 mL of human plasma.

PROCEDURE

1. To 2 mL plasma in a centrifuge tube, 200 pg D_6-PGE_1/D_4-PGE_0 and 500 pg D_6-15-keto-PGE_0 as internal standards are added to the sample and then adjusted to pH = 3.0–3.5 with 200 μL 3% formic acid.
2. The sample thus prepared is applied onto a C18 column—which has been preconditioned with methanol (6 mL) followed by redistilled water (6 mL)—and slowly sucked through under vacuum.
3. Subsequently, the column is washed with 3 mL redistilled water and with 6 mL hexane.
4. The pressure drop is increased to 80 kPa, and the column is sucked dry for 10 min.
5. The analytes are eluted[3] with 3 mL ethyl acetate.
6. The eluate is evaporated to dryness at room temperature.
7. The residue is incubated with PFBBr (10 μL) and DIPEA (20 μL) in acetonitrile (70 μL) for 10 min at 40°C.
8. The sample is evaporated to dryness and converted to the methoxime with 200 μL of MOX reagent for 30 min at 70°C.
9. After evaporation to dryness, the sample is transferred three times by 1 mL dichloromethane to a Bond Elut Si cartridge, prewashed with 6 mL of dichloromethane.
10. The cartridge is washed with ethyl acetate-dichloromethane (5:95, v/v).
11. The analytes are eluted with 3 mL of ethyl acetate.
12. The extract is incubated with 100 μL of BSTFA for 60 min at 50°C.
13. It is evaporated to dryness at room temperature.
14. Subsequently, the sample is taken up in 40 μL of BSTFA, 1 μL of which is injected.

[3]Recovery over the concentration range validated: 95–100% for PGE_1, 86–89% for PGE_0, and 91–93% for 15-keto-PGE_0.

30.4.3 GC-MS/MS System

Finnigan TSQ 700 triple-stage mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with a Hewlett–Packard HP 5890 Series II gas chromatograph, an HP 7673A autosampler (Hewlett–Packard, Palo Alto, CA, USA), and a temperature-programmable split/splitless injector (Gerstel, Mühlheim/Ruhr, Germany).

Column: Fused-silica capillary column, Hewlett–Packard Ultra 2, 12 m × 0.20 mm inner diameter (ID)

Injection: Splitless mode

Gases: Carrier gas (helium); inlet pressure 30 kPa

Reagent gas (methane); ion source pressure 120 Pa

Collision gas (argon); collision cell pressure 0.2 Pa

Temperatures: Injection port (programmed) from 150°C to 300°C at 10°C/s (held for 5 min)

Oven program:

Initial temperature: 150°C (held for 0.1 min)

Heating rate: 40°C/min

Final temperature: 300°C (7 min)

The transfer line, the manifold, and the ion source were kept at 300°C, 70°C, and 150°C, respectively.

Detection: The mass spectrometer was operated in the negative-ion mode with an emission current of 300 μA and an electron energy of 85 eV

Sample size: 1 μL

Analysis time: About 9 min per sample

For quantitation in the multiple ion detection (MID) mode, the precursor → product ions monitored in the negative-ion mode were m/z 626.4 → m/z 346.2 = $[P–2(CH_3)_3SiOH]^-$ion for PGE_1, m/z 483.3 → m/z 393.2 $[P–(CH_3)_3SiOH]^-$ion for 15-keto-PGE_0, and m/z 528.4 → m/z 438.2 = $[P–(CH_3)_3SiOH]^-$ ion for PGE_0.

EVALUATION

Linear calibration curves were obtained over the concentration range 2–100 pg/mL (PGE_1 and PGE_0) and 10–500 pg/mL (15-keto-PGE_0) of human plasma. The LLOQ (lower limit of quantitation) of the assay, i.e., the concentration with an accuracy and a precision $\leq$20%, was 2 pg/mL (PGE_1 and PGE_0) and 10 pg/mL (15-keto-PGE_0). The precision and the accuracy of the method were determined by analysis of blank human plasma (2 mL) spiked with PGE_1/PGE_0 in the concentration range 4–200 pg and with 15-keto-PGE_0 in the concentration range 20–1,000 pg. In any case, both the precision and the accuracy were <17% and indicated good reproducibility. The method has been applied successfully for the determination of PGE_1, PGE_0, and 15-keto-PGE_0 in human plasma after a 2-h IV infusion of 60 µg of PGE_1in order to investigate the pharmacokinetics of PGE_1, PGE_0, and 15-keto-PGE_0 in healthy volunteers.

CRITICAL ASSESSMENT OF THE METHOD

A highly selective and sensitive routine method for the simultaneous determination of PGE_1, PGE_0, and 15-keto-PGE_0 in human plasma is described. Compared to the recently developed GC-NICI-MS/MS assay (Schweer et al. 1994), the described method with a modified purification step of the PFB ester methoxime derivatives by means of Bond Elut Si cartridges allows the processing of at least 24 samples per day applying the available solid-phase extraction unit.

References and Further Reading

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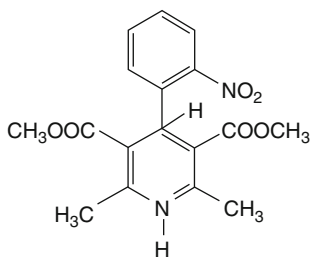
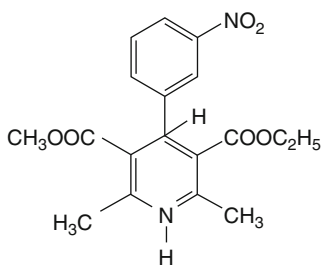
30.5 Determination of Calcium-Blocking Agents Using Gas Chromatography with Electron-Capture Detection (GC-ECD)

PURPOSE AND RATIONALE

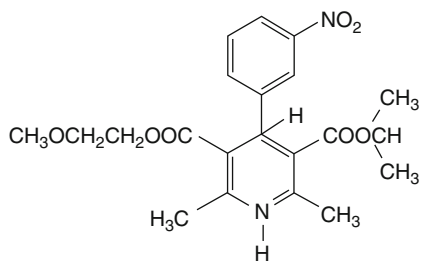
Nifedipine (NF), nitrendipine (NT), and nimodipine (NM) belong to the dihydropyridine group of calcium-blocking agents. The main indications are treatment of angina pectoris and for the treatment of arterial hypertension (Krebs 1982); the main indication for NT is the treatment of hypertension (Stoepel et al. 1981). NM acts mainly on cerebral vessels (Langley and Sorkin 1989). The assay was developed by Rämsch et al. (1986) to investigate the pharmacokinetics and the metabolism of calcium-blocking agents like nifedipine (NF), nitrendipine (NT), and nimodipine (NM) using capillary gas chromatography with electron-capture detection (ECD).



nifedipine (NF)



nitrendipine (NT)



nimodipine (NM)

Note: The ECD detector consists of a sealed stainless steel cylinder containing radioactive ^{63}nickel. The ^{63}nickel element emits beta particles (electrons), which collide with the carrier gas molecules, ionizing them in the process. This forms a stable cloud of free electrons in the ECD cell. When organic molecules that contain electronegative functional groups, such as halogens, phosphorus, and nitro groups, pass by the detector, they capture some of the electrons and reduce the current measured between the electrodes. The ECD is by far more sensitive than the FID (flame-ionization detector) but has a limited dynamic range (about 10^4-fold) and finds its greatest application in analysis of halogenated compounds, like pesticides, herbicides, and drugs.

30.5.1 Reagents

The 1,4-dihydro-2,6-dimethyl-4-(2- or 3-nitrophenyl)-3,5-pyridine-dicarboxylate derivatives nifedipine (NF), nitrendipine (NT), and nimodipine (NM) were supplied by Bayer AG (Wuppertal, Germany). Analytical grade reagents were used at all times; toluene AR (Riedel-de-Haën AG, Seelze, Germany) was distilled over a 50-cm Vigreux column before use. Sodium hydroxide, 0.5 mol/L, was purchased from J.T.Baker Chemical Co. (USA), and the amber screw-cap autosampler bottles (2 mL) were obtained from Pierce Biotechnology Inc. (Rockford, IL, USA) and rinsed with acetone before use.

30.5.2 Assay

Nifedipine, nitrendipine, or nimodipine were extracted from alkalized plasma directly into toluene containing the respective internal standard: nitrendipine or nimodipine (NT for the quantification of NF and NM for the detection of NT). Thereby, it is important to take into account that the samples have to be protected from daylight and from fluorescent light to prevent formation of photodecomposition products (Ahnoff and Persson 1990; Le Guellec et al. 1992). Under the action of daylight and of fluorescent light, e.g., nifedipine is converted into the corresponding nitrosophenylpyridine (Testa et al. 1979).

Subsequently, the compounds were analyzed by capillary gas chromatography using an electron-capture detector.

PROCEDURE

1. To 0.5 mL plasma in a 2-mL autosampler bottle, 50 μL 0.5 mol/L NaOH and 1 mL toluene containing 50 ng of the respective internal standard (NT for the quantification of NF and NM, and NM for the detection of NT) are added.
2. The bottle is covered with a piece of aluminum foil instead of the usual silicon or rubber disk and closed with the screw cap.
3. Subsequently, the bottle is shaken for 5 min.
4. The bottle is transferred without opening—or any centrifugation—directly into the autosampler.
5. 2 μL of the upper toluene phase is injected into the gas chromatograph.

Using 300-μL micro-vials and one tenth of the reagents, 50 μL of plasma can be extracted to give the same sensitivity!

30.5.3 GC System

Hewlett–Packard 5840A gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD), an HP 7672A autosampler (Hewlett–Packard, Palo Alto, CA, USA), and a temperature-programmable split/splitless injector (Gerstel, Mühlheim/Ruhr, Germany). The needle tip of the autosampler dipped only into the upper third of a sample bottle to prevent the injection of any of the aqueous phase into the GC column.

Column: Fused-silica capillary column, Durabond DB-1 (0.1-μm film thickness), 30 m × 0.32 mm inner diameter (ID); (J & W Scientific, Folsom, CA, USA)

Injection: Splitless mode (1 min)

Gases: Carrier gas (helium), 3 mL/min

Electron-capture detector gas (argon/methane): 50 mL/min

Temperatures: Injection port 250°C

Detector: 300°C

Oven program:

Initial temperature: 160°C. Splitter closed; duration 1 min

Heating rate: 10°C/min

Final temperature: 270°C (20 min)

Detection: ECD detector
Sample size: 2 μL
Analysis time: About 32 min per sample

EVALUATION

The pharmacokinetics of the three dihydropyridine calcium blockers, nifedipine, nitrendipine, and nimodipine, was investigated using the GC assay presented above. The retention times of the three compounds amounted to 18.6 min for NF, 20.8 min for NT, and to 24.2 min for NM. The detection limits were at least 1 ng/mL, but for NT and for NM, limits of 100 pg/mL could also be achieved.

In spite of the structural and physiochemical similarity of the three compounds, they differ in their pharmacokinetic behavior. NF, a relatively polar molecule with the lowest volume of distribution and highest plasma levels in relation to dose, has the greatest bioavailability. On the other hand, NT and NM, with a threefold to fourfold lower solubility in water, have a low bioavailability, low plasma concentrations, and a larger volume of distribution (Rämsch et al. 1986).

CRITICAL ASSESSMENT OF THE METHOD

Analogous to the majority of dihydropyridines, nifedipine's, nitrendipine's, and nimodipine's chemical structures—a 2- or 3-nitrophenyl substituent in the 4-position combined with the dihydropyridine diester structure—results in a high response in electron-capture detection (ECD), thus allowing high detection sensitivity and sufficient assay specificity toward endogenous compounds, metabolites, or common co-medications (Mück and Bode 1994).

However, because it contains 5 mCi of ^{63}nickel, the ECD is covered by a "General License" requiring a periodic "wipe test" and the filing of a form with the respective state's Department of Health or with another adequate authority. Its advantages are (Venn 2000):

- It is selective for halogens, nitro groups, peroxides, and quinones.
- It is extremely sensitive.
- It is nondestructive (in contrast to the NPD, FID, or MS).

Its disadvantages are:

- A limited dynamic range, 10^4-fold.
- It is prone to contamination.
- It is radioactive; leakage must be checked regularly.

MODIFICATION OF THE METHOD

Since the calcium channel blockers are highly potent drugs, their concentration in human plasma and other biological fluids is generally low (high pg/mL to low ng/mL range) thus requiring analytical techniques of high sensitivity and specificity. The numerous publications, which have appeared until the end of the eighties describing various setups of gas and liquid chromatographic procedures, have been thoroughly reviewed by M. Ahnoff and Persson (1990). Their conclusion may be summarized that a simple liquid/liquid extraction followed by gas chromatography with electron-capture detection (GC-ECD) or mass spectrometric detection (GC-MS), preferably in the negative-ion chemical-ionization mode (NICI) (Fischer et al. 1986) is usually an adequate and efficient way to provide drug concentrations for pharmacokinetic evaluation and therapeutic drug monitoring. If not restricted due to its generally inferior limit of quantification, high-performance liquid chromatography with UV or amperometric detection (HPLC-UV resp. HPLC-ELCD) can be an attractive alternative for the quantification of dihydropyridines in biological fluids (Mück and Bode 1994).

However, as already mentioned earlier, high-pressure liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) has evolved in the last few years as the major bioanalytical technique for the bioanalysis of analytes in biological matrices. This is reflected also in a number of LC-MS/MS assays for the determination of dihydropyridine calcium antagonists in biological fluids (Carvalho et al. 2001; Schug et al. 2002; Kang et al. 2004).

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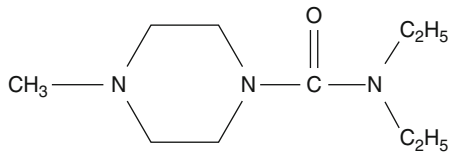
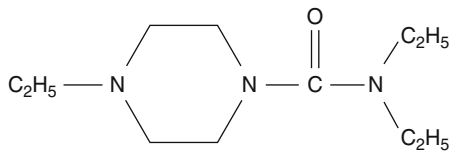
30.6 Determination of Diethylcarbamazine (DEC) Using Gas Chromatography with Flame-Ionization Detection (GC-FID)

PURPOSE AND RATIONALE

Single doses of diethylcarbamazine (DEC) in combination with albendazole have been found safe and efficacious for the treatment of *Brugia malayi* infection (Shenoy et al. 1999), and single-dose treatment of DEC with ivermectin is effective against adult *Wuchereria bancrofti* (Dreyer et al. 1998). However, since DEC is a compound that lacks a chromophore, spectroscopic methods of analysis that utilize chromophores, like HPLC with UV detection, are not suitable for the bioanalytical determination of the compound. In order to achieve the sensitivity and specificity for the determination of DEC in plasma, a number of gas chromatographic methods have been developed (Bogan 1977; Nene et al. 1984; Lee et al. 1997), partly with nitrogen phosphorus detection. To overcome the regular maintenance that is required for the nitrogen–phosphorus detector (NPD), which made it difficult to apply the method to clinical pharmacokinetic studies with high-sample throughput requirements, a sufficient sensitive, selective, accurate, and reproducible gas chromatographic assay using flame-ionization detection (FID) for the determination of DEC in human plasma was reported in 2001 by Miller and Fleckenstein (2001).



diethylcarbamazine (DEC)



1-diethylcarbamyl-4-ethyl-piperazine (IS)

Note: The FID detector was the first successful universal GC detector to be developed and remains the most widely used. The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons, which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. The FID is a useful general detector for the analysis of organic compounds[4]; it has high sensitivity, a large linear response range, a low noise, and it shows a similar response for most analytes. It is generally robust and easy to operate, but because it uses a hydrogen diffusion flame to ionize compounds for analysis, it destroys the sample in the process.

30.6.1 Reagents

All solvents and chemicals were HPLC grade. Organic solvents, sodium carbonate (anhydrous), and sodium

[4]It responds to any molecule with a carbon–hydrogen bond, but not at all or poorly to compounds such as H_2S, CCl_4, or NH_3.

bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Carbonate buffer, pH = 10.0, was prepared using the sodium carbonate (anhydrous), and sodium bicarbonate. A solution of 0.1% triethylamine in methanol was made for the elution solvent. Diethylcarbamazine citrate was obtained from Sigma and used to make stock solutions (St. Louis, MO, USA). The internal standard, 1-diethylcarbamyl-4-ethylpiperazine (E-DEC), was synthesized by the Division of Medicinal and Natural Products Chemistry at the University of Iowa, College of Pharmacy. Ultrapure analytical grade type I water was produced by a Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA). For the extraction of DEC and of its internal standard, Alltech Extract, Clean C18 cartridges, 500 mg with a 2.8 mL reservoir, and an SPE vacuum manifold (Alltech, Deerfield, IL, USA) were used.

30.6.2 Assay

DEC and the internal standard (IS), 1-diethylcarbamyl-4-ethyl piperazine HCl (E-DEC), were extracted from human plasma—that has been alkalized with carbonate buffer—after loading onto a conditioned C18 solid-phase extraction cartridge, rinsed with water, and eluted with methanol. After evaporation under a stream of nitrogen and reconstitution in methanol, 3 μL was injected into the GC system and detected using a flame-ionization detector (FID). The retention time for DEC was 5.5 min, and for the internal standard (E-DEC), it was 7.28 min.

PROCEDURE

1. To 0.5 mL human plasma in a disposable centrifuge tube, 25 μL of the working solution of the internal standard (21 μg E-DEC/mL) and 1 mL of carbonate buffer pH = 10.0 are added, and the sample is mixed on a vortex mixer for 30 s.
2. The sample thus prepared is applied onto a C18 column—which has been activated by aspirating 1 cartridge volume (~3 mL) methanol followed by 1 cartridge volume of HPLC grade water—and slowly sucked through under vacuum (at about 20-kPa pressure drop).
3. Subsequently, the column is washed with 2.5 mL of HPLC grade water at the same reduced pressure and allowed to dry for 10 min under vacuum.
4. For sample elution, 2 mL of 0.1% triethylamine in methanol is added to each cartridge and allowed to pass through the cartridge into 5 mL disposable centrifuge tubes under low vacuum.
5. After elution, the eluate is evaporated to dryness at 40°C under a gentle stream of N_2 gas.
6. The residue is taken up in 50 μL methanol and mixed on a vortex mixer for 60 s.
7. 3 μL of the reconstituted residue is injected into the GC.

30.6.3 GC System

Hewlett–Packard 5890 Series II Plus gas chromatograph equipped with a flame-ionization detector (FID) and an HP 7673 autosampler (Hewlett–Packard, Palo Alto, CA, USA).

Column: Fused-silica capillary column, Heliflex AT-35 capillary column (0.25-μm film thickness), 30 m × 0.32 mm inner diameter (ID); (Alltech, Deerfield, IL, USA)

Injection (*split*/*splitless*): No information available

Gases: Carrier gas (helium): 1.5 mL/min; inlet pressure 11 psi

Detector makeup (helium): 25 mL/min

Detector (hydrogen): 35 mL/min

Detector (air): 420 mL/min

Temperatures: Injection port 180°C

Oven: 160°C

Detector: 240°C

Detection: FID detector

Sample size: 3 μL

Analysis time: About 22 min per sample

EVALUATION

Calibration curves for DEC in human plasma were linear using unweighted linear regression in the concentration range of 100–2,000 ng/mL, with correlation coefficients greater than or equal to 0.9934 for all curves. The limit of quantification (LOQ) in human plasma was accepted as 70 ng/mL. Plasma samples were spiked to a nominal concentration of 70 ng/mL with DEC working solution and internal standard and carried through the extraction procedure. At the LOQ, the CV ($n = 6$) of the measured concentration was 4.5%, and the deviation of the mean of the measured concentrations from the nominal value was −6.1%.

Precision, accuracy, and recovery were evaluated by conducting repeated analysis ($n = 6$) of spiked plasma samples at three different concentration levels: 120, 1,000, and 2,000 ng/mL. For an intra-day run ($n = 6$), the coefficient of variation of DEC at 120, 1,000, and 2,000 ng/mL had been shown to be 4.5%, 1.3%, and 1.6%, respectively. The deviation of mean values from nominal ($n = 6$) was –4.4%, 2.0%, and 1.4%, for DEC concentrations 120, 1,000, and 2,000 ng/mL, respectively. The CV results for inter-day precision at the same concentrations were all less than 7% ($n = 12$). The deviation of mean values from nominal ($n = 12$) was –1.7%, 2.6%, and 0.8%, for DEC concentrations 120, 1,000, and 2,000 ng/mL, respectively.

Recovery was tested at low, medium, and high concentration of DEC and internal standard. Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for DEC and the internal standard were 84.8% and 85.5%, respectively.

Autoinjector stability was carried out for over 13 h by repeated injection of the same extracted plasma sample at room temperature (nominally 25°C); the results showed that the extracted specimens remained stable over the course of the study. QC samples containing 120 and 2,000 ng/mL DEC in plasma were subjected to 3 freeze/thaw cycles. Samples were frozen at −20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were transferred back to the original freezer and kept refrozen at least 24 h. Freezing and thawing of the QC samples appeared to have no effect on quantitation of the analyte. In addition, QC samples containing 120 and 2,000 ng/mL DEC in plasma were subjected to storage at −20°C for 12 weeks. Plasma samples ($n = 6$) were taken for DEC analysis at 0, 2, 4, 9, and 12 weeks. The QC samples stored in a freezer set to maintain −20°C remained stable for the duration of the study period.

CRITICAL ASSESSMENT OF THE METHOD

The assay has been validated, and the results of validation demonstrate that the standard curve is linear over the concentration range of 100–2,000 ng/mL. The assay is reproducible and accurate, with recovery of the analyte and internal standard in the range of 80–90%. The analysis requires 0.5 mL of plasma and has a limit of quantification of 70 ng/mL. The stability of plasma samples stored at −20°C has been demonstrated for up to 12 weeks. Autoinjector stability has been demonstrated for over 13 h, and freeze–thaw stability has been demonstrated for 3 freeze–thaw cycles. The procedure has a sample throughput of at least 30 specimens per day. The assay meets the guidelines for bioanalytical methods validation for human studies (Shah et al. 1991).

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Bioanalytical Assays LC-MS/MS

31

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PURPOSE AND RATIONALE

Powerful analytical techniques are one key requirement for successful drug research and development. The concentration of drugs, prodrugs, and metabolites has to be determined in very diverse matrices such as plasma (blood), urine, feces, and also in different organ tissues (depending on the nature of the drug and on the targeted organs).

A wide set of analytical tools has been used for this purpose. Gas chromatography, in combination with different detection techniques such as mass spectrometry, FID (flame ionization detector), and others, has been successfully used for this purpose (Jennings 1987) as well as liquid chromatography (LC), in combination with UV or fluorescence detection and others (Dorschel et al. 1989; Chu et al. 1999; Jin et al. 2004; Abu-Qare and Abou-Donia 2001).

However, these techniques have certain limitations. One prerequisite for a successful GC analysis is often the need for one or several chemical derivatization steps, since most analytes are not volatile enough for GC separation. Liquid chromatography, in combination with, for example, UV-, fluorescence-, or radioactivity detectors, is usually sensitive and does not require any sample derivatization. The only common drawback in this method is the lack of analyte specificity and in some cases also sensitivity.

The technique which is currently used predominantly is liquid chromatography coupled to mass spectrometry (LC-MS or LC-MS/MS). Consequently, this chapter describes the use of LC-MS in detail. However, it should be mentioned here that other very powerful techniques such as immunoassays, ELISAs, and chromogenic enzyme assays are used as well, and these types of bioassays are coming more and more into focus since biomolecules are being developed in a rapidly increasing number, which fuels the need for assays that specifically allow the sensitive analysis of biomolecules. The increasing number of biomolecules such as proteins in drug development also brought the possibility of analyzing these compounds by LC-MS/MS into focus. The possibilities and limitations of LC-MS/MS in this application are also discussed in this chapter.

PROCEDURE

31.1 Ionization

A big step in resolving this issue was achieved by the introduction of electrospray ionization (ESI) mass spectrometry and atmospheric pressure chemical ionization (APCI) mass spectrometry (and recently also atmospheric pressure photo ionization—APPI; Bruins 2000; Raffaelli and Saba 2003) as a detection system for liquid chromatography. These techniques, established in the late 1980s (Lim and Lord 2002; Dole et al. 1968; Whitehouse et al. 1985; Cole 1997; Gaskell 1997; Robb et al. 2000) have very rapidly become the method of choice for drug quantification throughout the drug research community and in the pharmaceutical industry (Tiller et al. 2003; Hopfgartner and Bourgogne 2003).

The basic principle is a soft ionization (no or almost no fragmentation of the analyte molecules is usually happening during the ionization step) of samples out of the liquid phase (ionization occurs in the liquid phase in case of ESI or in the gas phase after solvent evaporation in case of APCI). Samples are separated and purified using liquid chromatography. The eluent of the LC is transferred into the mass analyzer via a capillary. Usually, no sample derivatization is needed. As a result of the ionization process, analytes will form predominantly protonated or deprotonated molecular ions ($[M + H]^+$ or $[M-H]^-$) and also adducts such as $[M + Na]^+$, $[M + NH_4]^+$, and other adducts. In addition to that, and depending on the size of the molecules, multiple charging might also occur (Lim and Lord 2002; Cole 1997; Gaskell 1997).

31.2 Mass Analyzers

A large variety of mass analyzers has been tested and is in use for all kinds of bioanalytical assays. The type of detector predominantly used throughout the pharmaceutical community for drug quantification is the triple-quadrupole mass spectrometer. This type of mass analyzer has several key advantages for the analysis of biological samples in combination with liquid

chromatography. Triple quadrupole mass analyzers allow for the selection of one or several analytes, which can be filtered in the first quadrupole, allowing only ions of a certain mass to charge ratio to pass this first quadrupole (first mass filter). In a second step, the filtered ions can be fragmented in a collision cell by collisions with background gas molecules. The fragmentation pathway is a characteristic property of a chemical compound or chemical compound class, which allows the use of fragment formation as a fingerprint for a specific compound or compound class. In a third step, the fragments formed during this process can be filtered again in order to let only one specific fragment ion reach the detector of the mass analyzer. In this way, a substantial reduction of background is achieved in combination with a very high selectivity against interference of endogenous compounds in the analyte matrix. Multiple compounds can be analyzed in one run since the instruments are able to switch within milliseconds from one ion/fragment to the other and back. As a result, a separate chromatogram is yielded for each and every analyte, which can be integrated and processed (Venn 2000; Willoughby et al. 1998).

Besides the triple quadrupole instruments, other types of mass spectrometers might be used as well. Examples for these types of instruments are ion traps, time of flight mass spectrometers, and also single quadrupole mass analyzers. Due to the characteristic and specific advantages and disadvantages of different instrument types, the overall assay performance (e.g., sensitivity, dynamic range, and selectivity) may vary quite a bit from one instrument type to the other.

A new technique that has been introduced recently is the "FAIMS" (High-*F*ield *A*symmetric waveform *I*on *M*obility *S*pectrometer) technology (Guevremont 2004). The FAIMS interface works in combination with ESI and APCI ion probes in order to increase selectivity in challenging assays in combination with LC-MS/MS. FAIMS is an atmospheric pressure ion separation technology. In FAIMS, the ions are separated according to their properties while drifting in very high electric fields. Simply stated, each type of ion has an ion mobility which is a constant in low electric fields. At high fields, the mobility of each ion deviates from its low-field value. The extent of that deviation is the key to ion separation in FAIMS. Using FAIMS can help to overcome selectivity issues which are not solvable by LC-MS/MS alone. Although signal intensities with FAIMS are typically lower, the elimination/reduction of chemical noise might still provide a better sensitivity through an improved signal-to-noise ratio.

31.3 Internal Standards

In order to compensate for variations during sample analysis (e.g., thermal instabilities, variability in flow rate, and also electronic instability in the mass analyzer), samples are usually analyzed together with an internal standard, which is always added to the sample in the same amount. All measured peak areas or peak heights can be normalized on the signal of the internal standard, which helps to eliminate fluctuations during the individual measurement and compensate for matrix effects and recovery variations.

Two different types of internal standards are used. The first and usually ideal choice is a stable isotope of the analyte itself. In most cases, ^{13}C or ^{2}H isotopes are used for this purpose. It is important to note that the number of atoms replaced by the stable isotope should be large enough in order separate the isotope distribution of the internal standard from the natural isotope distribution of the analyte. The replacement of 6 ^{12}C or 6 ^{1}H atoms by ^{13}C or ^{2}H is usually a good choice for most pharmaceutical analytes ($m/z < 500$). Such isotopes are deemed ideal as internal standards since they will have the best probability for similar properties not only in terms of the ionization efficiency and sensitivity, but also in terms of the sample preparation procedure (solubility, extraction rate, and so forth). They will have an identical retention time and will therefore correct any fluctuations on the chromatography to the best possible extend (Venn 2000). Caution should be applied when dealing with deuterated ($^{2}H-$) standards. In some cases, a small shift in retention time is observed, which can limit the effect of such a standard. Unfortunately, the synthesis of labeled compounds can be difficult, time consuming, and expensive. In case that no stable isotope of the analyte is available, another compound has to be chosen as standard. For this purpose, one should look for a compound with similar structure, preferably from the same compound class, since such a molecule will

have the best chance for similar physical and chemical properties like ionization efficiency, retention time, and so forth. The difference in retention time should be small in order to correct for fluctuations in the LC-MS/MS system. However, in case of multiple analytes, this might not be achievable. Therefore, it will be necessary for some analytical problems to use more than one internal standard.

The use of an analog compound as internal standard will be the method of choice when it comes to the analysis of biomolecules, such as proteins, by LC-MS/MS since a stable isotope labeled standard is typically not available due to the fact that a large number of, for example, hydrogen atoms would need to be replaced by deuterium in order to achieve a mass difference which is large enough to prevent an overlay of the natural isotopic distribution with the stable isotope pattern. An iodine-labeled compound would be an option to overcome this hurdle. Otherwise, an analog standard will be the choice, which, for example, has a difference in one amino acid.

31.4 LC Conditions

In drug analysis, LC-MS usually means reversed phase liquid chromatography (RP-LC) coupled to mass spectrometry. Although normal phase LC can be used as well (especially in combination with atmospheric pressure chemical ionization—APCI), predominantly RP-LC is used in drug research and drug analysis due to the typical physical and chemical properties of the analytes (e.g., polarity, size).

Gradients of aqueous and organic mobile phases are typically used for LC-MS/MS analysis of drug compounds and metabolites. The most common aqueous solvents are water with 0.1% formic acid or 0.1% acetic acid (v/v) or volatile buffers like 5 mM ammonium acetate or ammonium formate often adjusted to a certain pH value with the corresponding acid or base (the pH of the eluents will have to be optimized with respect to the polarity of the analytes, since ionic species will have very low or no retention on the reversed phase LC-columns). Other volatile buffers can be used as well. Nonvolatile buffers are typically not useful; they will deposit in the ion source housing and will eventually lead to system contamination/breakdown. Phosphate buffer should also be avoided, since it will cause suppression of the ionization and thus lead to very bad analytical performance (Venn 2000). Reagents like triethylamine should also be avoided as mobile phase or as part of mobile phases. They induce ion suppression as well. In terms of the organic solvents, methanol and acetonitrile are very widely used and they are very well suitable for LC-MS. Other solvents can be used as well, as long as they are compatible with the materials used in the LC-MS system.

The gradients used are typically as short as possible (often less than 5 min) in order to realize a short analysis time and high sample throughput. A chromatographic separation of all components is usually not required since the analyzer is mass selective and very specific. The LC-method is mainly necessary for sample cleanup, which in most cases means the separation of matrix-related compounds from the analyte molecules. In terms of the flow rates, a very wide range can be used. Depending on the instrument used and on the source design, flow rates can typically vary between 10 or 20 µL/min and 5,000 µL/min or more. LC-columns should be selected with respect to the flow rate that is going to be used (inner diameter, particle size and length of the column). A very broad variety of packing materials is in use. However, C_{18}-reversed phase columns are probably the basic standard columns that are in use (Venn 2000; Willoughby et al. 1998).

The newest developments in LC techniques have significantly shortened analysis time without compromising chromatographic resolution or sensitivity. For that purpose, short LC columns with small particles (e.g., C_{18} columns with 50 mm length, a particle size of 1.8 µm and an inner diameter of about 3 mm) are used. These kind of columns are used at higher temperatures of about 60°C in order to reduce back pressure. Using this techniques, analysis times per sample can be reduced to less then 2 min in many cases, allowing for high sample throughput. This type of LC, often referred to as "UPLC" or "Rapid Resolution HPLC" (Hüsgen 2006; Swartz 2005), needs special LC-equipment in order to keep up with the higher pressure (>400 bar) that is caused by the higher flow rates.

31.5 Sample Preparation

Sample cleanup and sample preparation is a crucial step for a successful analysis. Three major approaches are used on a routine basis in many assays, which have

been reported in the literature (O'Connor 2002; Venn 2000; Chambers et al. 2007).

(a) Protein precipitation/dilution (PP)
(b) Solid phase extraction (SPE)
(c) Liquid-liquid extraction (LLE)

31.5.1 Protein Precipitation/Dilution

Protein precipitation is a very simple method of sample preparation. The sample (typically a plasma or urine sample) is spiked with internal standard solution and, in case of the calibration standard or quality control samples also with analyte solution. In case of unknowns, pure solvent is added instead of the analyte solution. Following this step, samples are diluted with an organic solvent (in most cases acetonitrile or methanol), which leads to protein precipitation. Samples are typically centrifuged after this step and the resulting supernatant is either analyzed directly by LC-MS/MS, or a dilution step is implemented prior to sample analysis (Beck et al. 2004; De Jonge et al. 2004; Viberg et al. 2004; Crommentuin et al. 2004; Stovkis et al. 2004; Hou et al. 2004; Kasel et al. 2004; Jamal et al. 2000). In many cases, this kind of sample preparation proved to be sufficient. However, more advanced sample cleanup might be necessary, depending on the matrix and analytes that need to be handled.

31.5.2 Solid Phase Extraction (SPE)/Mixed Mode SPE

Another simple and effective method for sample preparation is the solid phase extraction (SPE). In a typical approach, samples will be mixed with aqueous internal standard solution and with a small amount of acid (typically 0.2% formic acid). The resulting sample will be loaded on to the SPE extraction column (columns need to be conditioned before use, typically by flushing with methanol and water). After loading the sample on to the column, the loaded column is washed with water. Finally, the sample is washed off using an organic solvent combination such as $CHCl_3$/methanol (e.g., 2:1 v/v with 0.1% formic acid). The resulting sample solution is lyophilized in order to yield the solvent-free sample. The dry sample is reconstituted with mobile phase and is now ready for LC-MS/MS analysis. In many cases, an online approach is used as well, where the sample is eluted from the SPE cartridge directly onto the analytical LC-column (Venn 2000; Sottani et al. 2004; Pichini et al. 2004; Ding and Neue 1999; Kollroser and Schober 2002). However, using SPE might not always be sufficient in order to remove endogenous matrix constituents (such as proteins and phospholipids) or exogenous compounds (e.g., drug dosing vehicles such as PEG 400 or Tween 80), which can lead to ion-suppression or ion-enhancement effects during LC-MS/MS analysis. Mixed-mode SPE uses a dual retention mechanism to extract ionizable drugs from biological fluids. This approach allows a rigorous interference elution procedure to be used, selectively removing interfering compounds from the SPE column, prior to elution of drugs of interest. Mixed-mode SPE can significantly improve sample cleanup compared to SPE based on a single retention mechanism (Chambers et al. 2007). However, the more complicated sample workup for mixed mode SPE is not in general justified. It should be considered on an assay by assay basis.

31.5.3 Liquid-Liquid Extraction (LLE)

A general recipe for sample preparation by liquid-liquid extraction is not available, since the necessary procedures (solvents, pH, etc.) are depending on the chemical nature of the analyte that needs to be extracted (e.g., pK_a; it could be an acidic or basic compound or might be neutral) and of course also on the properties of the matrix that is present. However, when a basic compound needs to be extracted out of plasma samples, the following steps might be appropriate in many cases. Typically, in a first step, the internal standard is added to the unknowns. In case of the calibration standards and quality control samples, the blank matrix samples should be spiked with the analyte as well. This will guarantee that all extraction steps following this step will be applied on the standard and analyte. In case of a basic analyte, the sample pH should be basic. This can be achieved by adding, for example, 0.05% NH_3-solution. In case of acidic compounds, the use of formic acid or acetic acid is recommended. Following the addition of the acid or base, the samples can be extracted with CH_2Cl_2 (or another organic solvent). After shaking and centrifugation, the aqueous phase should be removed and the remaining organic phase (which should contain

the analyte) could be evaporated in order to yield the purified dry sample. The sample will then be reconstituted by adding a suitable solvent (e.g., starting mobile phase for the LC) (Stovkis et al. 2004; Keller et al. 2003; Bonato et al. 2003; Baker et al. 2004; Xia et al. 1999; Laurito et al. 2004). In any case, a recovery experiment should be performed in order to assess the efficacy of the extraction procedure. Recovery can be assessed by comparing the results for an extracted sample of known concentration with an unextracted sample, containing the theoretical concentration (assuming 100% recovery) in the mobile phase. In cases where fat or fatty tissues need to be analyzed, a washing step for the samples (e.g., with pentane) might be implemented as well in order to remove as much of the fat as possible (Getie and Neubert 2004). In these cases, one needs to make sure that the analyte is not too lipophilic. Otherwise it might be extracted as well. Methods for the determination of compound levels in different tissues are also often needed. Liquid-liquid extraction is used in these cases very often (Getie and Neubert 2004; Boner et al. 2003; Barratè et al. 2004; Bogialli et al. 2003; Hows et al. 2004; Ito et al. 2004). Recovery considerations are of special importance in these cases in order to get an idea on the completeness of the compound extraction procedure.

Eleven Steps on Method Development

1. Compound: Obtain information on the test article: solubility, purity, polarity, and stability in order to avoid analytical problems due to compound precipitation or compound decomposition. One should also estimate, which lower limit of quantification (LLOQ) will be required for the assay and it should be estimated which calibration range is desirable (the calibration range should reflect the expected sample concentration range).
2. Tune compound on your mass spectrometer: Optimize the intensity of the precursor ion as well as the selected product ion. If necessary, try positive and negative ionization as well as different ionization sources such as electrospray, APCI, APPI, and also the combination with FAIMS if available. Usually, the most intense fragment ion is selected as the product ion mass. Make sure that the selected product ion mass is not too close to the mass to charge ratio (m/z) of the precursor ion (e.g., loss of water, -18, is not characteristic and might question the selectivity of the method. Fragments with very low mass to charge ratio are also less characteristic and might sacrifice specificity.
3. Solvent selection: Select solvents for method development. A mixture of 0.1% formic acid/acetonitrile is usually a good starting point.
4. Optimize chromatography (column, solvents, flow rate, gradient) using a solution of the analyte in mobile phase.
5. The response for the selected transition of analyte(s) and internal standard(s) should be optimized by repeated flow injections of a dilute solution in the mobile phase (resulting in a weak signal of may be 10:1 signal-to-noise) of analyte and standard. All instrument parameters (gas flows, temperature, source position, etc.) should be optimized for maximum response according to the specific instrument type that is used.
6. Sensitivity in different ionization modes and with different ion sources should be tested as well in order to choose the best setup for the method.
7. Sample preparation: Depending on the analyte (SPE, Liquid-liquid, protein precipitation, dilution).
8. Run first matrix samples in order to identify LLOQ, dynamic range, analyte recovery and confirm suitability of the chromatographic setup.
9. Tests on sample stability, carryover, specificity, matrix interference, sample stability (freeze/thaw stability and so forth).
10. Run validation samples (batch to batch reproducibility, within batch reproducibility).
11. Validation report.

31.6 Sample Preparation for "Large Molecules"

Large molecules (MW $>$ 500 Da) are becoming more and more important in research and development of drug candidates as well as biomarker candidates (van den Broek 2008; Ezan 2009). Peptides, proteins, and certain sugars are usually analyzed by immunological methods due to their high sensitivity and rapid sample throughput, but one major disadvantage is their potential for cross-reactivity (e.g., metabolites, other compounds). Mass spectrometry is much more selective but cannot compete with the sensitivity of the immunological methods in general. However, mass

spectrometrical methods are increasing in the literature since the sensitivity of immunoassays is not always required and new LC-MS/MS instruments are performing much better (Zhang 2008). Nano-LC in combination with LC-MS/MS results in an increase of sensitivity, but with longer run times as compared to the usual HPLC methods.

Sample cleanup of large molecules is rather challenging (Yang 2007). Proteins and peptides adsorb on various surfaces during sample extraction and inside the LC-MS/MS-systems. It should be tested, if dilutions of the stock solution are linear and if the analyte binds to extraction vials (usually glass or polypropylene) or pipette tips. Binding inside the LC-MS/MS-systems results in a severe carryover problem. The adsorption can be controlled by using other solvents, pH, surfactants, ionic strength, other material (glass <-> polypropylene), or addition of serum albumin. For compounds, which bind to polypropylene tubes and glass tubes, low-binding polypropylene might be an option. The lower the concentration of the protein or peptides in aqueous solutions, the higher is the adsorption rate to tube surfaces in general.

The major extraction techniques for small molecules (protein precipitation, solid phase extraction, and liquid-liquid extraction) can be used for large molecules, but the use of organic solvents can result in an unintentional precipitation and loss of the analyte. In addition to the three basic extraction methods, immunoaffinity purification is frequently used to extract and clean the analyte from biological matrices (Dubois 2008; Thevis 2008; Whiteaker 2007). In many cases, an antibody which is directed against the analyte is immobilized on a surface (e.g., magnetic beads) or on a gel matrix. The biological sample will be incubated with the antibody which binds the analyte. After several washing steps at physiological pH, the analyte is eluted from the matrix with an acidic pH. The resulting eluate can be used directly for LC-MS/MS-analysis or will be further cleaned up by SPE, PP, LLE, TFC (turbo flow chromatography), or a 2D approach. Since the presence of "antidrug-antibodies" in the biological matrix can reduce the unbound fraction of the analyte/drug, immunoaffinity purification extracts only the non-antibody-bound fraction of the analyte/drug. Depending on the selectivity of the antibody, structural analogs of the analyte, ^{127}I labeled analyte, or stable isotopic labeled internal standard (SILS) can be used as internal standard. A second approach is the quantification of an antibody itself where a receptor has been coupled to magnetic beads (Dubois 2008).

The most sensitive triple quad mass spectrometers are limited to 1,250–1,500 m/z, but most proteins and peptides have a molecular mass above 1,500 Da. One solution is the detection of multiply charged ions (e.g., $[M + 3H]^{3+}$ to $[M + 8H]^{8+}$) of the intact protein or an enzymatic digestion of the protein. The first approach may be used with smaller peptides (MW < 7,000–10,000 Da) and the second approach must be used with peptides and proteins with a MW > 10,000 Da. During the enzymatic digestion the peptide is incubated with a proteolytic enzyme (e.g., trypsine, pepsine, Lys-C, Glu-C, or others) under its specific cleavage conditions and the much smaller peptide fragment can be quantified by LC-MS/MS, usually with an increased sensitivity. The optimal internal standard is of course the stable isotopic labeled internal standard to cover the whole cleavage process, but another possibility is to add an SILS for the smaller peptide fragment after the enzymatic digestion has been performed. In the second way, the SILS does not cover the enzymatic reaction.

31.7 Sample Preparation from "Dried Blood Spots"

Another method of sample preparation for LC-MS/MS analysis is the so called "Dried Blood Spot" (DBS) technique. In this technique, a droplet of blood is spotted onto a paper card and dried. Small pieces of paper can be cut out (punched out) of these dried blood spots and the analyte might be extracted from these dried blood spots using an appropriate solvent. This technique is currently under investigation in many pharmaceutical companies and it is already very likely that this technique will replace at least some part of the classical blood sampling as it is currently done on a routine basis. There are several potential advantages of this technique: Only a very low volume of sample is needed (only one or a few droplets of blood per sampling time compared to typically 1 or 1.5 mL of blood per time point). Another advantage might be the sample handling, since the resulting papers are a lot easier to store, handle, and ship compared with frozen blood samples. Moreover, the DBS technique might also

allow the sampling of other populations, such as children, where it otherwise might be difficult for ethical reasons. The number of experiments and analyses per subject might potentially also be increased due to the low volume of blood that is consumed. Preliminary tests also indicate that samples might even be more stable in dried blood spots than in frozen plasma. However, there are also limitations: Due to the limited amount of blood per blood spot, the sensitivity of a DBS assay is typically not as good as the corresponding plasma assay. In addition to that, a thorough validation needs to be done in order to cover variability induced by different types of sampling cards and a potentially uneven distribution of blood over the dried blood spot or by variations in the hematocrite.

31.8 Evaluation

31.8.1 Validation

LC-MS/MS methods are usually subjected to a validation procedure before they are used for routine analysis. In case of GLP studies or clinical studies, a validation is considered to be mandatory. During the validation procedure, the assay is evaluated with respect to the overall performance. The following tests might be considered as a standard set of tests for the validation of a bioanalytical method (EMA Guideline on bioanalytical method validation 2012; Shah et al. 2000; FDA Guidance for Industry 2001; Viswanathan et al. 2007; Shah 2007). However, additional tests or modifications of tests might be necessary since the regulatory requirements are not identical in all countries, although the guidances from the FDA are widely accepted:

- Determination of the regression model and weighting factor
- Assessment of assay accuracy and precision (assay variability)
- Analyte stability in plasma at 37°C over 24 h (or other appropriate temperature)
- Investigation of ionization efficiency (effect of analyte matrix on ionization)
- Efficiency of extraction procedure
- Assay specificity and selectivity
- Test of matrix variability (different lots of plasma or other analytical matrix)
- Test of assay robustness (largest batch size)
- Stability of analyte in stock solutions and working solutions
- Whole blood stability of test compound
- Vacutainer effect/container binding (investigation of, e.g., potential adsorption effects)
- Stability over typically three or more freeze/thaw cycles
- Show the ability to dilute samples with blank matrix in order to extend the calibration range
- Characterization of autosampler carryover
- Effect of hemolysis on analysis (in case of plasma samples)
- Multicomponent analysis test in order to show that different analytes do not influence each other
- Assess the effect of potential co-medications on the analysis of the compound of interest
- Signal-to-noise ratio (5:1 for lowest calibration standard or better)
- Long-term frozen stability of spiked samples
- Incurred sample reproducibility
- Incurred sample long-term frozen stability

CRITICAL ASSESSMENT OF THE METHOD

31.9 General Acceptance Criteria

31.9.1 Accuracy and Precision

Accuracy is defined as the percent difference (%D) from nominal at each concentration level, according to the following equation:

$$\%\mathrm{D} = \frac{(\text{Observed concentration} - \text{Expected concentration}) \times 100}{\text{Expected concentration}}$$

Mean percent differences (M%D) are reported for all data sets.

Precision is defined as the percent coefficient of variation (%CV) for each data set and will be calculated by the following equation:

$$\%\mathrm{CV} = \frac{\text{Standard deviation} \times 100}{\text{Mean}}$$

%CV values will be reported for all data sets.

31.9.2 Assay Variability

No more than 33.3% of individual validation samples within a given concentration level may be greater than ±15.0% of nominal, except at the LLOQ, where the acceptance criterion is ±20.0% of nominal. The point estimates for accuracy (bias) and variance (precision) for each validation level cannot be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%. Within-run, between-run, and total variances are estimated by equating observed and expected mean squares with a one-way random effects analysis of variance (ANOVA) for each concentration separately. A statistical test (Lund) for outliers can be performed and points determined to be outliers can be removed prior to ANOVA analysis. If an outlier is observed, data should be reported with and without the outlier.

31.9.3 Assay Robustness

If a data point appears to be anomalous and if an n of 6 or greater per observation is obtained, the Lund test for outliers will be performed and points determined to be outliers will be removed prior to calculation of mean statistics. No more than 33.3% of individual validation samples within a given concentration level may be greater than ±15.0% of nominal, except at the LLOQ where the acceptance criterion is ±20.0% of nominal. The mean estimates for accuracy (bias) and variance (precision) for each validation level cannot be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%.

31.9.4 Matrix Variability

The acceptance criteria for the matrix variability test apply to each individual lot. For the individual lot, no more than 33.3% of individual samples can be greater than ±20.0%, that is, no more than two out of the six within a lot can be outside the specifications. The mean, SD, %CV and M%D will be calculated for each matrix lot. The point estimates for accuracy (bias) and variance (precision) cannot be greater than ±20.0%. If a data point appears to be anomalous, the Lund test for outliers will be performed and points determined to be outliers will be removed prior to calculation of mean statistics.

The acceptance criteria for the analysis of the blank samples from the six individuals are based on the raw peak areas found at the retention times of the analyte and internal standard. No more than 10.0% of the blank samples can have raw peak areas that are greater than 20.0% of the average peak area of the analyte in the LLOQ validation samples.

31.9.5 Acceptance Criteria for Stability, Dilution, Hemolysis, and Concomitant Medications

For the tests listed below, no more than 33.3% of individual validation samples within a given concentration level could have been greater than ±15.0% of nominal, except at the LLOQ where the acceptance criteria is ±20.0% of nominal. The point estimates for accuracy (bias) and variance (precision) for each validation level should not be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%.

- Stability in stock solutions
- Freeze/thaw stability
- Stability at 37°C for 24 h
- Frozen stability
- Stability in extraction buffer
- Processed sample stability
- Dilution
- Hemolysis
- Specificity against concomitant medications
- Binding
- Multicomponent analysis (if applicable)

31.9.6 Blood Stability

The limit of stability is estimated as the time at which the 90% two-sided lower confidence bound for the estimated stability from time zero intersects the lower specification limit of 85%. If blood cell partitioning is high, an upper specification limit of 115% will be considered in the event that significant blood cell lysis occurs.

31.9.7 Vacutainer Effect

At each nominal concentration, the blood collection tubes will be determined to be equivalent to the

polypropylene (control) tubes if the 90% confidence interval for the estimated ratio falls entirely within the 15% specification limits (0.85, 1.15).

31.9.8 Autosampler Carryover

No more than 33.3% (i.e., two out of six) of individual LLOQ-1 samples (injected immediately after a HIGH QC sample) should be greater than ±20.0% of the nominal. The point estimates for accuracy (bias) and precision (variance) for all LLOQ-1 samples may not be greater than ±20.0%.

31.9.9 Process Efficiency

There is no fixed criterion for extraction (process) efficiency as well as the matrix effect. However, both effects should be determined and they should be on a constant level over the entire concentration range. Matrix effects might be acceptable to some extent when stable isotope labeled standards are used, since this should correct for these effects.

31.10 Matrix Effects

Although there is usually no need for any chemical derivatization, caution has to be applied when LC-MS/MS data are reviewed. The ionization of analytes might be affected and altered by endogenous compounds, which can be present in the matrix and which might coelute together with the analyte or internal standard. This can lead to ion suppression (predominantly observed with ESI ionization) as well as ion enhancement, which more often is observed when APCI-ionization is used. Matrix effects can lead to false results if analyte and the corresponding internal standard show different matrix effects. Such matrix effects are typically well compensated when a stable isotope internal standard is used. In case of analog compounds, which are often used in case no stable isotope labeled standard is available, the likelihood of an uncompensated matrix effect is a lot higher. For this reason, the usage of stable isotope labeled internal standards is becoming more and more a routine procedure.

Matrix effects are sometimes not obvious to recognize, which is one of the major pitfalls when using LC-MS/MS. The most practical experiment is probably a recovery experiment. A spiked matrix sample is analyzed and the result is compared to a spiked solvent sample. If no matrix effect is present, the same analytical response for the analyte and internal standard should be found in both samples.

In case the matrix effects are present (or in case the absence of matrix effects should be shown), samples can be diluted and reanalyzed. Matrix effects are usually concentration dependent. Lowering the sample concentration in many cases helps to minimize matrix effects. If this does not help, other measures have to be taken in order to eliminate matrix suppression. Such measures could be the use of a different sample preparation/sample cleanup procedure or change of LC-column or the LC-conditions (gradient, solvents). One important source of matrix effects could be the drug formulation as well. Especially when plasma samples originating from intravenous administration are analyzed, effects of the vehicle (e.g., PEG-400 or Transcutol, Solutol and others), which can be present in the samples of the first time points in substantial amounts, should be considered. These compounds can falsify the analytical results. A reanalysis of the samples in dilution should be considered in order to reveal a potential matrix effect (Dams et al. 2003; Pascoe et al. 2001; Annesley 2003; Hopfgartner and Bourgogne 2003; Schuhmacher et al. 2003; King et al. 2000; Liang et al. 2003).

One way to compensate for matrix effects is also the use of stable isotopes as internal standards. Since the standard will coelute with the analyte, the signal suppression or enhancement should have the same effect on analyte and standard, which will usually compensate quite well for the matrix effect.

31.11 Sample Analysis (Routine Application of the Assay)

A series of unknown samples is usually measured together with two sets of calibration standard samples (covering the concentration range for the assay, usually two sets of six or more calibration standards) and two or more sets of quality control samples. The calibration standard samples will be used to establish the calibration for the unknowns. Quality control samples (usually at least 5% of number of unknowns) are

matrix samples of known concentration, which are equally distributed over the analytical run (usually two sets of three different concentration levels; two to three times the LLOQ, mid-concentration range, and close to the upper limit of quantification). They establish a set of control samples in order to verify the assay performance within the run. Typically, the calibration standards and quality control samples should be within ± 15% of the nominal value. However, in typical assays, it is considered to be acceptable, if 75% of the standards are within the ± 15% criteria. Outliers will not be used for the calculation of the calibration curve. Not all standards at one concentration should be excluded. A similar criterion is applied for the quality control samples: two out of three of the quality control samples should be within ± 15% of their nominal value. In any case, at least 50% of the QCs at one level must be acceptable. This leads to the conclusion, that it is not advisable to use odd numbers of QC sample sets, since three QC sets will still fail the criteria in case more than one QC at one level is out of criteria.

Special consideration should be given to the placing of quality control samples (concentration levels), especially in cases where most of the unknown samples have concentration levels between the same two QCs. In these cases, it can be necessary to revalidate the assay with a narrower calibration range, or additional QC levels might be introduced in order to better characterize the calibration in the concentration range of interest (Viswanathan et al. 2007; Shah 2007).

Processing of analytical data such as chromatograms is another new and very critical quality criterion. Usually, data are processed by integration of chromatograms and subsequently by applying a standard regression and weighting to the data. For these steps, the same parameters should be used, which were established during method development and method validation. However, it might be necessary to change integration parameters on a run-by-run basis in order to achieve proper integration of all data. If this is done, it is considered to be mandatory to document the data before and after manual modification of the integration. Moreover, it is typically not accepted if more then 30% of the samples in a run are modified. In order to avoid problems in routine analysis, the quality of chromatography (e.g., peak shape) should be an important point to consider during method development.

31.11.1 Incurred Sample Reproducibility (ISR)

A rather new topic, which is now already considered to be a regulatory requirement for GLP studies as well as clinical studies, is the conduct of the so-called ISR-Test, incurred sample reproducibility test (and also ISA and ISS – incurred sample accuracy and incurred sample stability) (EMA Guideline on bioanalytical method validation 2012; Viswanathan et al. 2007; Shah 2007). The term "Incurred samples" describes samples that are obtained after actual dosing of the compound of interest. ISR samples are natural samples which are not artificially prepared (spiked). In fact, incurred samples are nothing else but regular specimens from a GLP study or clinical study.

The samples that are used during method validation are typically spiked samples which do not contain any metabolites of the drug administered. In addition to that, variability in the matrix of patients might be high, which could lead to analytical problems as well. For these reasons, it is now considered to be mandatory to perform the ISR.

Assessment of incurred specimen reproducibility (ISR) is usually performed for each analyte and matrix (plasma, serum, urine, etc.) in several studies and with the respective validated assay:

- Primary rodent toxicology study (typically rat)[1]—with an exploratory assay
- Primary non-rodent toxicology study (typically dog)—with an exploratory assay
- Primary rodent GLP toxicology study (typically rat)
- Primary non-rodent GLP toxicology study (typically dog)
- All first-in-man studies with healthy volunteers—single dose
- All first-in-man studies with healthy volunteers—repeated dose
- All studies with special patient populations, for example, renally or hepatically impaired patients
- All drug-drug interaction studies
- All bioequivalence studies

[1]Depending on the assay validation strategy, at the time of the exploratory non-GLP study only an exploratory assay may be available.

Ten percent of the total numbers of study samples of each matrix should be selected for ISR testing in studies with up to 1,000 samples. In studies with more then 1,000 samples, 5% of samples in excess of 1,000 should be selected in addition to the 100 samples selected up to 1,000 samples (e.g., 150 samples for a study with 2,000 samples). Based on the precision of the validated assay, a statistical approach may result in a different number of study samples which should be used for ISR testing. ISR testing is described in the study plan and reported in the bioanalytical phase report for the study.

The ISR is performed during or at the end of the study and the same validated assay (in case of primary non-GLP studies this could be exploratory assays) have to be used for the selected ISR samples and the study samples. The selected samples for ISR must have an acceptable analytical result which has been obtained without dilution of the respective sample and the result should be within three times the LLOQ and the ULOQ of the validated assay. A sufficient volume to perform the ISR should be left and samples must be within the documented stability period. Additionally, the selected samples should origin from different animals or subjects and should represent the pharmacokinetic profile of the respective analyte. The ISR concentrations are only used for ISR assessment and are not used for pharmacokinetic or toxicokinetic analysis.

$$\%\mathrm{D} = \frac{(\text{repeat concentration} - \text{original concentration}) \times 100}{\text{mean concentration}}$$

After acceptance of the run acceptance criteria for calibration and quality control samples, no more than 33% of the ISR samples from each study can have a concentration greater than ± 20% (physicochemical) or ± 30% (bioassay) of the mean of both determination. Any individual ISR sample with a concentration outside of the acceptance limit will not be reanalyzed, provided that the total number of ISR samples failing does not exceed 33%. However, in those cases where more than 33% of ISR results exceed the acceptance limit, an investigation into the cause of ISR failure will be initiated. The impact of ISR failure on existing specimen data must be assessed and documented.

31.11.2 Incurred Sample Accuracy (ISA)

Assessment of short-term incurred specimen analyte stability (ISA) is usually performed for each analyte and matrix (plasma, serum, urine, etc.) in several studies and with the respective validated assay:

- Primary rodent toxicology study (typically rat)[2]—with an exploratory assay
- Primary non-rodent toxicology study (typically dog)—with an exploratory assay
- Primary rodent GLP toxicology study (typically rat)
- Primary non-rodent GLP toxicology study (typically dog)
- All first in man studies with healthy volunteers—single dose
- All first in man studies with healthy volunteers—repeated dose
- All studies with special patient populations, for example, renally or hepatically impaired patients
- All drug-drug interaction studies

Due to limited volume of study samples, the investigation of short-term stability of incurred specimen stability on individual incurred samples is not feasible and therefore a pooled stability sample is used for ISA assessment instead. ISA testing is described in the stability study plan and reported in the stability report.

The ISA is performed at the end of the study which includes acceptance of all study concentration data of the respective matrix and successful ISR testing with the same validated assay (in case of primary non-GLP studies this could be exploratory assays) as was used for study samples and ISR assessment. The selected samples for ISA must have an acceptable analytical result and must be representative of the study design (multiple time points, dosing days and dose groups, multiple subjects/animals, dosed subjects/animals only, impaired subjects only, etc.). As far as the study and ISR are accepted, the ISA samples are pooled to one bulk stability sample. The concentration of the bulk stability samples should be within the LLOQ and the ULOQ of the validated assay.

The ISA samples must be transferred into the same type of tubes which are used for the long-term storage of the study samples. Replicate aliquots ($n = 9$) of the

[2]Depending on the assay validation strategy, at the time of the exploratory non-GLP study only an exploratory assay may be available.

freshly prepared pooled bulk stability sample should be stored frozen (t_{0h} at approx. −20°C or other validated storage temperature) and the remainder stored for 24 h at room temperature (t_{24h}). Both the t_{0h} and t_{24h} samples are analyzed within the same run to reduce inter-assay variability.

After acceptance of the run acceptance criteria for calibration and quality control samples, the mean ratio of data at $t_{24\ h}$ versus the data at t_{0h} and its 90% confidence interval will be calculated using Fieller's theorem for each study type. Incurred short-term specimen analyte stability will be assured if the 90% confidence interval for the estimated ratio falls entirely within the 15% specification limits (85%, 115%).

The results of short-term incurred specimen stability, including the statistical results, are documented in the stability report.

MODIFICATIONS OF THE METHOD

The following table lists a selection of method changes that are often required during the course of drug development. These changes usually require some degree of method revalidation. The listed activities are only a guide. In all cases, judgment of the bioanalyst will be the key factor to determine the necessary steps in order to cover the required method change.

Change to validated method	Test required
Reduce LLOQ of assay, while maintaining similar dynamic range of assay (e.g., change a 1–500 ng/mL assay to a 0.1–50 ng/mL assay)	Perform full assay validation, including calibration model, core studies and all satellite studies.
	Perform and report as a new study
Increase LLOQ of assay, while maintaining similar dynamic range of assay (e.g., change a 1–500 ng/mL assay to a 10–5,000 ng/mL assay)	Perform calibration model, assay variability test, autosampler test, and dilution test.
Verification of analyst competency to perform assay (multiple species of same assay are deemed equivalent)	Assay verification test, e.g., run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Verification of instrument (same platform, e.g., API4000 to API4000) to perform assay	Assay verification test, e.g., run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Verification of instrument (new platform, e.g., API4000 to API5000 or API5000 to Quantum Ultra)	Full assay variability with statistical analysis. Consideration being given to performing calibration model if significant differences in sensitivity observed between platforms.
Change of autosampler	Perform autosampler test
Changes of assay consumable (tube type and or vendor, solvent vendor, plate vendor, etc.)	Assay verification test, e.g., run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Additional freeze/thaw stability	Perform freeze/thaw stability test
Additional biological fluid stability	Perform additional biological fluid stability test
Additional processed stability	Perform additional processed sample stability
Additional dilution factor required	Perform dilution factor test
Additional major concomitant medication	Perform concomitant medication test
Chromatographic modification to resolve in vivo interference (gradient, flow, or modification of column geometry such as length, diameter, or particle size)	Assay verification test, e.g., run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Change to assay specificity (e.g., chromatographic or extraction chemistry)	Full assay variability with statistical analysis
	Full matrix variability
Increase signal-to-noise (e.g., increase injection volume)	Assay robustness test
Decrease signal-to-noise (e.g., reduce injection volume)	Full assay variability with statistical analysis.
Increase run size	Assay robustness test
Additional anticoagulant (same species)	Full calibration curve with three to six replicates at the LOW and HIGH validation levels against a calibration curve prepared in the original anticoagulant.
Additional nonclinical species (same matrix)	Perform partial assay validation, including core study and all appropriate satellite studies.
	Perform and report as a new study.
Additional matrix (same species)	Perform full assay validation, including calibration model, core studies, and all satellite studies.
	Perform and report as a new study.
Physiological proxy matrices	Full assay variability with validated matrix as calibration samples and physiological proxy matrix as validation

(*continued*)

Change to validated method	Test required
	samples. Includes statistical analysis.
Addition of metabolite	Perform full assay validation, including calibration model, core studies, and all satellite studies.
	Perform and report as a new study.
Effect of stable labeled analyte (e.g., ^{2}H, ^{15}N or ^{13}C) on validated assay	Full assay variability with statistical analysis and cross-over test.
Effect of radiolabeled analyte (^{14}C) on validated assay.	Use ^{14}C standard as test article. Alternative approaches, such as ion summing, theoretical correction factor, etc., may be used when proven.
Change SRM transition in assay (either for analyte or stable labeled internal standard)	Full assay variability with statistical analysis. Full matrix variability
Transfer of assay from site to site	Perform full assay validation, including calibration model, core studies, and all satellite studies.
	Perform and report as a new study

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Bioanalytical Assays: RIA/EIA

32

Heinz Jürgen Skrzipczyk and Patrick Verdier

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32.1 Introduction

Immunoassays have become the most valuable analytical tool of medicinal in vitro diagnostics and are routinely employed for the detection of a wide range of analytes (e.g., hormones, peptides, proteins, viruses, pharmaceuticals). Further important areas of application are environmental pollutants and food analysis.

The radioimmunoassay (RIA) for the determination of insulin developed by Yalow and Berson (1959) combined for the first time the high sensitivity of a radioactively labeled compound with the high specificity of an immunological reaction by the formation of a thermodynamically stable antigen-antibody complex (dissociation constants are typically 10^{-9} to 10^{-12} M). In this way it was possible to detect quantitatively substances in the attomolar range even in the presence of an excess of other similar foreign substances without prior enrichment. The theoretical and practical aspects of RIA and related techniques including numerous examples are described by Moss et al. (1976), Jaffe et al. (1979), Travis (1979), Langone et al. (1981, vol. 73/74), Langone et al. (1983), Odell et al. (1983), and Chard (1990).

Antibodies are prepared by immunization of animals (e.g., rabbits, guinea-pigs, sheep, goats) with the analyte of interest. However, direct immunization is only possible with relatively large compounds (approximately $>$ 1,000 Da). Since small molecules are not capable to trigger an immune response, the immunization is performed with hapten (compound)-carrier protein conjugates. Typical carrier proteins are keyhole limpet haemcyanin (MW $= 4.5 \times 10^5$ to 1.3×10^7 Da), bovine serum albumin (68 kDa) and ovalbumin (45 kDa). The haptens are coupled to the carrier proteins via amino or carboxyl groups using appropriate reagents.

The immunization procedure (at least 12 weeks with booster injections, addition of oil based adjuvants to stimulate the immunogenic response) gives rise to an antiserum representing a mixture of polyclonal antibodies which are produced by different immunocompetent cells and thus have different binding sites and affinities.

Monoclonal antibodies generated by fusing mouse spleen cells to myeloma cells can be considered as a pure single reagent that is available in unlimited quantity. Drawbacks are high costs, clonal drift and often low affinity.

Further details to polyclonal/monoclonal antibodies are found in Langone et al. (1981, vol. 73/74), Langone et al. (1983), and Szentivanyi et al. (1986).

The use of a radioactive label (mainly ^{125}I) not only attributed to the excellent sensitivity of RIA but also to the excellent flexibility and precision of radiochemical labeling and its high robustness with regard to interferences because of the "hard" signal.

However, the disadvantages of radioactivity such as the required handling licenses, disposal costs, short half-life (60 days for ^{125}I), short shelf-life of labeled reagents, restricted sensitivity due to limited signal emission during the measuring time of usually only 1 min led to the development of nonisotopic immunoassays (e.g., enzyme/fluorescence/chemiluminescence/bioluminescence immunoassay – EIA/FIA/CIA/BIA) described by Engvall and Perlmann (1971), Langone et al. (1981, Part B/C), Langone et al. (1983), Van Dyke (1985, Vol. I/II), Hemmilä (1989), Schaap et al. (1989), and Stanley and Kricka (1991).

Nevertheless, RIA is still the first choice for research assays provided the required handling licenses are available and not the ultimate sensitivity is required.

Some of the new labels have not only facilitated replacement of radioisotopes but also a breakthrough in sensitivity (zemptomolar range attainable).

The most widespread alternative to RIA is EIA (Tijssen 1985). Since the introduction of EIA at the beginning of the 1970s a tremendous number of techniques have become available by using different enzymes (horseradish peroxidase – HRP, alkaline phosphatase – AP, β-D-galactosidase – GAL, luciferase) and substrates (chromogenic, fluorogenic, luminogenic).

EIA has the potential for higher sensitivity than RIA because enzymes produce a lot of signal-generating species. In practice, however, the sensitivity is determined by the signal to noise ratio. Time resolved fluorescence detection is a powerful technique to overcome this problem by using fluorophores (rare earth chelates) with a long fluorescent time (μs–ms) compared to the background fluorescence (ns) (Janssen 1997; Read 2001). In many enzyme immunoassays, specific signal and noise are amplified to a similar

extent with the consequence that no improvement in sensitivity compared to RIA could be achieved. EIAs are in principle less robust than RIAs because enzymes are bulky and can disturb the immunological reaction. Furthermore, enzymes are prone to interferences. The implementation of sophisticated techniques and automation (assays are usually carried out in the 96/384 well format under controlled conditions) resulted in comparable assay quality independent of the label used.

In routine analysis, EIA and other nonisotopic immunoassays have displaced the RIA in many fields more and more during the past years.

32.1.1 Immunoassay Principles

(a) Competitive Immunoassay with Analyte Tracer

Analyte and analyte tracer (structurally similar to analyte, mostly ^{125}I-analyte) compete for a small number of antibody binding sites in an equilibrium reaction. The lower the concentration of the analyte, the more antibody-analyte tracer complexes can be formed. Selective measurement of the signal emitted from the antibody-analyte tracer complex requires prior separation of uncomplexed analyte tracer.

This assay principle can be applied for compounds of low and high molecular weight.

(b) Competitive Immunoassay with Antibody Tracer

The main difference compared to the method described before is that the antibody carries the label (antibody tracer). Analyte (from the sample) and an unlabeled analyte derivative (sometimes only analyte bound to a solid phase) compete for a small number of antibody tracer. The lower the concentration of the analyte, the more analyte derivative-antibody tracer complexes can be formed.

This assay principle is advantageous in cases when labeling of small antigens renders difficult or leads to different immunological properties compared to the unlabeled antigen.

(c) Sandwich Immunoassay

This principle requires two different antibodies (capture and tracer antibody).

Both antibodies bind to the analyte at different sites (epitopes) and thus form a sandwich complex. An excess of the two antibodies is employed in order to shift the equilibrium in favor of the sandwich complex.

Sandwich immunoassays have distinct advantages in comparison to competitive immunoassays: Higher specificity (two antibodies for analyte recognition) and better sensitivity (favorable equilibrium due to the excess of reagents). Theoretically, every analyte molecule present in the sample can be bound to a tracer antibody allowing extremely limits of detection. Often a straight-line calibration curve can be achieved over many orders of magnitude. In addition, higher and easier labeling of an antibody (in particular with nonisotopic labels) compared to a small analyte is possible.

The sandwich principle achieved its breakthrough when monoclonal antibodies became available because of the need of higher amounts of antibody compared to competitive assays.

The major disadvantage of this method is the restricted applicability to only analytes with MW >approx. 5 kDa), since two antibodies cannot bind to a small compound for steric reasons.

32.1.2 Separation Methods

The selective measurement of labeled immune complexes necessitate a prior separation of the unbound analyte tracer (a), of antibody tracer not bound to the analyte derivative (b), or of unbound antibody tracer (c).

The first separation methods involved purification steps (chromatography or electrophoresis). Better manageable, but nowadays rarely found in commercial kits (however still used in research assays) are the methods in which the immune complexes are precipitated, e.g., by addition of salts, organic solvents or a second antibody directed against the first antibody. All these techniques require a cumbersome centrifugation step.

Modern methods employ solid phases (e.g., tubes, particles, microtiter plates).

Based on solid phase technologies numerous fully automated immunoassay systems which allow the determination of a broad spectrum of analytes are nowadays commercially available. While very

successful in the case of nonisotopic methods, attempts for automation in the RIA field more or less failed due to the properties of the radioactive label.

Alternatives for simple handling of immunoassays without the need of expensive instrumentation have been developed.

In the homogeneous immunoassays there is no separation step because they are based on a changing signal by formation of the immune complex. The first assay of this type was the EMIT method (enzyme-modulated immunoassay technology) described by Engvall et al. (1970). The main disadvantages of homogeneous assays are low sensitivity and a more pronounced susceptibility to interferences.

Dry tests and test strips described by Morris et al. (1987) and Litman (1985) contain all the reagents required for a quantifiable test on a strip or filter. Evaluation can be performed visually or be read out by a pocket reflectometer. Automated systems for a higher sample throughput are also available. The sensitivity of these tests is rather low (mg–µg/mL range) but sufficient for application as quick tests in clinical chemistry.

32.1.2.1 DATA EVALUATION

Competitive immunoassays give a sigmoidal standard curve with a linear concentration-binding range of about two orders of magnitude. Due to the sigmoidal shape, sophisticated curve-fitting and interpolation software is required. 4-PL logistic fitting is the most widely proposed whatever immunoassay device manufacturers.

A straight-line calibration curve over many orders of magnitude can often be found in the case of sandwich immunoassays with deviations from linearity only in the lower and higher concentration range.

32.1.3 Special Features

1. Immunoassays can allow the quantification of enantiomers of a racemic chemical entity – by using a stereospecific tracer and a serum raised against the mixture of enantiomers.
2. The immunogen design generating an antibody for a product-class immunoassay can also be applied for a wide spectrum immunoassay enabling high throughput in research phase or in early stage of development for chemical series reaching the chemical optimization phase. In principle, the "constant" scaffold of different compounds are being used as immunogen resulting in a serum containing antibodies for these compounds. In cases where high throughput combined with highest analytical sensitivity is required, a wide spectrum immunoassay (WSIA) could be the method of choice.

32.1.3.1 Future Trends in the Immunoassay Field

Chemical sensors utilize the immunological recognition principle by coupling with optical, electrochemical, or other transducer (signal transfer) described e.g., by Eggins (1996) and Rogers et al. (1998). A tendency to miniaturized formats ("chips") as part of the nanotechnology can be observed.

Phage libraries for antibodies enable the access to "monoclonal like" antibodies in unlimited quantities which can be produced within a period of 2 weeks without using animals (Marks et al. 1996). These antibodies are less immunogenic than monoclonal antibodies (mouse-human chimeras) because the amino acid sequences are entirely human.

A promising technique for the determination of small molecules in sandwich immunoassays is the use of monoclonal anti-idiotypic antibodies which recognize unique V region of antigen-binding antibody and mimic internal image of antigen (Vogel 2000). In recent years, antigen mimicry has been shown on a molecular level for some xenoantigens.

Molecular imprinting molecular imprinting is a technique in which the shape of a template molecule (analyte) is imprinted in a polymer, e.g., described by Kriz et al. (1997), Reid et al. (1998), Yano (1999), and Yan (2002). The imprinted polymer can be used as an antibody mimic for an immunoassay.

32.1.4 Advantages of Immunoassays

The immunoassay can be considered as the most sensitive bioanalytical method.

Its selectivity is very high (but limitations with regard to metabolites).

Immunoassays are easy to handle and allow high throughput in contrast to HPLC-based methods.

The throughput can be further increased by semi-or fully automated systems.

The running costs of immunoassays are distinctly lower compared to other sensitive bioanalytical methods.

32.1.5 Disadvantages of Immunoassays

The production of antibodies needs about 3 months/6–12 months for polyclonal/monoclonal antibodies.

Matrix effects lead to a decrease in binding of analyte to antibody resulting in reduced sensitivity and falsely increased or decreased concentration depending on the assay principle. The matrix effects can be reduced or eliminated by diluting the sample.

Cross-reactivity (e.g., due to metabolites) can only be eliminated by collecting appropriate fractions from HPLC separations or applying solid-phase extractions (SPE) preceding the immunoassay procedure.

The high-dose hook effect (HDH) evident as a decrease in signal with very high antigen concentration can be observed in sandwich immunoassays (in particular in single incubation assays). HDH can be prevented by diluting the samples or applying more steps in the assay.

A cross-validation with an accepted analytical method (e.g., GC, HPLC, LC-MS/MS) is required if the immunoassay could be subject to interference from matrix or metabolites.

32.1.5.1 Terms and Abbreviations

Terms and abbreviations used in the immunoassay field are sometimes ambiguous.

Radioimmunoassay (RIA)

(a) Competitive immunoassay with a radioactive analyte tracer ("classical" RIA)

(b) Generic term for all immunoassays with a radioactive label

Enzyme immunoassay (EIA)

See RIA, it is distinguished only by the type of label used.

Enzyme-linked immunosorbent assay (ELISA)

(a) Excess reagent assay

(b) Generic term for all immunoassays with an enzyme label

2-site IXMA: A synonym for sandwich assay; "immuno-x . . . metric assay" (e.g., IRMA – immunoradiometric assay). The expression immunometric means that it is an assay with excess reagent.

1-site IXMA: Refers to a competitive assay with antibody tracer. The designation is not uniform because in 2-site IXMA, immunometric means an assay with excess reagent.

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32.2 Competitive RIA of Apidra (Insulin Analogue) with Double Antibody Precipitation

PURPOSE AND RATIONALE

Apidra (HMR 1964) is a synthetic short-acting insulin (polypeptide) which is structurally very similar to human insulin and has an approximate molecular weight of 5,800 Da.

Quantitative insulin determination (Yalow et al. 1959) is useful for the diagnosis of diabetes mellitus and for monitoring therapy.

In the early development of Apidra a commercially available radioimmunoassay for the determination of rat insulin (Linco Research Inc. 14 Research RD, St. Charles, MO 6330, USA) was used for the analysis of Apidra in human serum because specific antibodies were not yet available.

Several RIAs for insulin had been compared before aiming at 100% cross-reactivity with Apidra. The rat insulin RIA from Linco showed similar reactivity for Apidra and human insulin. Therefore, this assay was used with only slight modifications; rat insulin standards were replaced by Apidra standards in an appropriate human serum matrix for calibration.

The assay described below in more detail uses the principle of a competitive immunoassay with a double antibody precipitation (Chard 1990). The antibody (from guinea pig) bound tracer is separated from the free tracer by the second antibody present in the precipitating reagent (goat anti–guinea pig antibody). After decanting the free tracer, the activity of the antibody-bound fraction is measured (B) and calculated as a fraction (B/B_0) of the noncompetitive binding (B_0). A standard curve is set up with increasing calibrator concentrations by plotting the measured B/B_0 fraction against concentration, and from this curve, the concentration of antigen in unknown samples can be calculated.

PROCEDURE

32.2.1 Reagents

Reagents of the Linco rat insulin RIA are used except the standards and control sera.

The test kit comprises reagents for 250 determinations. It contains:

One vial of ^{125}I-Insulin <185 KBq, lyophilized
One vial of guinea pig anti-rat insulin antiserum in assay buffer, 26 mL
One vial of assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% RIA grade BSA), 40 mL
One vial of label hydrating buffer (assay buffer containing normal guinea pig IgG as carrier), 27 mL
One vial of precipitating reagent (goat anti–guinea pig IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05 M phosphosaline, 0.025 M EDTA, 0.08% sodium azide), 260 mL
Standards: stock solution: 20 ng/mL Apidra in insulin-free human serum; standard matrix: insulin-free human serum
Controls K1–K3 (low, medium, high): Apidra in human serum, concentration stated.

32.2.2 Preparation of Reagents

^{125}I-insulin tracer is dissolved in entire volume of label hydrating buffer.
Antiserum, assay buffer, and precipitating reagent are ready to use.
Standards: The stock solution of Apidra is prepared by dissolving about 500 µg (exact weight) Apidra in

5 mL of 0.1 M Tris/HCI buffer, pH 9. This solution (100 µg/mL) is further diluted in insulin-free human serum to a concentration of 20.0 ng/mL (nominal value). The standards (10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 ng/mL) are prepared by serial dilution of the stock solution in insulin-free human serum matrix (e.g., WBAG Resources GmbH).

Controls: Control samples are prepared by spiking human sera (insulin content below 0.5 ng/mL) with Apidra stock solution to a concentration of about 0.5, 1.5, 5.0 ng/mL.

32.2.3 Assay Procedure

100 µl of standard/control/sample is incubated in a tube with 100 µl of ^{125}I-insulin tracer and 100 µl of antiserum for 20–24 h at 2–8°C.

Separation of the antibody-bound and free radiolabeled ligand is done by dispensing 1 mL of cold precipitating reagent in each tube. The tubes are vortexed and incubated for 20 min at 2–8°C. The precipitate is then centrifuged down at >1,500 g for 60 min at 2–8°C. The supernate is decanted immediately, and the remaining solution is allowed to drain by standing the tubes upside down on adsorbent tissue up to 15 s. Invert tubes only once. Pellets are fragile and slipping may occur.

The radioactivity is counted in a gamma counter for 1 min.

Total activity and nonspecific binding (blank) are also determined in each run.

EVALUATION

Control/sample concentrations are read-off from the standard curve by use of the immunoassay data management program MultiCalc (Wallac Oy/Berthold).

Fitting algorithm = 4–PL weighted

Blank correction is applied. NSB (nonspecific binding) counts are subtracted from each average counts, except total counts, prior to final data reduction.

32.2.4 Validation

Precision (within day): 2.2–7.2% (4.9% near LOQ).

Precision (between day): 1.6–4.7% (2.3% near LOQ).

Accuracy (between day): 91.8–101.4%.

Linearity: The test shows a good linearity over the whole concentration range as shown by dilution of serum samples with assay buffer.

Limit of quantitation (LOQ): 0.2 ng/mL (corresponding to ED-80 of the standard curve).

Specificity: No specific determination of Apidra (cross-reactivity with human/rat/porc insulin is 80.2/106.9/102.2%, respectively).

CRITICAL ASSESSMENT OF THE METHOD

The method described above could not differentiate Apidra from naturally occurring insulin/metabolites and precursors. This lack of specificity renders the characterization of the pharmacokinetics difficult. Therefore, a specific assay for the determination of Apidra was required for its further development.

MODIFICATION OF THE METHOD

Aventis Pharma contracted Linco Research Inc., 14 Research RD, St. Charles, MO 6330, USA, to design and produce a highly specific RIA for the accurate and precise routine determination of Apidra in human serum in commercial kit form ("Insulin Analog HMR 1964 RIA Kit").

This kit is very similar to the method described before with the exception that a highly specific antibody for Apidra (guinea pig anti-HMR 1964 antiserum prepared by Linco) is used. The antibody meets the specificity requirements (cross-reactivity, e.g., to human insulin <0.001%) and the sensitivity requirements (<0.2 ng/mL).

References and Further Reading

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32.3 Competitive RIA of Free Anti-Insulin Antibodies with PEG Precipitation (Semiquantitative Assay)

PURPOSE AND RATIONALE

Anti-insulin antibodies (AIA) develop in the serum of many patients who are receiving insulin treatment (Berson et al 1956). In addition, insulin autoantibodies (IAA) are detected in insulin-dependent diabetes

mellitus (IDDM) or type I diabetes before insulin therapy (Palmer et al 1986).

The determination of circulating anti-insulin antibodies is of clinical importance for the following reasons:

1. Presence of free anti-insulin antibodies in serum interferes with the determination of insulin by immunoassays (Kuzuya et al. 1977).
2. High *titers* of AIA may induce a state of insulin resistence.
3. AIA may influence the quality of the glycemic control in diabetic patients by prolonging the half life of insulin.

The RIA described (in-house assay) is intended for the semiquantitative determination of free AIA in serum by the binding of the corresponding ^{125}I-Tyr-A 14-insulin tracer and precipitation of the complex by polyethylene glycol. It is useful for monitoring the development of AIA during immunogenicity studies in animals, toxicokinetic studies in animals, and clinical studies with patients receiving (synthetic) insulin.

PROCEDURE

32.3.1 Reagents

32.3.1.1 Controls

A blank control (TO) containing no anti-insulin antibodies is used to determine the nonspecific binding (NSB). Three titer controls (T1–T3) containing low, medium, and high levels of anti-insulin antibodies (guinea pig anti-porc insulin antiserum, e.g., Scantibodies Laboratory Inc. T 531 B, Part. 3 AK 025 in buffer solution) are measured in each run.

Buffer: 0.05 M phosphate buffer, pH 7.4, 0.5% BSA, 0.4% bovine IgG, 0.075% NaN_3

Preparation of Controls:

Stock solution: anti-insulin antiserum is diluted 1:100 in buffer.

Titer control T3 (high): stock solution is diluted 1:100 in buffer.

Titer control T2 (medium): titer control T3 is diluted 1:4 in buffer.

Titer control T1 (low): titer control T2 is diluted 1:8 in buffer.

Blank control TO: buffer.

The titer controls are stored in polystyrene tubes at −20°C. They are stable for at least 12 months.

^{125}I-Tyr-A14-insulin (e.g., human, porcine or bovine insulin) is prepared according to routine procedures. Aliquots in buffer solution (0.05 M phosphate buffer, pH 7.4, 1% BSA, 0.05% NaN_3) are lyophilized.

When stored at 2–8°C the tracer can be used for at least 6 weeks.

32.3.1.2 Specification

Specific activity: 13.320 MBq/mg (360 mCi/mg)
Activity: 37 kBq/mL (1∼Ci/mL)
Concentration: approx. 2.8 ng/mL

32.3.1.3 Assay Buffer

7.8 g $Na_2HPO_4 \times 2H_2O$ and 0.75 g KH_2PO_4 are dissolved in 800 mL of water. After pH value has been adjusted to 7.4, 0.75 g NaN_3 is added and filled up to 1,000 mL with distilled water. 5.0 g BSA is added and dissolved.

32.3.1.4 Dilution Buffer

For sample dilution assay buffer containing 4 mg IgG/mL (bovine immunoglobulin) is used.

Storage:

Assay buffer can be stored at 2–8°C for at least 4 weeks.

Dilution buffer can be stored up to 1 week at 2–8°C.

32.3.1.5 PEG Solution

The PEG solution consists of 17.5% (w/v) polyethylene glycol (PEG 6000) in phosphate buffer (0.05 M, pH 7.5) containing 0.075% sodium azide and 0.5% bovine serum albumin.

It can be stored at 2–8°C for at least 6 months.

32.3.1.6 Preparation of Reagents

^{125}I-insulin tracer lyophilized is dissolved in 20 mL H_2O bidest. It can be stored up to 24 h at 2–8°C after reconstitution.

The titer controls (T0–T3) are thawed and homogenized.

Assay buffer and PEG solution are ready to use.

32.3.1.7 Assay Procedure

100 µl of control/sample is incubated with 200 µl of ^{125}I-insulin tracer and 200 µl of assay buffer for

19–24 h at room temperature. Separation of the antibody-bound and free radiolabeled ligand is performed by dispensing 1 mL of PEG solution in each tube. The tubes are vortexed several times until mixing is complete. The rapidly formed precipitate is then centrifuged down at 1,500 g for 15 min at room temperature. The supernate is decanted and the remaining PEG solution is allowed to drain by standing the tubes upside down on adsorbent tissue. The radioactivity is counted in a gamma spectrometer for 1 min.

EVALUATION

32.3.1.8 Calibration

Due to the different nature of insulin antibodies that can be formed a defined analyte does not exist and a calibration is not possible. The tracer binding test is a semiquantitative test without a standard curve. Three titer controls (T1–T3) containing low, medium and high levels of free anti-insulin antibodies and a blank control (TO) to determine the non-specific tracer binding (NSB) are measured in each run. Total activity of 200 µl tracer is also measured in each run.

32.3.1.9 Calculation of Results

The mean of duplicate determinations is calculated, rejecting obvious outliers. In cases CV >15%, analysis is repeated. The binding percentage of ^{125}I-insulin is calculated, according to the formula:

$$\mathrm{B/T}(\%) = (\text{sample counts}/\text{total counts}) \times 100$$

32.3.1.10 VALIDATION

Precision (within day): 1.4–8.1% (in the range from 5% to 90% B/T in 10 determinations)

Precision (between day): 4.9–11.1% (for titer controls T1–T3, T0 and two human sera from 15 determinations on separate occasions

Accuracy: cannot be determined in a semiquantitative assay

Linearity: cannot be tested since no standard curve exists

Matrix effects: cannot be tested since no standard curve exists

Specificity: due to structural similarity of insulin analogues, cross-reactivity of antibodies is usually high

Limit of quantitation: cannot be defined for a semiquantitative method (precision of double determinations is below 15% down to the nonspecific binding and between-day precision of the negative serum controls (about 4% B/T) is below 10%

REFERENCE RANGE

The binding values of 20 healthy blood donors were determined e.g., with ^{125}I-human insulin tracer. It can be considered that a binding value higher than the mean value plus three standard deviations (e.g., 4.1% B/T found with a tracer lot for human-insulin tracer binding) indicates the presence of antibodies.

For evaluation of antibody positive samples a negative control has to be determined in each individual run.

An absolute change in binding values >10% B/T from baseline to endpoint (in clinical studies) when measured in the same run is considered as significant.

APPLICATION DATA

The binding values for Type II diabetic patients without prior insulin therapy ranged from 2.5% to 8.7% B/T (mean 3.6% B/T), whereas Type I diabetic patients that had been pretreated with insulin showed significantly elevated binding values before and after treatment ranging from 2.7% to 83.8% B/T (mean 21.5% B/T).

This is in agreement with published data that insulin antibodies are formed in IDDM (Type I diabetic patients).

Note: Insulin levels are determined to be lower in Type I compared to Type II patients since antibodies bind to the insulin administered and it is not available as free and acting insulin. On the other hand, the results cannot be interpreted as free insulin concentration because antibodies also interfere with the immunological determination of insulin by competition with the antiserum. Determination of free insulin (not bound to antibody) requires a pretreatment of serum with PEG.

METHOD COMPARISON

A commercially available RIA for determination of free anti-insulin antibodies (CIS bio international) was used for method comparison with insulin tracer binding test for Type I diabetic patients. The CIS test uses a ^{125}I-porcine insulin tracer for insulin antibody determination. Linear regression analysis yielded a correlation coefficient of 0.94 for human insulin antibodies. The absolute binding values were 10–15% lower in the commercial test than in the in-house test.

Note that absolute binding values cannot directly be compared between different tests due to the semiquantitative nature of the tracer binding test.

CRITICAL ASSESSMENT OF THE METHOD

A quantitative test for the determination of anti-insulin antibodies is not possible due to the different nature of insulin antibodies that can be formed. The criteria for evaluation of antibody positive samples are somewhat arbitrary, e.g., selected pool of healthy blood donors, definition of mean +3 standard deviations, varying binding with different tracer batches, consider an absolute change in binding values >10% B/T in clinical studies from baseline to endpoint when measured in the same run as significant.

MODIFICATION OF THE METHOD

A slightly modified method (MDS Pharma Services Switzerland AG, CH-8602 Wangen) was used in the development of Apidra (a synthetic short acting insulin).

This method is based on the same principle as described above with only minor changes in the assay procedure.

References and Further Reading

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32.4 Immunoradiometric Assay (IRMA) of Ferritin with Bead Separation

PURPOSE AND RATIONALE

Ferritin, a macromolecule with a molecular weight of ~440,000 Da, is an iron storage protein which occurs ubiquitously in the organism. A protein shell (apoferritin) surrounds a nucleus containing iron oxyhydroxide phosphate. Every molecule can absorb up to 4,000 atoms of iron and can contain up to 20% of its weight as iron when saturated.

Functionally, ferritin, in conjunction with transferrin, regulates the movement of iron from the gastrointestinal tract to various tissues responsible for iron storage as well as to the bone marrow.

The ferritin found in each tissue has a characteristic structure and exists as isoferritin. Of special interest is the serum ferritin, which is considered to be different from any specific isoferritin. The serum ferritin level reflects the iron status of the body.

More detailed information to ferritin is given by Crichton (1973) and Jacobs et al. (1975).

The RIA-gnost Ferritin kit (no more commercially available) from formerly Behringwerke AG, Radiochemical Laboratory described below uses the principle of an immunoradiometric assay (IRMA). It is a two-site solid phase assay of the sandwich type, based on a plastic bead as solid phase to which the anti-ferritin antibody adheres. The antibody solid phase is incubated with standards or serum samples containing ferritin and in this process the ferritin in the solution is bound quantitatively to the solid phase via the antibody. The amount of ferritin bound to the solid phase is then determined by a reaction with ^{125}I-labeled anti-ferritin antibody. An antibody-ferritin-^{125}I-antibody complex is thus formed.

The RIA-gnost Ferritin kit uses anti-ferritin antibodies of two animal species, which have been raised against different organ isoferritins. Since the immunological characteristics of the ferritin circulating in the blood are not precisely known, it is necessary for the assay method to have broad specificity for organ isoferritins, measuring them all with practically equal potency. The antibodies used in RIA-gnost Ferritin meet this requirement.

It is typical for an immunoradiometric assay that a “high dose hook” effect occurs in the region of very

high concentrations. Therefore, sera with concentration above the highest standard S7 have to be diluted with the dilution serum included in the kit.

PROCEDURE

32.4.1 Reagents

The kit consists of:
100 plastic beads with anti-ferritin immunoglobulin from sheep ("anti-ferritin beads")
One anti-ferritin immunoglobulin from rabbit
Iodine-125-labeled, <4 μCi ("anti-ferritin-^{125}I-antibody"), lyophilized
Eight ferritin standards, S0–S7, concentration in the range of 0–400 ng ferritin/mL (related to the protein fraction of the molecule), lyophilizates from serum
One test serum, ferritin concentration declared, lyophilized
One buffer, pH 8.6, protein carrier solution, lyophilized
One diluent serum, lyophilized
One pair of forceps

32.4.2 Assay Procedure

- Number sufficient round-bottomed incubation tubes (3–5 mL) for 1 test serum, up to 41 serum samples and total activity. Assay duplicate samples.
- Place one anti-ferritin bead in each tube (exception: total activity) by means of the forceps contained in the kit.
- Add 200 μl buffer solution to each tube (exception: total activity)
- Dispense the standards, when completely dissolved, after mixing briefly on a rotary mixer, in aliquots of 100 μl into the tubes. Use a new pipette tip for each standard.
- Dispense 100 μl of test serum/serum sample, after mixing on a rotary mixer, into the tubes. Use a new pipette tip for each serum sample.
- Mix all tubes briefly on the rotary mixer; cover the whole test tubes rack with Parafilm and place the rack for the incubation on a horizontal shaker, shake for 2 h at room temperature (22 ± 5°C) with ~300 rpm (250–350 rpm).
- Carefully aspirate the incubates; it is best to use a Pasteur pipette (or a pipette tip) connected to a suction pump. The beads should remain in the tubes.
- 2 mL distilled water are dispensed into each tube (exception: total activity) in such a manner that each bead is agitated. Aspirate the water.
- Dispense 300 μl of the anti-ferritin-^{125}I-antibody solution into each tube and shake the test tubes rack briefly by hand. Then cover the rack with Parafilm and place it on the horizontal shaker and shake for 3 h at room temperature (22 ± 5°C) with ~300 rpm (250–350 rpm).
- Carefully aspirate the incubates (exception: total activity); the beads should remain in the tubes.
- Dispense 2 mL distilled water into each tube (exception: total activity) in such a manner that the bead is agitated. Aspirate the water.
- The tubes containing the beads and "total activity" are measured directly in a gamma scintillation counter for 2 min. Between 40,000 and 60,000 cpm are to be expected for the "total activity" tubes. This depends on the age of the kit and the efficiency of the counter.

EVALUATION

The cpm of the standards S0–S7 are calculated as a percentage of the cpm of the "total activity" and are plotted as individual values against the appropriate ferritin concentration. The "best fit" standard curve is drawn through these points.

The mean values are calculated for the two counts for the test serum and the patient serum samples. The ferritin concentrations per mL serum are read from the standard curve.

CRITICAL ASSESSMENT OF THE METHOD

The method described above was cumbersome in handling because of placing each individual anti-ferritin bead into the tubes by means of the forceps.

A data evaluation program with an appropriate fitting algorithm was not yet available.

MODIFICATION OF THE METHOD

Since the commercial distribution of the RIA-gnost Ferritin kit (beginning of the 1980s), improvements regarding easier handling, more sophisticated data evaluation and higher throughput have been made.

For instance, the next generation of RIA-gnost Ferritin was based on the coated tube technology which saved the manual transfer step of the anti-ferritin beads into the tubes. Further development led to semi- and fully automated assays based on enzyme/fluorescence and chemiluminescence immunoassays commercially available from several companies.

References and Further Reading

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32.5 Specific Radioimmunoassay of 314d Isomer of Beraprost in Human Plasma

PURPOSE AND RATIONALE

Beraprost (*CAS*: 88475-69-8) is an equimolar mixture of two racemates (four isomers), APS-314d,l and APS-315d,l. Radioimmunoassays of the isomers of beraprost (an orally active prostacyclin PGI_2 analog which has antiplatelet and vasodilating effects) were carried out by combining a serum from a rabbit immunized with beraprost conjugated to bovine albumin and radioiodinated tracers prepared from each isomer. The main biological activity is contributed to the APS-314d isomer and the clinical daily dosing is in the range of 40–120 μg. APS-315d has a lower activity whereas APS-314 l and APS-315 l have almost no activity. The anti-beraprost serum contains three families of antibodies specific for the APS-314d and APS-315d isomers and for the mixture of the two l-isomers. The binding with either APS-314d, APS-315d or APS-315 l tracers made it possible to determine the plasma concentrations of APS-314d isomer, APS-315d isomer, and of the mixture of the two l-isomers during clinical studies (Mouren 1995).

Each assay method is specific with respect to the other stereoisomeric components of beraprost and related metabolites and also sensitive. The limit of quantification can be taken as 3 pg/mL for APS 314d, 12 pg/mL for APS 315d and 10 pg/mL for APS 314 l +APS 315 l, when applied to 0.1 mL of human plasma.

32.5.1 Reagents

- APS-314d Na
- Sodium chloride (Prolabo)
- Sodium azide (Merck)
- Sodium dihydrogen phosphate monohydrate (Carlo Erba)
- Anhydrous sodium hydrogen phosphate (Prolabo)
- Powdered gelatin (Merck)
- Triton X-100
- Sodium salicylate (Prolabo)
- Rabbit γ-globulins
- (Cohn's fraction II/III, G-0261, Sigma)
- Freund's complete and incomplete adjuvants (Difco)
- Human blank plasma

32.5.2 Preparation of Reagents

32.5.2.1 Immunogen and Rabbit Antiserum

Beraprost was linked to bovine serum albumin according to Erlanger et al. (1957).

A serum containing an antibody to APS-314d was produced in New Zealand rabbits. The immunogen (10 mg) was dissolved in 10 mL of a 0.9% sodium chloride solution and emulsified with 10 mL of Freund's complete adjuvant by repeated passages through a syringe. Each rabbit received 1 mL of emulsion (500 μg of immunogen) subcutaneously in 10 sites on the back. Three booster injections were given at 4-week intervals with the same dose of immunogen but using Freund's incomplete adjuvant. Blood sera were collected 3 weeks after the last booster and checked for titer, affinity and specificity without any purification.

The sera can be stored, either undiluted at −30°C for several years or diluted 1:100 in a buffer at +4°C for up to 6 months, without significantly losing activity. At the time of the assay, the selected serum is diluted 1:50,000 in assay buffer containing sheep anti-rabbit γ-globulins and rabbit γ-globulins.

32.5.2.2 Sheep Anti-Rabbit Gamma Globulins for Separation Step

An antibody to rabbit γ-globulins was raised by immunizing sheep with rabbit γ-globulins.

Each sheep initially received 5 mg of protein in multiple injections at intervals of 5 weeks until a serum with a satisfactory precipitating potency was obtained. Generally, two booster injections were sufficient and blood was withdrawn 15 days after the last booster. The serum was purified by precipitation of the immunoglobulins with half a volume of a saturated ammonium sulfate solution and then dialyzed for 4 days against a 0.9% sodium chloride solution which was replaced every day. It was stored at −30°C.

32.5.2.3 ^{125}I-APS-314d

The radioactive tracer used was obtained by iodine 125-labeling of the conjugate of APS-314d and histamine, according to Hunter and Greenwood's method (1962). Stored at −30°C, the iodinated tracer was stable for at least 3 months without any loss of performance. It was diluted extemporaneously in assay buffer to obtain a solution containing 80,000 cpm per mL.

32.5.2.4 Assay Buffer

The assay buffer was prepared by dissolving 9 g of sodium chloride, 1 g of sodium azide, 1 g of gelatin, and 2.5 g of sodium salicylate in 1 l of a 0.1 M phosphate buffer containing 0.01% (v/v) of triton X-100. It was stored at ambient temperature.

32.5.2.5 APS-314d Standard Solutions

A 100 μg/mL solution (M1) of APS-314d Na was prepared by dissolving the compound in demineralized water. This was stored at +4°C. Diluted 1:100 in phosphate-gelatine buffer, it yielded a solution (M2) of 1 μg/mL from which were obtained by 1:100 and 1:40 dilutions and then by successive 1:2 dilutions, solutions (A to J) containing 250–0.49 pg of APS-314d Na per mL of buffer. These solutions were prepared extemporaneously on the day of the assay.

PROCEDURE

Assays could be carried out with 0.1 mL of undiluted plasma samples or after dilution in human blank plasma. The standard curve was established in the presence of 0.1 mL of human blank plasma. The operating procedure is as follows (Table 32.1):

For calibration: to three replicates of:

0.1 mL of assay buffer (maximum binding Bo) or 0.1 mL of the M2 solution (nonspecific binding) or 0.1 mL of the standard solutions (i.e., 0.049–25 pg) were added in 12 × 65 mm glass test tubes, 0.1 mL of human blank plasma, and 0.2 mL of assay buffer.

For plasma samples: to three replicates of:

0.1 mL of undiluted plasma samples or to 0.1 mL of diluted plasma samples were added in 12 × 65 mm glass test tubes, 0.3 mL of assay buffer.

In all cases, the tubes were allowed to stand for 1 h at room temperature, following which iodinated APS-314d (4,000 cpm) was added in 0.05 mL of assay buffer to all tubes, as well as to three tubes labeled "total radioactivity." With the exception of the latter, the tubes were vortexed for a few seconds and then they received 0.05 mL of a mixture containing anti-beraprost serum (1:50,000 dilution), sheep anti-rabbit γ-globulins (200 μl/mL), and rabbit γ-globulins (200 μg/mL) (the quantity of rabbit γ-globulins involved depends on the quality of the purified sheep anti-rabbit γ-globulins serum and must be checked with each preparation of this second antibody).

After mixing the reagents, incubation was carried out at +4°C for 3 days. The bound fractions were then sedimented by centrifugation for 30 min at 3,000 rpm (about 2,200 g) at +4°C. The supernatant phases were eliminated by inverting the tubes which were then allowed to stand on cotton wool for 30 min.

The total amount of radioactivity involved and the amounts bound to the antibody were measured for 2 min on a well-type gamma counter. The found values were corrected for nonspecific binding, and the concentrations of APS-314d in samples were obtained from the calibration curve smoothed by a 4-PL model.

32.5.2.6 Validation and Cross-Reactivity Check

The assay method was validated and assay performance characterization established.

Accuracy: the absolute value of the difference within and between day was lower than or equal to 9.0%, and the limit of quantification can be taken as 3 pg/mL.

Precision: the within-assay coefficient of variation was between 4.0% and 10.1%, and the between-assay coefficient of variation was between 6.8% and 10.4% over the range 3.13–100 pg/mL.

Table 32.1

	Standard point	Samples	Human blank plasma	Assay buffer		Iodinated APS-314d	Mixture containing rabbit anti-beraprost serum, sheep anti-rabbit γ-globulins, and rabbit γ-globulins
Total radioactivity (three replicates)					Allow to stand for 1 h at room temperature	0.05	
Maximum binding Bo (three replicates)			0.1	0.3		0.05	
Nonspecific binding (three replicates)	0.1 (M2)		0.1	0.2		0.05	0.05
Standard curve (three replicates)		0.1		0.3		0.05	0.05
Samples (three replicates)		0.1		0.1		0.05	0.05
Quality controls (three replicates)							

Specificity: determined by Abraham's method (1974) at 50% inhibition of maximum binding, the percentage cross-reactions of the APS-315d Na, APS-314 l Na, and APS-315 l Na isomers in the assay of APS-314d Na were 2.4, 0.5 and 0.1%, respectively. This establishes, by comparing to the results using the beraprost (four isomers) as radiolabeled reagent, the complete fall down of the cross-reactivity and allows its use after administration of beraprost sodium in human.

CRITICAL ASSESSMENT OF THE METHOD

This way of using a single isomer labeled with high purity is an efficient alternative in order to achieve a stereospecific immunoassay. This immunoassay gives reliable information in terms of safety pharmacokinetics. The application in pharmacokinetic studies of each isomer can be made. Only few amounts of isomer for tagged molecule and standard curves are requested.

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32.6 Enzyme Sandwich Immunoassay with Monoclonal Antibodies for the Detection of HBeAg and anti-HBe

PURPOSE AND RATIONALE

The commercially available Enzygnost HBe monoclonal kit (distributed by Dade Behring) can be considered as an example for an immunoassay which combines many favorable features, e.g., nonisotopic label, sandwich immunoassay principle, use of monoclonal antibodies, determination of antigen and anti-antigen antibodies in a single test, microtiter plate format, possibility for semi- and full automation.

HBeAg and the corresponding antibody anti-HBe are found exclusively in connection with hepatitis B virus infection.

Seroconversion from "HBeAg positive" to "anti-HBe positive" is a favorable sign indicative of convalescence and an uncomplicated course.

Further information is found in Hollinger (1990), Bruss et al. (1988), Carman et al. (1989), and Brunetto et al. (1990).

The Enzygnost HBe monoclonal kit is based on the sandwich principle. HBe antigen (HBeAg) in the sample binds to monoclonal anti-HBe antibodies to the surface of the walls in the microtitration plate. Unbound sample constituents are then removed by washing, peroxidase-conjugated monoclonal antibodies to HBeAg are added and bind to the remaining free antigen determinants.

The excess conjugate is washed out, hydrogen peroxide substrate with chromogen is added and reacts with the bound peroxidase producing a blue color. This enzymatic reaction is stopped by the addition of a stopping solution and the resulting yellow color is measured. The resultant color intensity is proportional to the concentration of HBeAg in the sample.

In the test for anti-HBe, anti-HBe antibodies in the sample block the HBeAg pipetted with the positive HBeAg reagent. When the resulting mixture is then used in the HBeAg test, no HBe antigen or minimal HBeAg is detected if the sample is positive for anti-HBe. The color intensity of the sample is inversely proportional to the concentration of anti-HBe.

PROCEDURE

32.6.1 Reagents

Enzygnost HBe monoclonal (test plate): microtitration plate coated with monoclonal antibodies to HBe antigen

Anti-HBe/POD Conjugate: Monoclonal anti-HBe, conjugated with peroxidase (POD), preservative: phenol (max. 1 g/L)

HBeAg reagent, positive: genetically engineered HBe antigen, stabilized with bovine serum albumin and Synperonic, lyophilized, preservative: phenol (max. 1 g/L)

Anti-HBe Control, positive: Human serum containing monoclonal antibodies to HBe antigen, preservative: phenol (max. 1 g/L)

HBe Control, negative: Negative human serum for the HBeAg and anti-HBe tests, preservative: phenol (max. 1 g/L)

Washing Solution POD (concentrate): Phosphate buffer solution (90 mmol/L) containing Tween (18 g/L), preservative: phenol (max. 1 g/L)

Buffer/Substrate TMB: Hydrogen peroxide (approx. 0.1 g/L in acetate buffer solution (25 mmol/L)), preservative: 1-butanol (max. 10 mL/L)

Chromogen TMB: Tetramethylbenzidine dihydrochloride (5 g/L)

Stopping Solution POD: 0.5 N sulphuric acid

32.6.1.1 Preparation of the Reagents

For each test plate, dilute 20 mL washing solution POD to 400 mL with distilled or demineralized water.

Working Chromogen Solution: For each test plate, dilute 1 mL Chromogen TMB with 10 mL Buffer/Substrate TMB in the empty plastic bottle supplied with the kit and store closed and protected from light.

Working solution of HBeAg reagent, positive: Before the test reconstitute a vial of HBeAg reagent, positive with 13 mL distilled or demineralized water. Shake gently to mix and ensure that the reagent has completely dissolved before use (approx. 30 min).

32.6.1.2 Equipment

Incubator: Covered water bath (+37 ± 1°C) or comparable incubation methods

BEP II: For automatic dispensing of reagent and washing

BEP III: For automatic processing of the test after dispensing the samples as well as for evaluation

BEP 2000: For fully automatic processing and evaluation of the test

32.6.2 Assay procedure

32.6.2.1 Procedure for HBeAg test using the BEP II (detection of HBeAg):

1. Dispense samples: Pipette 100 µL/well HBe Control, negative into four wells, 100 µL of HBeAg Reagent, positive into the next well and then fill the following wells with 100 µL of undiluted sample. At the end of the series/plate, pipette 100 µL of HBeAg reagent, positive once more and cover with foil.
2. Incubate: Incubate at 37 ± 1°C for 60 ± 2 min.
3. Wash: Remove the foil, aspirate the wells, and wash two times with approx. 0.3 mL/well of diluted washing solution. Proceed immediately to the "Dispense conjugate" step.
4. Dispense conjugate: Fill each well with 100 µl of anti-HBe/POD conjugate, cover with fresh foil, and immediately place into the incubator.
5. Incubate: Incubate at 37 ± 1°C for 60 ± 2 min, then proceed immediately to the "Wash" step.
6. Wash: Remove the foil and wash four times as described in "3. Wash."
7. Dispense substrate: Pipette 100 µL of the working chromogen solution into each well and cover the plate with fresh foil.
8. Incubate: Incubate at +18 to +25°C for 30 ± 2 min, protected from light.

9. Stop reaction: Remove the foil and add 100 µL of stopping solution POD to each well, keeping to the same timing as in "8. Dispense substrate."
10. Read: At 450 nm within 1 h.

The use of a photometer with two wavelengths (measurement and reference beams) is to be recommended. The absorbances of the control samples and patient samples are to be measured at a wavelength of 450 nm. The wavelength recommended for the reference reading is 650 nm (if necessary between 615 and 690 nm).

32.6.2.2 Procedure for Anti-HBe Test Using the BEP II (Detection of Anti-HBe)

1. Dispense samples: Pipette 25 µL/well HBe control, negative into four wells, 25 µL of anti-HBe control, positive into the next well, and then fill the following wells with 25 µL of undiluted sample. At the end of the series/plate, pipette 25 µL of anti-HBe control, positive once more.
2. Binding of the anti-HBe: Directly after dispensing the samples, add 75 µL of HBeAg reagent, positive, (working solution) into each well containing the 25 µL of sample or control, then seal with foil.

Perform the subsequent steps as described for the HBeAg test, i.e., continue processing starting at "2. Incubate."

32.6.2.3 Test Procedure Using the BEP III

Before using the BEP III, perform the sample dispensing steps (Sect. 32.6.2.1 from "procedure using the BEP II"). Immediately afterward, place the uncovered test plates into the BEP III. The test is then processed fully automatically.

32.6.2.4 Test Procedure Using the BEP 2000

The sample dispensing steps and the subsequent processing of the test are performed fully automatically by the analyzer.

EVALUATION

The evaluations are performed automatically if the BEP III or BEP 2000 is used. The following sections apply if the measurements are carried out without a software.

32.6.3 HBe Test

Calculate the mean absorbance value of the negative controls, then calculate the cut-off value by adding 0.050:

$A_{(neg)} + 0.050 = \text{cut-off}$

Based on the criteria of the test, the samples are classed as follows:

1. $A_{sample} < \text{cut-off-10\%} = \text{HBeAg negative}$
2. $\text{cut-off-10\%} \leq A_{sample} \leq \text{cut-off} + 10\% = \text{HBeAg equivocal}$
3. $A_{sample} > \text{cut-off} + 10\% = \text{HBeAg positive}$

32.6.4 Anti-HBe Test

Calculate the mean absorbance value of the negative controls, then calculate the cut-off value by multiplication with 0.5:

$A_{(neg)} \times 0.5 = \text{cut-off}$

Based on the criteria of the test, the samples are classed as follows:

1. $A_{sample} > \text{cut-off} + 10\% = \text{anti-HBe negative}$
2. $\text{cut-off-10\%} \leq A_{sample} \leq \text{cut-off} + 10\% = \text{anti-HBe equivocal}$
3. $A_{sample} < \text{cut-off-10\%} = \text{anti-HBe positive}$

Samples with equivocal results must be retested in double determinations. If in the retest, the mean value of double determination is greater than or equal to the cut-off, or less than the cut-off, the initial equivocal result can be ignored and the sample to be considered reactive or negative as appropriate.

32.6.5 HbeAg

Precision (within day): 3.8–6.4%
Precision (between day): 8.7–11.4%

32.6.6 Anti-Hbe

Precision (within day): 3.2–7.4%
Precision (between day): 3.4–6.3%

CRITICAL ASSESSMENT OF THE METHOD

The method involves a lot of incubation and washing steps which is due to the use of the enzyme label. In addition, the incubation conditions (time and temperature) have to be kept very strictly to meet the product specifications. These requirements can hardly be fulfilled for longer sample batches by a manual procedure. Therefore, automation at least for the processes of dispensing of reagents and washing is

recommended. The best results, however, will be obtained with the analyzers optimized for product performance ("closed systems" with reagents and instrumentation from the same manufacturer). User-defined modifications are not supported by the manufacturer and may affect the assay results.

The example described above supports the general statement made in the introduction that EIAs which have to fulfill high-quality requirements with regard to sensitivity and robustness likewise need sophisticated techniques and/or automation.

MODIFICATION OF THE METHOD

The number of incubation and washing steps can be greatly reduced by employing "direct" instead of "indirect" labels (enzyme generates a signal only by reaction with a substrate). In addition to the radioactive label, nonisotopic alternatives with improved features (e.g., time-resolved fluorescent, chemiluminescent, electrochemiluminescent labels) have become available. Immunoassays based on these labels show an excellent performance even by manual procedure, automation is only required for a higher sample throughput. The advantages of luminogenic compared to chromogenic substrates (e.g., higher sensitivity and dynamic range of signal) are often used to replace the chromogens by luminophors in already available tests and systems.

An example for an anti-HBe test with a direct label is the electrochemiluminescence immunoassay "ECLIA" from Roche Diagnostics (automated on "Elecsys" immunoassay analyzer), whereas the Immulite anti-HBe test from Diagnostics Products Corp. represents an enzyme-amplified chemiluminescence immunoassay with sustained signal (automated on the Immulite chemiluminescent system).

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Bioanalytical Assays – Toxicokinetics

33

Karl-Heinz Lehr

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PURPOSE AND RATIONALE

Toxicokinetics is defined as "the generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure. These data may be used in the interpretation of toxicology findings and their relevance to clinical safety issues" (ICH Guidance Toxicokinetics 1994).

PROCEDURE

33.1 Main Group or Satellite Animals?

Whenever possible, toxicokinetic measurements are performed in all animals of the toxicity study. This is the most representative approach, and it allows the individual PK data to be directly correlated with the toxicological findings. The second choice is the toxicokinetic measurement in representative subgroups or in satellite groups. Satellite groups are animals that are treated and housed under conditions identical to those of the main study animals. The use of satellite animals is indicated, for example, in small animals, where the collection of a relatively large volume of blood may influence the toxicological findings.

33.2 Number of Animals and Time Points?

In ICH Guidance Toxicokinetics 1994, it is stated: "The number of animals to be used should be the minimum consistent with generating adequate

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toxicokinetic data," and "The area under the matrix level concentration-time curve (AUC) and/or the measurement of matrix concentrations at the expected peak-concentration time C_{max}, or at some other selected time $C_{(time)}$, are the most commonly used parameters." In large animals (e.g., dogs), the number of animals is usually fixed by the number of animals, which is necessary for safety evaluation. The withdrawal of a sufficient number of blood samples (6–9) per animal is not a problem. However, in small animals like rodents, it is recommended not to collect more than 10% of the blood volume during the AUC sampling interval (BVA/FrAME/RSPCA/UFAW Working Group of Refinement 1993; Cayen 1995). According to a new guidance from Diehl et al. (2001), the volumes per day are specified according to the recovery period. Diehl et al. (2001) limit the total daily volumes of multiple sampling to 7.5% of the circulatory blood volume at a recovery period of 1 week, 10–15% at a recovery period of 2 weeks, and 20% at a recovery period of 3 weeks. The optimum number of time points is always a compromise between blood volume restrictions and reliable assessment of TK parameters (AUC and C_{max}). Pai et al. (1996) compared for three different compounds the AUCs from intensive (full) (10–15 time points with 4–5 rats/time point) with sparse sampling schemes (5 time points with 2 rats/time point). Using Monte Carlo simulation, Pai et al. (1996) could show that the deviation of AUC estimation of the sparse sampling scheme from the full sampling scheme was not larger than 10%. Thus, a sparse sampling scheme with 5–7 time points with 2–3 animals per time point is well suited for the reliable determination of systemic exposure in small animal toxicity studies.

The dried blood spot (DBS) technique was recently rediscovered and is under intensive investigation (Burnett 2011) and regulatory discussion (Beharry 2010), which may significantly impact the above-mentioned considerations. The dried blood spots approach to toxicokinetic and pharmacokinetic sampling requires less blood at each time point than for plasma or serum analysis, meaning that even for small animals, serial rather than composite TK profiles can be obtained from individual animals. A decision tree for choosing plasma or dried blood spot matrix depends on blood/plasma ratio and constancy in fraction unbound for plasma; constancy of hematocrit and blood cell affinity is given by G. Emmons and M. Rowland (2010).

33.3 Evaluation of Samples from Control Animals?

In note 8 of ICH Guidance Toxicokinetics 1994, it is stated: "It is often considered unnecessary to assay samples from control groups. Samples may be collected and then assayed if it is deemed that this may help in the interpretation of the toxicity findings, or in the validation of the assay method." In the introduction of the CPMP Guideline on the evaluation of control samples in nonclinical safety studies of the EMEA (2005) this topic is further elaborated in light of a survey conducted by the European Federation of Pharmaceutical Industry, which showed that controls contamination during toxicology studies often occurs. The guideline requests: "Controls sampling and analytical procedures should be integrated in the toxicokinetic evaluation normally conducted in support of nonclinical safety studies. The analysis of samples from controls may need to be performed simultaneously with those from dosed animals."

33.4 Analytical Methods

The analytical methods to be used in toxicokinetic studies should be specific for the entity to be measured and of adequate accuracy and precision. The limit of quantification should be adequate for the measurement of the range of concentrations anticipated to occur in the generation of the toxicokinetic data (ICH Guidance Toxicokinetics 1994).

33.5 Toxicokinetic Evaluation

The following aspects should be considered for toxicokinetic evaluation:

Pharmacokinetic profile of the compound (exposure)
Dose dependency of AUC and C_{max}
Chances of exposure during the course of the toxicity study
Gender differences

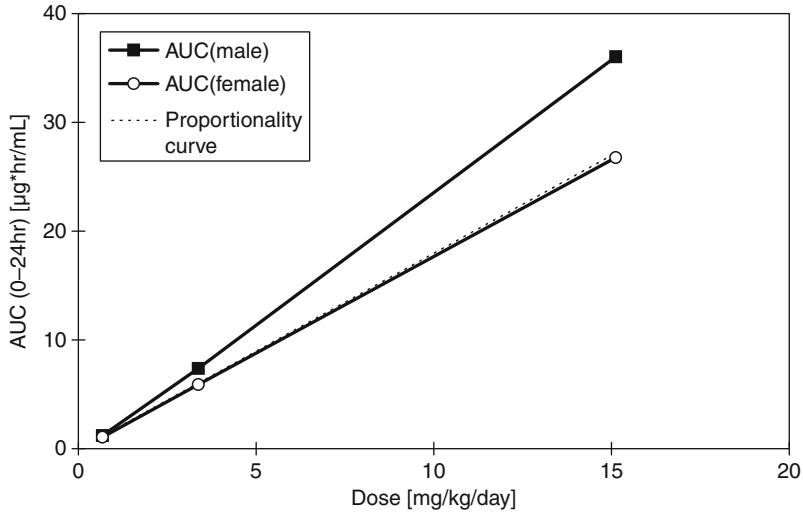

Fig. 33.1 Proportional dose dependency of AUC in an intravenous toxicity study in rat with the test compound A

33.5.1 Pharmacokinetic Profile of the Compound (Exposure)

For toxicokinetic purposes, it is usually sufficient to describe the systemic burden in plasma or serum of the test species with the test compound and/or its metabolites. The area under the matrix-level concentration-time curve (AUC) and/or the measurement of matrix concentrations at the expected peak-concentration time C_{max}, or at some other selected time (For example C (24 h) as trough value) $C_{(time)}$, is the most commonly used parameters. According to the supplementary notes in ICH Guidance Toxicokinetics 1994 for a profile (e.g., 4–8), matrix samples during a dosing interval should be taken to make an estimate of C_{max} and/or $C_{(time)}$ and area under matrix concentration time curve (AUC).

33.5.2 Dose Dependency of AUC and C_{max}

According to the ICH Guidance Toxicokinetics 1994, it is one of the primary objectives of toxicokinetics to describe the systemic exposure achieved in animals and its relationship to dose level.

At pharmacological and clinical doses, it can be generally assumed that most of the drugs show linear pharmacokinetics. Linear pharmacokinetics is given when exposure (AUC) is proportional to dose and principal pharmacokinetic parameters like bioavailability, elimination rate, volume of distribution, and clearance are independent on dose. For toxicokinetic studies, however, nonlinear pharmacokinetics is much more frequent than linear pharmacokinetics. This is mainly due to the fact that at very high doses, most systems in the body are likely to be stressed and, possibly, saturated to some degree. Thus, additional to the dose proportional increase of exposure (Fig. 33.1) and a less than proportional increase (Fig. 33.2) and a more than proportional increase (Fig. 33.3) is very frequently observed. For a less than proportional increase of exposure, a saturation of absorption processes or a concentration dependent change of volume of distribution should be considered as a potential cause. For a more than proportional increase of exposure saturation of metabolic elimination pathways, saturation of renal of biliary excretion of parent compound or a concentration dependent change of volume of distribution should be considered as a potential cause.

33.5.3 Chances of Exposure During the Course of the Toxicity Study

According to the ICH Guidance Toxicokinetics 1994, the description of the relationship of exposure to the time course of the toxicity study belongs to the primary objective of toxicokinetics. This objective may

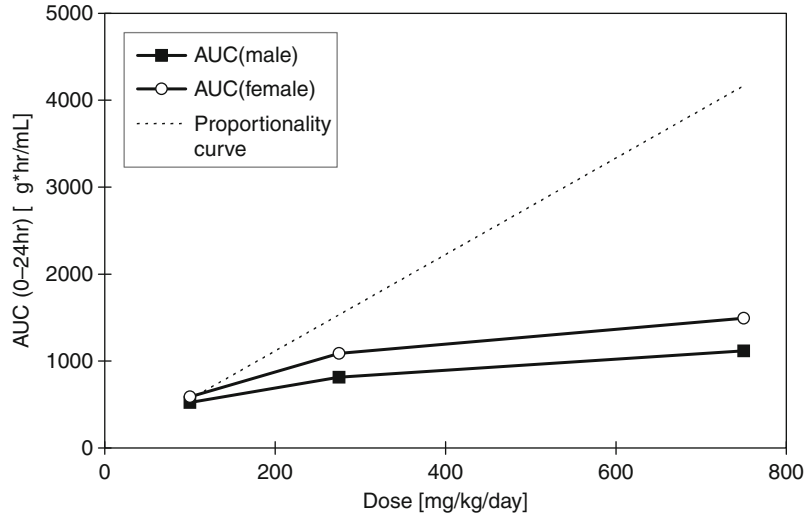

Fig. 33.2 Less than proportional dose dependency of AUC in an oral toxicity study in rat with the test compound B

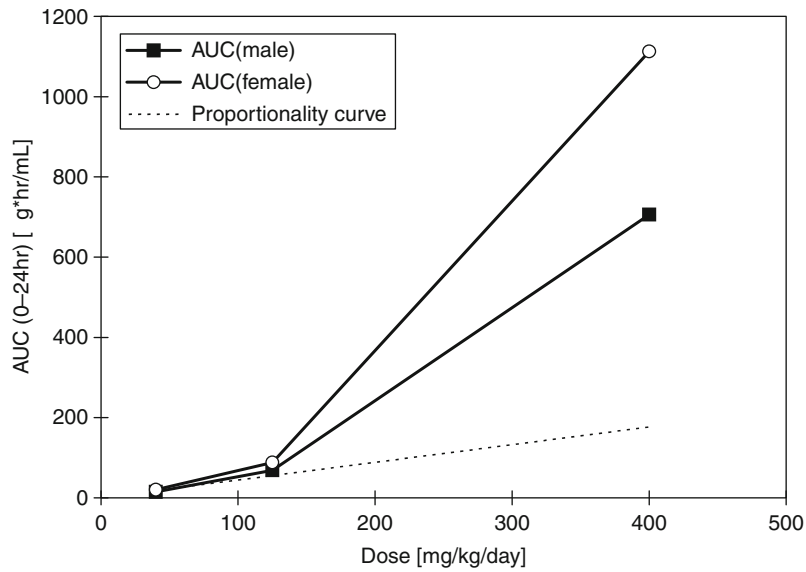

Fig. 33.3 More than proportional dose dependency of AUC in an oral toxicity study in dog with the test compound C

be achieved by derivation of pharmacokinetic parameters from measurements made at appropriate time points during the course of the individual studies. In the short-term studies (1 month or shorter), day1 and a day at the end of the toxicity study may be appropriate sampling days. In the long-term studies, day1, a day after one-third of the study duration, and a day at the end of the toxicity study may be appropriate sampling days. Increasing exposure may occur during the course of a study for those compounds, which have a particularly long plasma half-life. Conversely, unexpectedly low exposure may occur during a study as a result of auto-induction of metabolizing enzymes. However, other facts can also play a role in changes of exposure during the course of the study. Very often, rats and mice were used in an age at which they are not sexually matured, and during the study, the sexual maturation with their known impact on the rate and extent on metabolism takes place in the first 2 months. The harm of elimination pathways (e.g., nephro- or hepatoxicity) by the test compound can be another reason for changes in exposure. A more trivial

reason as the aging or the change of the administered batch with impact on bioavailability should also be considered.

33.5.4 Gender Differences

According to the ICH Guidance Toxicokinetics 1994, it is normal to estimate exposure in animals of both genders unless some justification can be made for not so doing. For evaluation, both genders should be evaluated separately. The assessment of exposure data of the two genders can be performed just by calculation of the ratio of AUC and C_{max} and elimination half-life in males and females. However, additional factors such as, for example, size of the investigated groups (with respect to random variation) and sexual maturity have to be considered. As a rule of thumb, it can be stated that in rodents, a gender difference is quite usual, when metabolism is involved as major elimination pathway, whereas in nonrodents, distinct gender difference is rather seldom.

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33.6 Practical Examples

33.6.1 HPLC UV Assay

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. The UV absorption properties of the compound are used to determine the compound of interest. Since many of the endogenous compounds also show absorption in that wavelength range, a tailored sample work-up has to provide additional selectivity for the compound of interest. In addition, the plasma proteins have to be removed by the work-up in order to avoid blocking of the HPLC columns. Three principles are generally applied to remove the proteins from the sample: Protein precipitation, liquid/liquid extraction and liquid/solid extraction.

PROCEDURE

A typical method using liquid/liquid extraction was described by Shum et al. (1994) for the determination of an ACAT inhibitor in rat plasma. Internal standard, 1 mL water and 1 mL of n-pentane were added to 0.25 mL volume of plasma. After shaking for 15 min and centrifugation for 15 min at approx. 1200 g, the upper organic phase was transferred and evaporated. The residue was reconstituted in 200 µL of mobile phase, and 100 µL was injected onto the HPLC column for analysis. The HPLC system consisted of a Bio_Sil ODS-5S column (150 × 4 mm; 5 µm particle size) and a UV detector operating at 250 nm. The mobile phase consisted of 55 % acetonitrile, 27 % methanol, and 18% water. The flow rate was 1.9 mL/min. The column temperature was ambient temperature.

EVALUATION

For calibration, a set of eight standards was prepared by fortifying blank plasma with concentrations ranging from 60 ng/mL to 8000 ng/mL. In addition, three sets of quality control samples are prepared of low, medium and high concentration within the range of standards. From the response of the eight standards a calibration curve is determined using the least-square linear regression using a weighting factor of 1/concentration squared. The response is generally the peak height or peak area ratio of analyte/internal standard.

MODIFICATIONS OF THE METHOD

For sample preparation, protein precipitation or solid phase extraction or any combination of the different principles can also be applied. Kim et al. (2002) described a simple toxicokinetic assay for a prodrug and its two consecutively hydrolyzed active antifungal compounds. A 0.2 mL aliquot of dog plasma was added to 0.6 mL methanol containing the internal standard compound in a 2 mL microcentrifuge tube. The tubes were vortexed at high speed and centrifuged at 4500 g for 5 min. The supernatant was transferred into a new tube and stored for a minimum of 12 h at −20 °C to complete the precipitation. The samples were again vortexed and centrifuged. 200 μL of the supernatant were injected onto the HPLC column. In order to cover all three analytes and the internal standard in a reasonable HPLC-run time, a gradient elution on an Ultrasphere ODS column was applied instead of isocratic elution.

An appropriate tool to enhance the selectivity and the feasibility of the work-up procedure using liquid/liquid extraction is the subsequent back extraction into aqueous phase. For example, Los et al. (1996) described a toxicokinetic assay for diltiazem and its two metabolites. To 0.2 mL rat plasma, internal standard solution and a pH 7.3 phosphate buffer and methyl-t-butyl ether were added. The analytes were extracted into the organic phase. The organic phase was transferred and the analytes back-extracted into 250 μL of 0.05 M phosphoric acid. The phosphoric acid was modified with 100 μL acetonitrile before injection onto HPLC.

Kawauchi et al. (2001) described a toxicokinetic assay for a thymidin phosphorylase inhibitor. This compound was water-soluble and not extracted with organic solvents. Protein precipitation failed also due to the appearance of a lot of interfering peaks. Therefore, solid-phase extraction on a strong cation exchange sorbent (= RPS) was used for sample preparation. To 0.1 ml of plasma 50 μL of internal standard solution and 0.7 mL of 0.01 M HCl were added and mixed. The mixed sample was loaded 5 onto a Bond Elut PRS column (1 cc/100 mg) set up at a Vac Elut SPS 24 (Varian) that prior to the sample loading had been conditioned with 1 ml of methanol, and then with 1 ml of water. After passing the sample, the column was washed with 1 ml of water and then with 1 ml of methanol. The eluate was collected with 2 ml of 2 % ammonia solution - methanol in a glass test tube, and dried under a stream of N gas at 37 °C. The residue was reconstituted in 0.2 ml of 10 mM acetate buffer (pH 4.3), and a 60 ml aliquot was injected onto the HPLC column. The HPLC system consisted of a Mightsil RP-18 column (150 × 4.6 mm; 5 μm particle size) and a UV detector operating at 276 nm. The mobile phase consisted of 9 % acetonitrile and 91 % 7 mM sodium 1-hexanesulfonate in 10 mM acetate buffer (pH 4.3). The flow rate was 1.0 mL/min.

CRITICAL ASSESSMENT OF THE METHODS

The described methods are typical HPLC UV assays for drug level determination for toxicokinetic purposes. However, the conditions of sample preparation, the choice of the internal standard substance, the choice of the HPLC stationary and mobile phase and the wavelength of the UV detector have to be adjusted specifically to the properties of the analytes. Particularly, the lipophilicity, the pKa value, and the pH stability of the analytes have to be considered. Regarding selectivity and sensitivity of the assay, HPLC UV assays are not the cutting edge technology. However, in most cases the sensitivity is adequate, since doses, and concomitantly the drug levels, are usually quite high in toxicity studies. A very important aspect for assays used in toxicokinetics is huge range of drug concentration, which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid time consuming and error prone dilution procedures. The assay in plasma described by Shum et al. (1994) was validated from 0.06 to 8 μg/mL (dynamic range of 133), the assay described by Kim et al. (2002) was validated from 0.05 to 50 μg/mL (dynamic range of 1000), the assay described by Los et al. (1996) was validated from 0.01 to 5 μg/mL (dynamic range of 400) and the assay described by Kawauchi et al. (2001) was validated from 0.05 to 40 μg/mL (dynamic range of 800).

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Shum YY, Black A, Chang T (1994) Determination of 2,2-dimethyl N-(2,4,6-trimethoxyphenyl) dodecanamide, C1-976, in rat plasma by reversed-phase high-performance liquid chromatography. J Chromatogr Biomed Appl 653(2):205–209

33.6.2 Fluorescence Assay for Analytes with Native Fluorescence

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. The native fluorescence of the compound is used, to determine the compound with a relatively high sensitivity and selectivity.

PROCEDURE

A typical method was described by Kim et al. (1999). for the determination of a dopamine D receptor antagonist in rat plasma. 0.2 mL volume of plasma was diluted with 1.5 mL of 0.25 M potassium phosphate buffer, pH 8, followed by the addition of 20 µL of the internal standard (1000 ng/mL in methanol), which was a methyl analog of the analyte. The diluted sample was slowly applied on a 1 mL disposable ethyl cartridge which had been successively prewashed with 2 mL of methanol, 1 mL of acetonitrile, 1 mL of water and 1 mL of 0.25 M phosphate buffer, pH 8, using a vacuum manifold. The loaded cartridge was washed with 2 mL of water, then dried by airflow through under vacuum for 5 min. The analyte was eluted from the cartridge with 2 × 0.6 mL of methyl-t-butyl ether (saturated with water and containing 8 % triethylamine). The eluate was evaporated to dryness under nitrogen. The residue was dissolved in 0.4 mL of mobile phase and a 90 µL aliquot was injected onto the HPLC column. The HPLC system consisted of a YMC basic column (150 × 4.6 mm; 5 µm particle size) and a fluorescence detector. The detector was set at an excitation wavelength of 260 nm and an emission wavelength of 400 nm. The mobile phase consisted of 35 % acetonitrile and 65 % 0.05 M ammonium acetate. The flow rate was 1 mL/min. The column temperature was ambient temperature.

EVALUATION

For calibration, a set of five standards is prepared by fortifying blank plasma with concentrations ranging from 5 ng/mL to 100 ng/mL. In addition, three sets of quality control samples are prepared of low, medium and high concentration within the range of standards. From the response of the five standards a calibration curve is determined using the least-square linear regression with or without weighting factor. The response is generally the peak height or peak area ratio of analyte/internal standard.

MODIFICATIONS OF THE METHOD

For sample preparation, protein precipitation or liquid/liquid extraction can also be applied instead of solid phase extraction. Gluth et al. (1988) described for a toxicokinetic assay for Sotalol a threefold combination of these principles. A protein precipitation using 5 M perchloric acid was followed by a liquid/liquid extraction into a mixture of n-pentanol-chloroform 1/3 at pH 9. Thereafter, the organic phase was transferred to another glass tube and the analyte back extracted into 0.05 M sulfuric acid.

For the case that two analytes with quite different native fluorescence and different concentration ranges have to be determined, Chollet et al. (1998) describe an elegant solution. They developed a gradient elution program which generated an elution order suitable for an automated wavelength change in respect to reliable peak integration. The carboxylate and lactone form of Irinotecan and its metabolite were detected at λ_{ex} = 362 nm/λ_{em} = 425 nm and λ_{ex} = 375 nm/ λ_{em} = 560 nm, respectively.

CRITICAL ASSESSMENT OF THE METHODS

The described method is a typical HPLC fluorescence assay for drug level determination for toxicokinetic purposes. However, the conditions of sample extraction, the choice of the internal standard substance, the choice of the HPLC stationary and mobile phase and the combination of excitation and emission wavelength has to be adjusted specifically to the properties of the analytes. Particularly, the lipophilicity, the pKa value and the pH stability of the analytes have to be considered.

Regarding selectivity and sensitivity of the assay, HPLC fluorescence assays are clearly preferable to UV assays, however a prerequisite is the native fluorescence of the analytes. A very important aspect for assays used in toxicokinetics is a huge range of drug

concentration, which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid time consuming and error prone dilution procedures. The assay in plasma described by Kim et al. (1999) was validated from 5 to 100 ng/mL (dynamic range of 20), the assay in plasma described by Gluth et al. (1988) was validated from 50 to 1500 ng/mL (dynamic range of 30) and the assay in urine was validated from 2 to 100 µg/mL (dynamic range of 50).

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33.6.2.1 HPLC DBS MS/MS Assay

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite can be determined in blood samples instead of plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. The currently most versatile approach to perform a drug level determination in blood is the bioanalysis in dried blood spots (DBS) followed by quantification using the LC–MS/MS technique. This approach requires very low blood volumes and has consequently the potential to significantly reduce the number of animals required in a toxicokinetic study. Typical animal numbers required using dried blood spot samples were compared with animal numbers required when generating toxicokinetic data from plasma samples by Burnett (2011).

PROCEDURE

A typical procedure using dried blood spots combined with HPLC-MS/MS was described by Barfield et al. (2008) for the quantification of acetaminophen in dog blood. Replicate (n = 3 for each time point) 0.15-mL volume aliquots from each plasma sample were spotted onto FTA® Elute blood spot cards (supplied by Whatman, Sanford, USA) and allowed to dry at room temperature for 2 h. These were stored and shipped at room temperature in a sealed plastic bag (Fisher, Loughborough, UK) containing a sachet of desiccant (Sud-Chemie, Northwich, UK). For DBS analyses, a 3-mm-diameter disk was punched from the center of the DBS into a clean tube. Methanol (0.1 mL) containing internal standard ($[^2H_4]$-acetaminophen) laws was added and the tube vortex was mixed for approximately 30 s. The tube was centrifuged for 5 min at 3000x g and the supernatant transferred to a clean tube and a portion injected onto the HPLC-MS/MS system.

The HPLC-MS/MS system consisted of a CTC HTS PAL autosampler (Presearch, Hitchin, UK) with fast wash, an Agilent 1,100 binary pump (Palo Alto, CA, USA) with integrated column oven and divert valve, and a Polaris Amide C18, 3 µm, 50 mm × 3.2 mm i.d, HPLC column (Varian Limited, Oxford, UK). The post column flow was diverted to waste for first 0.5 min of each chromatographic run. During this time, flow (0.25 mL/min, methanol:water (1:1, v/v)) was provided to the MS by a Knauer pump (Presearch, Hitchin, UK). The chromatographic separation was achieved using a solvent gradient employing the mobile phase 10-mM ammonium formate containing 0.3% ammonia (A) and methanol (B). Following sample injection (3µL), the mobile phase was held at 98% A for 0.2 min. A ballistic gradient at 5% A for 0.3 min was followed by an isocratic period at 5% for 0.8 min. The mobile phase was then returned to 98% A by 0.9 min and was held at this composition until 1.5 min, before the injection of the next sample. The flow rate was 1 mL/min; the column was maintained at 40°C. The HPLC effluent was split approximately 1:3 before entering the ion source. MS detection was performed on a Sciex API-3000 (Applied Biosystems/MDS Sciex, Canada) equipped with a TurboIonspray™ ion source. The source temperature was 450°C with a turbo gas flow of 7 L/min (N_2) and a nebulizer gas setting of 10 (N_2). The curtain gas and collision gas settings were 10 and 6, respectively (both N_2). The characteristic precursor $[M + H]^+$ to product ions transitions, m/z 152 → 110 for acetaminophen and 156 → 114 for the internal standard respectively, was used as selected reaction monitoring transitions. A dwell time of 200 ms was used for both transitions. The pause time was 5 ms.

EVALUATION

For calibration, a set of eight standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with blank whole dog blood to target concentrations ranging from 0.1–50 μg/mL. In addition, sets of quality control samples were prepared within the range of standards. When required, the DBS QCs were stored at room temperature in a sealed plastic bag containing desiccant until analysis. A calibration plot of analyte/internal standard peak area ratio versus the nominal concentration of acetaminophen was constructed, and a weighted $1/x^2$ linear regression was applied to the data.

MODIFICATION OF THE METHOD

One of the main considerations before starting the in-life phase of a safety study is confirmation of the type of DBS card to be used for sample collection. This requires some bioanalytical development and/or validation work to have been carried out in advance. Burnett (2011) has described in detail card selection and sample spotting considerations.

CRITICAL ASSESSMENT OF THE METHODS

Beharry (2010) from UK MHRA stated: "Dried blood spots is clearly a technique that falls into the remit of the replacement, refinement and reduction of animals in research (3Rs), which is fully supported by the MHRA." Given that this is a "relatively" new technique, it will be necessary to provide adequate details of analytical methods used, and these should conform to the requirements set out in ICH S3A.

Timmerman (2011) have outlined in a white paper the intensive discussion within the European Bioanalysis Forum (EBF) concerning the validation requirements needed to generate robust and reproducible concentration data from DBS experiments. They identified and evaluated the validation requirements needed in addition or in excess of the method validation practices established for liquid assays. In consequence, that means that the validation efforts using DBS are considerably higher.

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Distribution - In Vitro Tests - Protein Binding

34

Jens Riedel

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34.1 Introduction

The protein binding plays an important role in the pharmacokinetics and pharmacodynamics of a drug. Plasma binding can also be viewed as a circulating site of storage, releasing the drug to tissue with higher affinity. Only the unbound drug is thought to be able to diffuse across membranes and to interact with an effector site to produce a therapeutic response.

The extent of protein binding in the plasma or tissues controls the volume of distribution and affects both hepatic and renal clearance. The volume of distribution (V) depends on the fraction unbound in plasma (f_u), the fraction unbound in tissue (f_{uT}), the volume of tissue (V_T), and the volume of plasma (V_P) by the equation:

$$V = [f_u/f_{uT}]V_T + V_P$$

For all drugs with a volume of distribution value >30 L, changes in f_u therefore translate directly into changes in V (Rowland and Tozer 1995). According to their incidence on drug distribution, two types of drug plasma binding can be distinguished. It can be restrictive, when the quantitative distribution of the drug is limited, respectively vice versa nonrestrictive, when the distribution is not limited. A restrictive binding is characterized by blood concentrations higher than tissue concentrations. Large volumes of distribution values indicate a nonrestrictive plasma binding, especially if the binding capacity of some tissues is greater than that of the circulating protein and the greatest part of the applied dose penetrates into tissues (Urien et al. 2001).

The influences of plasma binding on drug elimination may be best understood by consideration of clearance.

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All organ clearance models incorporate a protein-binding term. For example, the conversion of the intrinsic clearance (CL_{int}) to the hepatic clearance (CL_{hep}) involves the use of equations describing the well-stirred and parallel tube models of hepatic clearance (Pang and Rowland 1977; Wilkinson and Shand 1975).

$$CL_{hep} = \frac{Q^{*}fu^{*}CL_{int}}{Q + fu^{*}CL_{int}} (\text{Well} - \text{stirred model}) \tag{34.1}$$

$$CL_{hep} = Q^{*}\left(1 - e\frac{-CL_{int}{}^{*}fu}{Q}\right) (\text{Parallel tube model}) \tag{34.2}$$

Q: hepatic blood flow

Taking into consideration that (hepatic) clearance is a product of (hepatic) blood flow and (hepatic) extraction ratio of the drug ($CL_{hep} = Q_{hep}{}^{*}E_{hep}$), two types of elimination can be distinguished. For very highly extracted drugs E_{hep} approaches the factor 1. As the product $CL_{int}{}^{*}f_u$ for these drugs is greater than Q, CL_{hep} approaches Q. Under this perfusion rate-limited condition, changes in fu have only small influence on the hepatic clearance. Conversely, the impact of the unbound fraction on the clearance is higher for drug with a small hepatic extraction ratio (Rowland and Tozer 1995).

Only the unbound fraction in plasma is filtered in the kidney. Therefore, the rate of filtration is the product of glomerular filtration rate and unbound plasma concentration. For a drug which is only filtered and all filtered drug is excreted into the urine, the renal clearance (CL_R) is the rate of filtration product:

$$CL_R = f_u{}^{*}GFR \tag{34.3}$$

The binding to plasma or subcellular liver fraction can be taken into account for the prediction of human pharmacokinetic parameters either from preclinical and/or in vitro metabolism data (Obach et al. 1997; Mahmood 2000). Obach (1999) showed by comparison the in vivo investigated clearance values and clearance values projected from in vitro intrinsic clearance data of 29 drugs that the inclusion of blood and liver microsomes binding values gave the best agreement.

The unbound fraction of the drug can be included for the calculation of the safety margin, based on the exposure of the drug from toxicological results in animal and the expected exposure in the first human study.

The binding of a drug, hence its amount of unbound fraction, can be changed by co-medication of another drug. The bound fraction of the primary drug is displaced by the secondary drug, which shows a higher affinity to the protein. The increase of free fraction can cause an increase of pharmacological effects, side effects, or/and toxicological effects. The quantitative and clinical importance of the drug-drug interaction from plasma protein binding depends on the total amount of the initial drug in the body that is bound to plasma before displacement, the extent of displacement, the extent of binding to tissue structures, and the apparent volume of distribution. However, the importance of displacement has been controversial and discussed by several authors. Rolan (1994), Sansom and Evans (1995), and Benet and Hoener (2002) have presented theoretic arguments about the limited cases when drug interaction has been regarded as significant. Rolan has shown with several examples that drug interactions, which have been attributed to plasma protein binding displacement, can be explained by other mechanism (e.g., inhibition of metabolism). He suggests an algorithm to determine the clinical significance of potential PPB displacement interactions. An interaction is likely and a clinical study should be performed to quantify the effects, if the drug of interest has a plasma protein binding above 90%, a narrow therapeutic index, the hepatic extraction ratio is high, and the drug is given intravenously.

The plasma protein profiles in humans are influenced by age, gender, and disease (Verbeeck et al. 1984; Grainger-Rousseau et al. 1989). Elderly tend to have decreased plasma protein concentrations. Disease may cause hypoalbuminemia as found in patients with burns, cancer, cardiac failure, cystic fibrosis, enteropathy, inflammations, liver impairment, malabsorption, nephrotic syndrome, renal failure, sepsis, and trauma. In acute renal failure the concentration of total plasma concentration, particularly of albumin, is significantly reduced (by approximately 25%). Significant changes in plasma composition have been observed in pregnancy and in neonates (Notarianni 1990). Pregnancy results in decreased drug-protein binding to albumin. Fetal albumin present in newborn

infants has less binding affinity for acidic drugs. Thus, free drug concentrations in neonates are higher than those observed in children or adults.

The PPB of racemic drugs is potentially stereoselective, as a consequence of chiral discriminative properties of the binding sites of the protein fractions. However, the limited available data on the binding of enantiomers indicate the differences are small (Pacifici and Viani 1992). The concentrations of total protein, albumin, and α-acid glycoprotein in plasma differ slightly between human and animal species (Davies and Morris 1993). Interspecies differences in the stereoselectivity of protein binding have been reported for various drugs (Lima 1988; Lin et al. 1990, 1991).

Beside the measurement in whole plasma, the PPB can be characterized according to the participated proteins. It may involve, in the simplest case one, or even several proteins. The most frequent situation is that of a drug-albumin complex since albumin is the protein having the highest concentration in plasma. It is by far the most important drug carrier. The plasma level of albumin in a healthy adult is 35–50 g/L (Rowland and Tozer 1995), with a mean value of 41.8 g/L (Davies and Morris 1993). It is mainly involved in acidic drug binding and has three major drug binding sites. Noncarboxylic acids (e.g., endol derivates) bind to site I that is also known as the warfarin site. Site II binds carboxylic acids through hydrophobic forces and is also selective for benzodiazepine and indole compounds (Fehske et al. 1981). Site III is specific for cardenolides and biliary acids (Kragh-Hansen et al. 1985; Kragh-Hansen 1990). Oral anticoagulants, oral hypoglycaemics and nonsteroidal anti-inflammatory drugs (NSAIDs) are examples of drugs that are extensively bound to albumin with a fraction unbound less than 10% and sometimes less than 1%.

The second main plasma protein fraction is α_1-acid glycoprotein. The normal plasma levels are 0.4–1.0 g/L (Rowland and Tozer 1995). As the protein contains an anionic charge, it attracts basic molecules (Muller 1989; Kremer et al. 1988). Drugs with high affinity to α_1-acid glycoprotein include synthetic opioids (e.g., fentanyl, methadone), antiarrhythmic agents (e.g., lidocaine, disopyramide) and tricyclic antidepressants (Jack 1992). Plasma concentrations of α_1-acid glycoprotein increase in the presence of stress, inflammation, malignancy, myocardial infarction, and various hematology disorders.

Other blood components, such as erythrocytes, lipoproteins, and α-, β- and γ-globulins, and tissue proteins, are also capable of drug-protein interactions.

The addition of anticoagulants to the plasma can alter the PPB and should be evaluated prior. Hence, the binding is determined in plasma (e.g., of human) collected using potassium EDTA, sodium citrate, and lithium heparin anticoagulants and compared against serum binding. The effect of an anticoagulant is considered acceptable, if the ratio of the free fraction in plasma to serum is within 0.8 and 1.25.

The affinity of drugs to proteins can be pH dependent. Hence, it is necessary to control the pH of the plasma prior. The pH value can be adjusted to an appropriate standard value (pH 7.4) by introducing carbon oxide or nitrogen gas. Also the stability of the drug in the matrix (e.g., plasma) should be verified prior to the assay. For instable compounds, the degradation (e.g., esterase) can be avoided by addition of appropriate inhibitor (e.g., sodium fluoride).

34.2 General Considerations

From the great variety of methods for the determination of protein binding three separation methods, equilibrium dialysis (ED), ultra filtration (UF), and ultracentrifugation (UC), and a non-conventional method with the binding to immobilized proteins has been chosen. The first methods are undoubtedly the most widely used because of their simplicity and general applicability to many different systems. Other methods, for example, size-exclusion chromatography, capillary electrophoresis, or spectroscopic methods, have been not described. Oravcová et al. (1995) gives a comprehensive review and comparison for these applications.

The methods, described in this chapter, are for the determination of adherent protein binding, which is reversible. Irreversible covalent binding (e.g., caused by reactive intermediates) has not been considered.

34.3 Analytics

The applied analytical method depends on the binding assay used, and on the phase of drug development when the protein binding is performed respectively, on the availability of radiolabelled substance.

Generally the highest purity of the compound is preferred, to avoid interferences with contaminants or degradation products.

In the stage of drug discovery, LC/MS-MS analytics is the method of choice to quantify the unbound drug concentration. The sensitivity can be increased by the use of radiolabelled substance. But, the radiochemical purity, isotope decay, if not ^{14}C-label is used; as well a sufficient specific activity must be taken into consideration (Wright et al. 1996). The concentrations of radioactivity in bound and unbound fraction are measured by liquid scintillation counting. The use of radiolabelled material allows easy examination of the potential of adsorption. However, the identity of the drug in unbound fraction should additionally be verified by HPLC-radiometry detection. In the early phase of drug discovery, high-throughput screening assays with 96 well plates, and fluorescence or UV readers are employed.

34.4 Evaluation of Binding Data

In characterizing the protein binding, several parameters of a drug can be experimentally determined—the unbound plasma concentration (c_u), the bound plasma concentration (c_b), the fraction bound (f_b), and the fraction unbound f_u. The determination can be performed at one defined concentration (e.g., 10 μM) or within a range including the pharmacological and toxicological relevant concentrations.

Additionally, the equilibrium association constant (K_A), equilibrium dissociation constant (K_D), and the number of protein binding per sites per class of binding site (n) are results of interest (Wright et al. 1996).

The drug protein association can be schematically represented as:

$$c_u + P \xleftrightarrow{K_A} c_b$$

P: protein concentration

whereas K_A is defined as $[c_b]/[c_u]^*[P]$, and $K_A = 1/K_D$

The equilibrium association constant K_A, or the corresponding dissociation constant (K_D) provides information on the affinity or the strength of the drug-protein association. A highly protein bound drug will typically have a K_A value ranging from 10^5 to 10^7 L/mol, and a drug with low to moderate protein binding will usually have a K_A value ranging from 10^2 to 10^4 L/mol (Wright et al. 1996).

Derivation of the second order equation for protein interactions yields an equation that describes c_b as a function of c_u:

$$c_b = \frac{n^*P^*K_A}{1 + K_A{}^*c_u} \tag{34.4}$$

This equation describes the bound drug concentrations as a function of unbound drug concentrations. A binding system with a single class of non-saturable binding sites can be described by:

$$c_b = n^*P^*K_A{}^*c_u \tag{34.5}$$

If the binding system consists of two classes of binding site, it is characterized by Eq. 34.6:

$$c_b = \frac{n_1{}^*P_1{}^*K_{A_1}}{1 + K_{A_1}{}^*c_u} + \frac{n_2{}^*P_2{}^*K_{A_2}}{1 + K_{A_2}{}^*c_u} \tag{34.6}$$

34.4.1 Graphical Methods

The graphical methods for the estimation of the protein binding parameters are limited to protein systems, which have only one class of binding sites. If the protein concentration P is known, the product n^*K_A can be calculated by using Eq. 34.4. The slope of the regression line is equal to $n^*K_A{}^*P$.

For saturable binding, the following graphical methods can be used after rearrangement and linearization of Eq. 34.5:

The Double–Reciprocal Plot (Fig. 34.1):

$$\frac{1}{v} = \frac{1}{n^*K_A{}^*c_u} + \frac{1}{n} \tag{34.7}$$

$$\text{Where } v = \frac{c_b}{P} \tag{34.8}$$

The Scatchard Plot (Fig. 34.2):

$$\frac{v}{c_u} = n^*K_A - v^*K_A \tag{34.9}$$

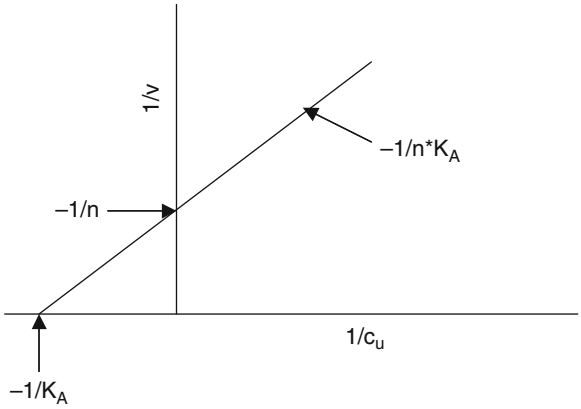

Fig. 34.1 Double reciprocal plot. The y-intercept is 1/n and the x-intercept is—$1/K_A$

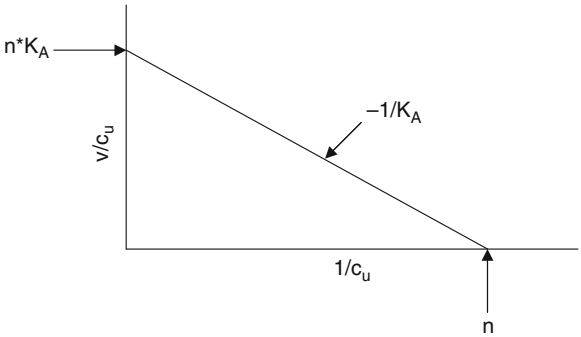

Fig. 34.2 Scatchard plot. The y-intercept is n^*K_A and the x-intercept is n. The slope is—K_A (Scatchard 1949)

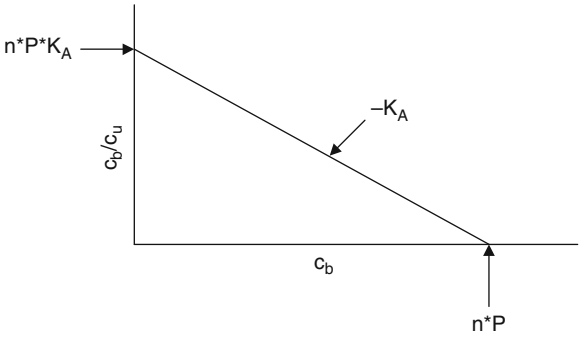

Fig. 34.3 Rosenthal plot. The y-intercept is $n^*P^*K_A$, the x-intercept n*P and the slope—K_A

$$\text{Where } v = \frac{c_b}{P} \tag{34.10}$$

The Rosenthal Plot (Fig. 34.3):
This modified Scatchard Plot is used if the protein concentration is unknown (Rosenthal 1967).

$$\frac{c_b}{c_u} = n^*K_A{}^*P - c_b{}^*K_A \tag{34.11}$$

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34.5 Ultrafiltration

PURPOSE AND RATIONALE

The ultrafiltration (UF) with semipermeable, size-exclusion membranes produces a separation of the free drug from macromolecules by employing a pressure gradient, which forces small molecules through the membrane. UF can either be used in an individual filtration unit or in a 96 well plate format system.

34.5.1 Conventional Ultrafiltration (Individual Devices)

34.5.1.1 Equipment and Material

- Plasma pool of different species.
- Stock solution of the drug prepared in an appropriate organic solvent (e.g., 10 mM, DMSO).
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl).
- Calibrated pH Meter.
- Nitrogen gas and carbon dioxide gas.
- Glass wool for lab purpose.
- 1.5 mL microcentrifuge tubes.
- Thermostatized centrifuge with fixes angle rotor (angle of inclination 33°) (e.g., Hettich Rotixa RP 001260, Tuttlingen, Germany).
- Disposable individual ultrafiltration unit (e.g., Centrifree Micropartition System No. 4104, Millipore, Bedford, MA, USA). It consists of two compartments—the sample reservoir and filtrate cup. A hydrophilic ultrafiltration membrane with a defined molecular cutoff (30 kDa) separates them.
- Single pipettes.

PROCEDURE

1. The frozen plasma is slowly thawed in cold water, or overnight in the refrigerator and filtered over glass wool. Protein binding of some drug is pH dependent. Therefore, the pH value of the freshly thawed plasma has to be adjusted to pH 7.3–7.4 by CO_2 or N_2. In the lab, carbon dioxide gas may be released by vaporizing small quantities of dry ice in an Erlenmeyer flask with plastic tube and small pipette for the introduction of the gas. Due to the strong pH lowering effect of the gas, the CO_2 should be introduced carefully and in small quantities. If the pH value falls below pH 7.3–7.4, it can be re-adjusted by degassing with nitrogen.
2. Aliquots of the stock solution diluted to 10 μM are added to 1 mL of pH-adjusted plasma, protein fraction, or buffer. The amount of organic content should not exceed more than 2% of the initial spiked plasma. Higher content could adversely interfere with protein binding.
3. The spiked sample (1 mL) is immediately placed in the ultrafiltration unit. Closing the sample reservoir with a provided cap before the centrifugation can prevent changes of the pH during the determination.

4. The sample is filtered through the membrane by centrifugation in a fixed angle rotor at 1,000–2,000 *g*. The flow rate depends on the protein concentration in the sample, which can differ between the species, the starting volume, the relative centrifugal forces, the rotor type, and the temperature. For the determination of the protein binding, the volume of the obtained filtrate should be a fifth (approx. 200 μL) of the initial volume of sample. Hence, for each matrix, the centrifuge time has to be determined beforehand.
5. In parallel, the nonspecific adsorption has to be investigated by using physiological PBS buffer or recommended, protein free serum filtrate.
6. The concentration of the free drug (c_u) in the ultrafiltrates or the concentration of bound fraction (c_b) in the retentate can be quantified by LC-MS/MS by means of a calibration curve. If radiolabelled substrate has been applied, liquid scintillation counting can be employed.

Complementary to the simple adsorption test, the mass balance of the separation could be investigated. This method is more accurate to determine the loss of free drug, which may occur due to variations in sample preparations methods, nonspecific protein binding, and to metabolism. The latter may also be tested in separate stability tests in plasma or applied matrix.

The mass balance is calculated by determining volumes by gravimetric and drug concentrations in both the retentate and ultrafiltrate.

34.5.2 Automated Ultrafiltration in a 96 Well Plate System

34.5.2.1 Equipment and Material

- Plasma pool of different species.
- Stock solution of radiolabelled or non-radiolabelled drug prepared in an appropriate organic solvent (e.g., 10 mM, DMSO).
- Phosphate buffered saline (PBS), pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl).
- Calibrated pH Meter with one bar measuring chain.
- Pure nitrogen (N_2) and carbon dioxide (CO_2). In the lab, carbon dioxide gas may be released by vaporizing small quantities of dry ice in an Erlenmeyer flask with stopcock, plastic tube, and small pipette for the introduction of the gas.
- Incubator.
- 96 well plate mixer.
- Centrifuge with swinging bucket rotor and sealed microtiter plate carriers.
- Liquid Scintillation Counter (LSC) or LC/MS-MS - equipment.
- MultiScreen Filter Assembly with Ultracel-PPB™ Membrane No. MAPPB1010 (Millipore Corp—Bedford, MA, USA).
- Single and multichannel pipetters.
- Non-sterile troughs.
- Microplate 96 deep-well (2 mL/well).
- 15 mL graduated centrifuge tubes.
- 1.5 mL microcentrifuge tubes.

PROCEDURE

1. Adjusting the pH of the plasma.
   The frozen plasma is slowly thawed in cold water, or overnight in the refrigerator and filtered over glass wool. Protein binding of some drugs is pH dependent. Therefore, the pH value of the freshly thawed plasma has to be adjusted to pH 7.3–7.4 by CO_2 or N_2. Due to the strong pH lowering effect of the gas, the CO_2 should be introduced carefully and in small quantities. If the pH value falls below pH 7.3–7.4, it can be re-adjusted by degassing with N_2.
2. Using a 1.5 mL microcentrifuge tube, prepare a 0.5 mM solution of each drug in PBS.
3. Add 80 μL of the drug solution to 7.92 mL of plasma or plasma fraction in a 15 mL centrifuge tube for a final test solution concentration of 5 μM.
4. Mix and incubate at 37°C for 60 min.
5. Transfer 300 μL/well of each plasma/drug mixture to one column of each of two Multi-Screen plates with Ultracel-PPB fitted with receiver plates. Cover the assemblies with sealed plate covers or the supplied extended centrifugal covers.
6. Centrifuge at 2,000 *g* (37°C) for 45 min.
7. Remove a 25 μL aliquot of the retentate volume (for purposes of determining the mass balance) and a 25 μL aliquot of the filtrate volume (for purposes of determining the concentration of free drug). It is possible that a concentrated protein layer forms on the membrane during filtration, so care should be taken to thoroughly mix the retentate prior to removing the aliquot.
8. The filtrate remaining in the receiver plate is then analyzed for volume determination using either spectrophotometry by determining the dilution

factor of a known aliquot of a chromophore added to the filtrate, or spectrophotometry by using the near infrared (NIR) absorption of water according to standard protocols, or gravimetric determination (Millipore Application Note AN1733EN00).

9. 25 µL of each sample or standard dilution is either analyzed by LC/MS-MS or LSC. The analytical method depends on the use of radiolabelled or non-radiolabelled drug. For good quantitative analysis, four to six point standard curves are generated.
10. Concentrations are determined for filtrates, retentates, controls, and standard dilutions.

EVALUATION

Final results are given as percentile fraction unbound (f_u) of percentile fraction bound (f_b) for a defined concentration.

$$f_u[\%] = \frac{c_u}{c_{initial}} * 100\% \quad (34.12)$$

$$f_{bd}[\%] = f_u[\%] - 100\% \quad (34.13)$$

c_u: concentration in ultrafiltrate [µM or µg/mL]
$c_{initial}$: concentration added [µM or µg/mL]

The fraction of nonspecific adsorption (f_{ads}) is calculated as:

$$f_{ads}[\%] = \frac{\text{conc.in filtrat of adsorption test}}{c_{initial}} * 100\% \quad (34.14)$$

Determination of Mass Balance (MB):

$$MB[\%] = \frac{c_u * V_u + c_{ret.} * V_{ret.}}{c_{initial} * V_{initial}} * 100\% \quad (34.15)$$

V_u: Volume of ultrafiltrate [mL]
V_{ret}: Volume of retentate [mL]
$V_{initial}$: Initial volume [mL]
$c_{ret.}$: Concentration in retentate [µM or µg/mL]

As well, the evaluation of equilibrium association constant (K_A) and the determination of the number of protein binding per sites per class of binding site (n) is possible, if different concentrations of the drug are applied (see Sect. 34.4).

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Rapid, efficient, and simple technique
- Commercially available kits
- Lack of sample dilution and volume shifts
- No requirement of a nonphysiological buffer
- Applicable for plasma and plasma fraction as well for other different types of biological matrices, including tissue homogenates
- If automated 96 well plate ultrafiltration system is used, high throughput with sample handling by robotic system

Disadvantages

- Potential of nonspecific binding (NSB) to filter membrane or plastic devices. Low recovery from either protein-filtrate or buffer indicates adsorptive losses and/or membrane rejection.
- Leakage of bound drug through membrane.
- Deviation of the drug protein binding equilibrium because of the change in the protein concentration, especially in case of high binding drugs. The concentration unbound may remain constant even though the proteins became concentrated in the upper reservoir as the amount of plasma water increases in the ultrafiltrate (Judd and Pesce 1982; Withlam and Brown 1981).

Ultrafiltration has been used to determine the protein bound fraction of many drugs, such as methadone (Wilkins et al. 1997), phenylacetate and phenylbutyrate (Boudoulas et al. 1996), etoposide (Robieux et al. 1997), doxorubin and vincristine (Mayer and St-Onge 1995), disopyramide (Echize et al. 1995), and ketamine and its active metabolites (Hijazi and Boulieu 2002). Schuhmacher et al. (2000) have shown the applicability for the determination of erythrocyte/plasma distribution. The method of UF has been applied in the measurement of free unaltered thyroxin or after displacement by salicylate as well after displacement by heparin in healthy people and in patients with non-thyroidal somatic illness (Faber et al. 1993). The protein binding of tritium labeled, anti-diabetic repaglinide and its displacement by warfarin, furosemide, tolbutamide, diazepam, glibenclamide, and nicardipine were determined by ultrafiltration (Plum et al. 2000).

The reliability and applicability of the UF has been done in comparison to the classical method of equilibrium dialysis for several drugs, like thiopental (Christensen et al. 1980), disopyramide (Norris et al. 1982), and valproic acid (Barre et al. 1985).

Although different filter material procedures were applied in these studies, the comparison gave comparable values for the free fraction of the drug for total concentrations between the two methods. Beside its use in in vitro assay, this method is widely applied for routine free drug monitoring of ex vivo PPB in clinical laboratories.

The implementation of new materials for filter membrane and plastics (e.g., polypropylene with PTFE) for the ultrafiltration units has diminished the disadvantages of nonspecific binding (NSB) and the break trough of protein, respectively protein bound drug. Eight radiolabelled drugs (Taxol, Digoxin, Prednisone, Testosterone, Warfarin, Propranolol, Methotrexate, Ibuprofen, and Mannitol) were analyzed for their drug recovery The applied Ultracel YM membrane with a nominal molecular weight limit of 10 K Da exhibits less than 2% NSB and >99.5% retention of serum proteins. (Millipore, Application Note AN1735EN00). With the improvement in the devices, the determination of the nonspecific binding of new drug by recovery experiment is recommended and taken into account by a correction factor, if necessary.

MODIFICATION OF THE METHOD

Pretreatment of the filter membrane (regenerated cellulose, Molecular cut off 10 KDa) with 5% Tween 80 or benzalkonium chloride showed significantly less nonspecific binding for compounds that had a tendency toward high membrane binding (Lee et al. 2003).

With rising temperature the drug binding decreases. The determination can be performed either at room temperature (approx. 22°C) or under more physiologically condition at 37°C. The chosen temperature should be maintained in the plasma sample, and during the separation by pre-warming the rotor or using a heated centrifuge.

The UF can either performed in a single ultrafilter unit or in a 96 well plate ultrafiltration system, latter in a semiautomatic high-throughput determination with sampling handling by a robotic system (Fung et al. 2003; Jordan et al. 2000).

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34.6 Equilibrium Dialysis

PURPOSE AND RATIONALE

The equilibrium dialysis (ED) is based on the establishment of an equilibrium state between a protein compartment and buffer compartment, separated by a membrane, which is permeable only for a low-molecular weight ligand. Although there is no "standard method" for binding measurement, ED is often regarded as the "reference method" for the determination of drug-protein-binding profile (Oravcová et al. 1995).

34.6.1 Equilibrium Dialysis (Individual Device)

34.6.1.1 Equipment and Material

- Fresh or frozen heparinized plasma of different species (pool), collected from fasted animals and humans. If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH at Sect. 34. 5).
- Stock solution of the radiolabelled, respectively non-radiolabelled drug prepared in an appropriate organic solvent (e.g., 10 mM ethanol).
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl).
- Equilibrium dialysis apparatus, for example, variable speed dialyser Dianorm-4 (Dianorm, Munich, Germany). The dialyser is equipped with 1 mL macro cells (4.5 cm working surface area) and cellulose dialysis membrane (63 mm diameter, molecular cutoff weight 10 KDa). The apparatus is placed in a water bath with a circulating system to obtain a constant temperature of 37°C in the system.

PROCEDURE

1. Preconditioning of Membrane
   Prior to dialysis, the membranes are preconditioned by soaking in water for 15 min, followed by soaking in PBS for 30 min. The cells are filled at ambient temperature, and when fully loaded, the Dianorm apparatus is placed in a water bath at 37°C and started at 12 rpm.
2. Determination of the time to equilibrium (ET)
   The ET is determined by dialyzing freshly spiked plasma containing the high (10 μg/mL) and intermediate (0.05 μg/mL) test concentrations against PBS. At time points 1, 2, 3, 4, and 6 h of dialysis, a small sample (50 μL) of plasma and PBS is removed from each cell and the concentration is analyzed by LSC or LC/MS-MS. The optimal ET is then determined.
3. Equilibrium Dialysis
   The ED is performed for five test concentrations (0.01, 0.05, 0.1, 1, and 10 μg/mL) for each of the test plasma samples against PBS. At each concentration, four cells, each containing 1 ml of spiked plasma and PBS, are prepared for each plasma type. At the time when the equilibrium is reached (e.g., 4 h), which was determined in the preliminary test, the dialysis is stopped. Aliquots of 200 μL plasma sample or PBS buffer are taken from each cell. To determine the concentration bound in plasma (c_{bd}) or the free or unbound fraction (c_u) in the buffer, the samples are analyzed by LC/MS-MS of LSC. The analytical method depends on the use of unlabelled or radiolabelled substance. The initial concentrations (c_{inital}) of each spiked plasma (aliquots of 200 μL) can also be determined.

EVALUATION

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u[\%] = \frac{c_u}{c_{initial}} * 100\% \qquad (34.16)$$

or if c_{intial} is not determined:

$$f_u[\%] = \frac{c_u}{c_u + c_{bd}} * 100\% \qquad (34.17)$$

$$f_b[\%] = \frac{c_{bd}}{c_{initial}} * 100\% \quad (34.18)$$

$$f_b[\%] = f_u[\%] - 100\% \quad (34.19)$$

c_u: concentration in buffer [µM or µg/mL]
$c_u + c_b$: concentration in plasma [µM or µg/mL]
$c_{initial}$: concentration added [µM or µg/mL]

If different concentrations are applied, the evaluation of equilibrium association constant (K_A) and the determination of the number of protein sites (n) are possible (see Sect. 34.4).

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Easy to set up and to use
- Temperature controlled

Disadvantages

- Long time to reach equilibrium, up to 24 h, potential degradation of test substance as a result of thermal or metabolic instability in plasma.
- Volume shift (Tozer et al. 1983)
- Donnan effects (Hindering of the passage of free ligand) (Mapleson 1987).
- Nonspecific adsorption to dialysis apparatus and membrane (Henricsson 1987; Henry and Mitchell 1981).
- Overestimation of the free fraction resulting from slight leakage of protein (or fragments of it) into the dialysate. The absence of protein in the dialysate should be verified by protein assay (Bowers et al. 1984).
- pH shift during ED due to continuous loss of CO_2, need of adjustment, and control (Brørs and Jacobsen 1985).
- High consumption of plasma.

34.6.2 24-Multiwell Plate Equilibrium Dialysis

34.6.2.1 Equipment and Material

- Freshly or frozen heparinized plasma of different species (pool), collected from fasted animals and humans. If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH at Sect. 34. 5).
- Stock solution of the radiolabelled, or non-radiolabelled drug is prepared in an appropriate organic solvent (e.g., 10 mM ethanol).
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl).
- Incubator.
- Plate shaker (orbital or reciprocating).
- Disposable 24-well BD Gentest™ Serum Binding System with 24 well dialysis insert, containing individual Spectrapor 2 membranes and 24 well BD Falcon™ Microplate (BD Biosciences, Bedford, MA, USA, Cat. No. 453700).
- Single and multichannel pipetters.
- Liquid Scintillation Counter (LSC) or LC/MS-MS-equipment.

PROCEDURE

1. Preconditioning of Membrane
   The plate is soaked for 20 min in deionized water thereafter in 30% absolute alcohol: deionized water. Then the plate is washed with water and soaked for 20 min in PBS used for the dialysis experiment.

Test well:	Bottom chamber: serum protein spiked with test compound
	Top chamber: PBS buffer only
Control well:	Bottom chamber: test compound in PBS buffer
	Top chamber: PBS buffer only

2. The bottom chamber is loaded with 742.5 µL of plasma/serum protein for the test well, and PBS buffer for the control wells. To both 7.5 µL of 100× concentrated compound (solved in DMSO) is added. To both top chamber of control and test well 250 µL of PBS buffer is added.
3. The plate is shaken for 20 h on medium setting: The shaker is placed in humidified incubator to reduce evaporation.
4. Aliquots (100 µL) from the upper chamber of the control and test wells are removed.
5. The concentration of test compound in the upper chamber of the test well ($c_{test\ well\ upper}$) and the concentration of compound in the upper chamber of the control well ($c_{control\ well\ upper}$) are analyzed by LC/MS-MS or LSC.
6. Alternatively, plasma protein binding can be determined without using a control well by comparing the concentration of compound in the upper

chamber ($c_{control\ well\ upper}$) to the total concentration (sum of $c_{control\ well\ upper} + c_{control\ well\ lower}$). This approach conserves test compound, but requires an analytical technique that can measure the concentration of compound in high protein samples (plasma).

EVALUATION

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u[\%] = \frac{c_{(test\ well)}}{c_{(control\ well)}} * 100\% \qquad (34.20)$$

Or (for single well approach, if control well is not applied):

$$f_u[\%] = \frac{c_{(test\ well\ upper\ chamber)}}{c_{(test\ well\ upper\ chamber)} + c_{(test\ well\ lower\ chamber)}} * 100\% \qquad (34.21)$$

$$f_b[\%] = f_u[\%] - 100\% \qquad (34.22)$$

Also evaluation of equilibrium association constant (K_A) and the number of protein binding per sites per class of binding site (n) is possible if different concentration are applied (see Sect. 34.4).

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Commercial available disposable 24-well system
- Easy to set and use
- High-throughput compatible with robotic systems
- Temperature controlled

Disadvantages

- Long time to reach equilibrium, up to 24 h and more
- Volume shift (Tozer et al. 1983)
- Donnan effects (Hindering of the passage of free ligand) (Mapleson 1987)
- Nonspecific adsorption to dialysis apparatus and membrane (Henricsson 1987; Henry and Mitchell 1981)
- Overestimation of the free fraction as result from slight leakage of protein (or fragments of it) into the dialysate, the absence of protein in the dialysate should be verified by protein assay (Bowers et al. 1984)
- pH labiality during ED due to continuous loss of CO_2 (Brørs and Jacobsen 1985)
- Potential degradation of test substance as a result of thermal or metabolic instability in plasma

MODIFICATION OF THE METHOD

An alternative ED method in 96 well plate format has been reported by Kariv et al. (2000). The authors used a disposable equilibrium dialyser with a 10 KDa ultrathin membrane, co-developed with Amika Corp. (Columbia, MD, USA). The binding of three well-studied drugs, propranolol, paroxetine, and losartan with low, medium, high binding properties, respectively, was tested to validate the method. The data of free fraction correlated with the published values determined by conventional ED.

The vertical design of 96 well dialysis block of Banker et al. (2003) allows the robotic system the access to the sample and dialysate site. The dialysis block with partially separated bars is reusable. The validity of the system was tested with ten different standard compounds, in comparison to standard ED, and literature data.

To equalize the osmotic pressure and therefore to attenuate the volume shift, dextran 2.5% (w/v) was added to the dialysate (Lima et al. 1983). However, this approach is inconvenient for high throughput. That the test compounds show no affinity to dextran has to be proven in an additional experiment.

Plum et al. (1999) showed the applicability of a step-wise ED for the plasma binding determination of the two strong adhesive ^{125}I-labeled proteins insulin aspart and insulin detemir.

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34.7 Ultracentrifugation

PURPOSE AND RATIONALE

The ultracentrifugation (UC) method achieves separation of free and protein bound drugs by centrifugation of the sample in a tube without a membrane. It makes use of the fact that sedimentation of solutes depends upon their molecular weight. For the first time Steinberg and Schachman (1966) demonstrated theoretically and practically the potential of the method.

PROCEDURE

34.7.1 Equipment and Material

- Freshly or frozen heparinized plasma of different species (pool), collected from fasted animals and humans. If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH at Sect. 34. 5).
- Stock solution of the radiolabelled, or non-radiolabelled drug prepared in an appropriate organic solvent (e.g., 10 mM ethanol).
- Centrifuge tubes (e.g., Ultra Clear tubes, Beckmann, Palo Alto, CA).
- Ultracentrifuge (e.g., Airfuge Beckmann, Palo Alto, CA).
- Single pipettes.
- Liquid Scintillation Counter (LSC) or LC/MS-MS-equipment.

PROCEDURE

1. Plasma samples are spiked with labeled or unlabeled drug to give specific concentrations (ranging from pharmacological to toxicological relevant concentrations). An aliquot (200 µL) is either transferred to LSC or LC/MS-MS to quantify the initial concentration (c_{intial}).
2. The centrifuge tubes are filled with identical volume (200 µL) of spiked plasma and loaded into the rotor of the ultracentrifuge. The cooled sample is centrifuged for 4 h at an air pressure of 30 psi and 150,000× g. The sample is cooled during and after the centrifugation to 4°C to avoid thermodynamic interferences. The time needed to obtain a "protein free" top layer should be determined in a series of preliminary experiments. Therefore, the centrifuge is loaded with a spiked plasma sample. The samples are spun at 150,000× g and the Airfuge stopped at different time intervals. An aliquot of 20 µL top layer is removed and the concentration is measured. The time where the concentration is constant is chosen for the experiment.
3. From the supernatant consecutive aliquots of 20 µL are taken without disturbing the remaining plasma sample. The concentrations of the top layers are quantified in the same manner like c_{intial} by means of a calibration curve.

EVALUATION

After the measurement of the concentration of the individual consecutive aliquots, the mean ($c_{supernatant}$) of the constant values is chosen. A significant higher amount in the first aliquot indicates a binding to lipoproteins.

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u[\%] = \frac{c_{(supernatant)}}{c_{(intial)}} * 100\% \tag{34.23}$$

$$f_b[\%] = f_u[\%] - 100\% \tag{34.24}$$

CRITICAL ASSESSMENT OF THE METHOD

34.7.1.1 Advantages

- Alternative method to ultrafiltration and equilibrium dialysis

- Elimination of the problems associated with free membrane effects like fewer nonspecific binding (NSB)
- No dilution of the sample, native status of plasma

34.7.1.2 Disadvantages

- High cost of ultracentrifuge.
- Time consuming (up to 18–24 h centrifugation time).
- Limited number of sample can be spun simultaneously.
- Intricate manual process, no automation applicable.
- Interferences according to sedimentation, thermal back diffusion, viscosity, and floating of lipoprotein in the supernatant fluid.
- Self-sedimentation of the test compound with molecular weight >300 Da.

Ultracentrifugation (UC) is an alternative to both equilibrium dialysis and ultrafiltration and enables the separation of the free and protein fraction in a more physiological environment. The UC is a method of choice for compound with a high affinity to nonspecific binding. It eliminates the problems associated with free membrane effects. Without the necessity to add a buffer system, a dilution observed at the equilibrium dialysis is avoided.

However several comparative studies showed quantitative discrepancies between the results obtained by UC compared to ultrafiltration, or equilibrium dialysis (Barre et al. 1985; Kurz et al. 1977). Verbeeck and Cardinal (1985) investigated the plasma protein binding of phenytoin, salicylic acid, propranolol, phetidine, and chlorpromazine using UC. The binding data was comparable to those obtained by equilibrium dialysis, except for chlorpromazine. The latter has been explained by the author in relation to the binding of the compound to a very low-density lipoprotein fraction, which floats after the centrifugation step. A micro scale UC was evaluated by Nakai et al. (2003). It showed a good correlation of the protein binding of ten compounds, with reported values determined by ultrafiltration and equilibrium dialysis.

MODIFICATION OF THE METHOD

Depending on the available ultracentrifuge different relative centrifugation forces have been used, ranging from 120,000 *g* up to 436,000 *g* (Nakai et al. 2003). Depending on the affinity of the test substrate for nonspecific binding, different material of ultracentrifuge tubes can be applied.

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34.8 Binding on Silica Beads Immobilized Protein Fraction

PURPOSE AND RATIONALE

This method is based on the affinity of the new chemical entity to a membrane, consisting of human serum albumin immobilized on Transil® beads. The silica beads are coated with egg yolk phosphatidylcholine and are commercially available under the trademark Transil® from Nimbus (Nimbus, Leipzig, Germany).

PROCEDURE

34.8.1 Equipment and Material

- Stock solution of drug prepared in an appropriate organic solvent, diluted to appropriate concentration (e.g., 10 µg/mL). The amount of the solubility modifier should be below 5% in the final concentration.
- Transil® "Ready to go plates" for 10 compounds per plate; for high precision assay, order no.: SP200096 (Nimbus Biotechnology, Leipzig, Germany).
- UV translucent plate (suitable for UV measurement above 250 nm).
- Multichannel pipette.
- UV plate reader and/or LC/MS-MS (needed for quantification of UV-inactive compounds).
- Centrifuge with rotor for 96 well plate or vacuum filtration device.

PROCEDURE

1. The plates are delivered in frozen state filled with a total volume of 270 µL of buffer for control well (column 1 and 7) and 270 µL of buffer/varying amounts of Transil for sample well. The plates are slowly defrosted immediately before measurement.
2. To each well (control and sample) 30 µL of compound solution has to be added. If UV spectroscopy method is used for quantification the wells H7–H12 have to use a reference sample to account for the absorbance of the UV plate (not necessary for LC/MS-MS).
3. Resuspend the liquid at least three to six times by swirling the beads around using a multichannel pipette to ensure complete mixing of the compound and the beads.
4. Separate the unbound fraction by short low speed centrifugation (15 min/10,000 *g*). Alternatively a vacuum filtration with collector plate can be applied.
5. Transfer a fixed volume (100 µL) of the filtrate into a UV translucent plate
6. UV plate reader and/or LC/MS-MS determines the concentrations of the unbound fraction at varying HSA concentration.

EVALUATION

The data is transferred into an excel workbook supplied by Nimbus to conveniently calculate the dissociation constant (K_D).

In the high affinity assay for each compound, five assay points are generated and evaluated via a Scatchard type analysis using a linearized binding equation.

Rearrangement of

$$c_b = \frac{n^* P^* K_A}{1 + K_A * c_u} \text{ with } K_A = 1/K_D \qquad (34.25)$$

leads to the dissociation constant (K_D)

$$K_D = c_u{}^* \frac{1 - \frac{c_b}{P}}{\frac{c_b}{P}} \qquad (34.26)$$

With the assumption $c_{protein} >> c_b$ simplifies Eq. 34.24 to:

$$K_D = \frac{P^* c_u}{c_{intial} - c_u} \qquad (34.27)$$

This condition is valid for compounds with low and medium affinity and also for compound concentrations larger than the protein concentration. Otherwise Eq. 34.24 has to be used, where the total compound concentration $c_{initial}$ has to be determined.

The bound fraction f_b is calculated by Eq. 34.26:

$$f_b = \frac{c_b}{c_{intial}} = 1 - \frac{1}{\frac{1+P}{K_D}} \qquad (34.28)$$

P: Concentration of the protein in the blood. For HSA it is usually 40 g/L, corresponding to 588 µM.

CRITICAL ASSESSMENT OF THE METHOD

34.8.1.1 Advantages

- Fast and easy, high-throughput assay, applicable to a robotic systems in 96 or 386 well plate format
- Simple quantification by hints of UV plate reader, MS measurement is only needed for UV-inactive compounds
- "Ready to go plates" are commercially available
- Determination of dissociation constant K_D and fraction bound
- Small amount of compound is needed
- Low influence of nonspecific binding for the determination of K_D

34.8.1.2 Disadvantages

- Only applicable to determine the binding to human serum albumin
- For UV-inactive compounds LC/MS-MS is needed, which diminishes the throughput

MODIFICATION OF THE METHOD

Schumacher et al. (2004) published a method, where Transil® beads in suspension have been used for the determination of protein binding in diluted plasma and plasma/erythrocyte distribution for strongly bound compounds. For HTS of the method 96 well plates with removable glass inserts filled with Transil suspension were applied. On comparison with the classical methods, ultrafiltration and equilibrium dialysis showed for eight compounds comparable precision and accuracy of the binding parameters.

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Distribution – In Vivo – Perfused Organs 35

Andreas W. Herling

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35.1 In Situ-Perfused Isolated Intestinal Segments and Bile Secretion in Anesthetized Rats

PURPOSE AND RATIONALE

The gut technique of in situ-perfused isolated intestinal segments in rats is used to determine the rates of intestinal absorption or intestinal secretion of a candidate compound, as well as in combination with a bile fistula to detect the hepatobiliary elimination of a candidate compound. By perfusion of different intestinal segments (duodenum, proximal and distal part of the jejunum, and ileum), the site of intestinal absorption or secretion of a candidate compound can be determined.

PROCEDURE

In general, rats are fasted overnight and are anesthetized with an intraperitoneal injection of 20 mg/kg ketamine plus 60 mg/kg pentobarbital sodium, and anesthesia is maintained by an s.c. infusion of pentobarbital sodium at a rate of 20 mg/kg/h for the desired length of the perfusion experiment. Animals are laparotomized by a median incision, and the common bile duct is cannulated with a polyethylene (PE) 10 catheter. Duodenum, or jejunal segments, or the ileum are cannulated for single pass perfusion or recirculated perfusion. After flushing the lumen of the respective intestinal segment with 40 ml of medium, perfusion is performed in situ at a very low continuous flow rate of 0.5 ml/min, ensuring a minimal perfusion pressure. The candidate compound is added to the perfusion medium at an appropriate concentration to reach detectable compound levels in blood (plasma) and bile. Body temperature

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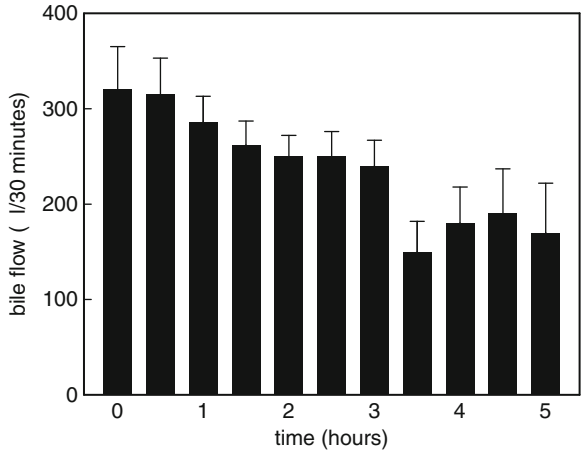

Fig. 35.1 Representative time course of bile flow in anesthetized rats with separate drainage of the bile. Values are mean ± SEM, $n = 4$

and perfusion medium are maintained at 37°C. Bile aliquots are collected at 10- to 30-min intervals. Blood samples (100 µl) are collected from the jugular vein every 30 min in EDTA tubes. At the end of the perfusion experiment, the livers are removed from all animals, total weight is determined, and the livers are immediately frozen for determination of tissue level of the compound in liquid nitrogen and then stored at −20°C until analysis. For analysis of tissue levels of the candidate compound, the frozen livers are homogenized in water (1 g tissue in 1 ml water (or solvent to extract the candidate compound from the tissue)) using an ULTRA-TURRAX. A continuous bile flow throughout the experiment is the best measure of the functional integrity of the organ (typically bile flow is shown in Fig. 35.1). Animals with an initial bile flow below 100 µl/30 min should be excluded from the final evaluation of the data.

EVALUATION

The concentrations of the compound are determined using appropriate analytical methods for the respective candidate compound in the perfusate at the end of the perfusion experiment, plasma, bile, and hepatic tissue. From the data, the site of intestinal absorption using different perfused intestinal segments in separate experiments as well as the degree of hepatobiliary elimination can be estimated.

Appropriate analytical methods with sufficient sensitivity are used for detection of the candidate compound in the perfusate, plasma, bile, and liver tissue. From the total amount of the candidate compound in the perfusate of the respective intestinal segment at the end of the perfusion experiment in relation to the total added amount of compound at time 0, the total enteral absorption rate can be estimated. The decline in the concentration of the candidate compound in the perfusate is a measure of the enteral absorption, uptake, metabolism, and elimination of the compound by the liver. The concentration in the plasma is a measure of the bioavailability. The appearance of the candidate compound in the bile is a measure of the hepatobiliary elimination of the compound. The tissue level of the candidate compound is a measure of the uptake and accumulation of the candidate compound in the liver. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the candidate compound in plasma, bile, and liver tissue.

In addition, the total hepatobiliary elimination of the candidate compound (sum of all collecting intervals (concentration × secreted volume per collecting interval)) can be calculated.

MODIFICATIONS OF THE METHOD

Various modifications are reported with respect to the animal species used as well as to the experimental setup (single pass or recirculated intestinal perfusion) and the site of blood collection, e.g., mesenteric vessels (DeGraw and Anderson 2004) or the portal veins (Gan et al. 2002) for estimation of the intestinal absorption rate when comparing the compound levels achieved in the mesenteric or portal veins and the concentrations in peripheral veins for estimation of systemic availability of the candidate compound. This method is widely used in rats, but also other animal species can be used and are reported, e.g., rabbits (Sawchuk and Awni 1986; Riad and Sawchuk 1991) and ferrets (Wang et al. 1993).

This method is often used for investigation of intestinal absorption of nutrients by using radioactive tracers (e.g., cholesterol, glucose) and their interference with the candidate compound (Arts et al. 2004). In addition, the secretion of the candidate compound into the intestine can be studied by peripheral administration of the compound into a peripheral vein and subsequent determination of the appearance of the

candidate compound in the intestinal perfusate (Merino et al. 2003; Berggren et al. 2004). Also variations are reported using chronically isolated intestinal loops in rats (Poelma et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

During anesthesia, intestinal motility is dramatically reduced, which secondarily might influence the intestinal absorption of the candidate compound. Bile flow is relatively constant during the first 2–3 h but declined during prolonged anesthesia (Fig. 35.1) due to the interruption of the enterohepatic circulation of bile acids.

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35.2 Isolated Perfused Livers

PURPOSE AND RATIONALE

Isolated perfused rat livers are used for physiological and pharmacological purposes to study intermediary metabolism of carbohydrates and lipids (e.g., gluconeogenesis, glycogenolysis, ketogenesis). This isolated organ preparation with separated cannulated bile duct can ideally be used for investigations of pharmacokinetic and of drug metabolism by elucidation of hepatic uptake and drug metabolic stability and its hepatobiliary elimination. The advantage of this perfused organ preparation over isolated hepatocytes is the maintained architecture of the hepatic structure (perivenous–periportal orientation).

PROCEDURE

Rats with a body weight between 250 and 300 g are anesthetized with 20 mg/kg ketamine plus pentobarbital sodium (60 mg/kg i.p.). The liver is exposed by longitudinal midline and transverse subcostal incisions, and the bile duct is cannulated as well as the portal vein with a venous cannula. The liver is immediately infused via the portal vein at 37°C with oxygenated saline containing heparin (70 units/ml). The vena cava caudalis is opened to allow a continuous flow of the saline/heparin solution for about 2 min. Then the liver is transferred into a heated (37°C) perfusion chamber and perfused via the portal vein in a recirculating manner at a constant flow rate of 35 ml/min with continuously oxygenated Krebs-Ringer bicarbonate buffer (100 ml total volume). The buffer consists of 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.72 mM NaH_2PO_4, 1.8 mM $CaCl_2$, and 0.5 mM $MgCl_2$ with 10 mM glucose, and is supplemented with 30% (v/v) washed bovine erythrocytes and 1.6% (w/v) bovine serum albumin. Experiments are routinely carried out on four livers simultaneously, and they are perfused for 2 h as total perfusion time for each liver.

The candidate compound is added to the perfusate before the isolated liver is transferred to the perfusion setup. After 5 min recirculation in the system without

the organ, a sample of the perfusate is collected for determination of the starting concentration of the candidate compound (value at time 0). Then the isolated liver is connected to the perfusion chamber, and subsequently samples of the perfusate are taken in 10- to 15-min intervals as well as bile samples are taken in 15-min intervals for the determination of compound levels. The volume of excreted bile per 30 min is determined gravimetrically (difference between tube weight without and with bile per collection period) with the assumption that 1 g is equivalent to 1 ml of bile. If radioactive-labeled candidate compounds are used, the intervals for bile collection can be much shorter (1–2 min). The liver is perfused for 2 h, and at the end of the perfusion experiment, the liver is removed and immediately frozen for determination of tissue level of the compound in liquid nitrogen and later on stored at −20°C until analysis. For analysis of tissue levels of the candidate compound, the frozen livers are homogenized in water (1 g tissue in 1 ml water (or solvent to extract the candidate compound from the tissue)) using an ULTRA-TURRAX.

Samples for determination of lactate dehydrogenase (LDH) activity are taken every 30 min, and they are a measure for the viability and integrity of the isolated organ. A continuous bile flow throughout the perfusion time is the best measure of the functional integrity of the organ (typically bile flow of four livers is shown in Fig. 35.2). Isolated livers without bile flow should be excluded from the final evaluation of the data.

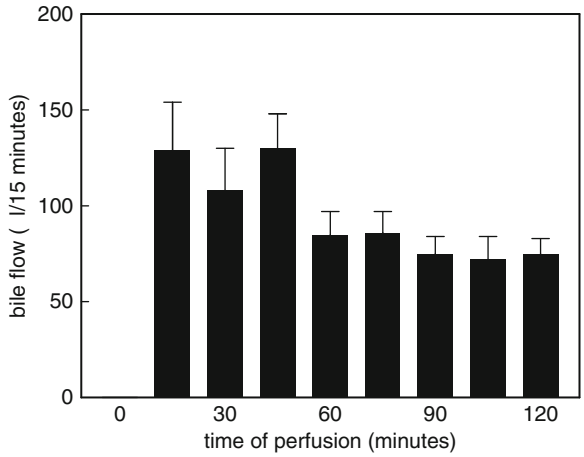

Fig. 35.2 Representative time course of bile flow of isolated perfused rat livers. Values are mean ± SEM, $n = 4$

EVALUATION

Compound levels in samples of the perfusate and bile are measured using appropriate analytical methods for detection of the candidate compound. The decline in the concentrations of the compound in the perfusate is a measure of the uptake, metabolism, and elimination of the compound by the liver. The appearance of the candidate compound in the bile is a measure of the hepatobiliary elimination of the compound. The tissue level of the candidate compound is a measure for the uptake and accumulation of the candidate compound in the liver. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the candidate compound in perfusate and bile.

From the total amount of the candidate compound and metabolites in the perfusate (100 ml) at the end of the perfusion experiment (after 2 h) in relation to the total added amount of compound at time 0, the total uptake into the liver can be calculated. In addition, the total hepatobiliary elimination of the candidate compound (sum of all collecting intervals (concentration × secreted volume per collecting interval)) can be calculated. From these values together with the remaining tissue concentration, a balance calculation can be set up, and the proportion of compound metabolism can be estimated. Representative data are shown for a candidate compound with a high first pass effect in Fig. 35.3.

MODIFICATIONS OF THE METHOD

This method has been widely used for studying carbohydrate and lipid intermediary metabolism (Herling et al. 1998) as well as drug metabolism (Milne et al. 1997, 2000; Vuppugalla and Mehvar 2004). Many variations have been reported predominantly with respect to the animal species used. Chaib et al. (2004) compared isolated perfused livers of rats with those of guinea pigs. den Butter et al. (1994) used livers from rabbits. Further modifications are related to the direction of perfusion via hepatic artery or portal vein or both simultaneously or in connection with the isolated jointly perfused small intestine (Stumpel and Jungermann 1997; Stumpel et al. 2000) as well as the continuous perfusion in a recirculated (see above) or open (nonrecirculated) manner (Lopez et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

Using an erythrocyte-containing medium for perfusion, one has to take into account the putative

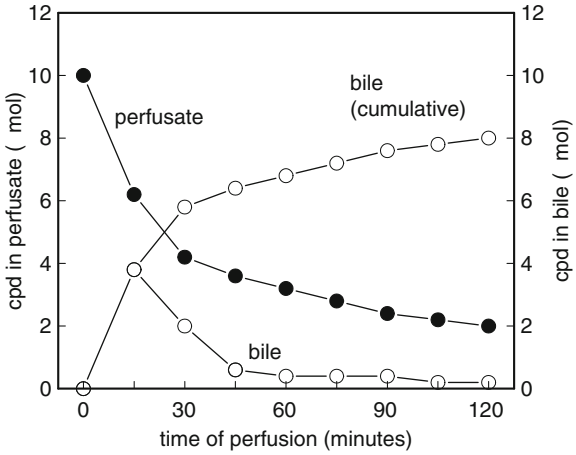

Fig. 35.3 Example for the time course of a compound with a high first pass effect. The values for the perfusate represent the total amount of compound in the total volume of perfusate (100 ml; nmol/ml × 100). The values for the bile represent the total excreted amount of compound in the bile per sampling period of 15 min (µmol/ml × bile volume per 15 min) and were expressed as the values measured per sampling interval and as cumulative excreted into the bile

involvement of the erythrocytes themselves with respect to uptake of the candidate compound. Therefore, not only the erythrocyte-free perfusate but also the erythrocyte fraction should be included in the analysis of the candidate compound, separately. In our hands, the model of isolated perfused liver is metabolically active for up to 3 h, and during this time, no decline in hepatic metabolic activity becomes obvious. However, bile flow declined during the perfusion experiments. Therefore, our total perfusion time of isolated livers in our standard experimental setup is limited to 2 h. The tissue level of the candidate compound analyzed after 2 h in the liver is a measure for the total amount of compound in the whole organ. This does not necessarily mean the presence of the candidate compound in hepatocytes but additionally in the capillary and biliary space of the liver.

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Wolkoff AW, Johansen KL, Goeser T (1987) The isolated perfused rat liver: preparation and application. Anal Biochem 167:1–14

35.3 Isolated Perfused Kidneys

PURPOSE AND RATIONALE

Isolated perfused rat kidneys are used for physiological and pharmacological purposes to study overall renal functions. For pharmacokinetic purposes, this preparation is used to study renal excretion profiles of the candidate compound and/or its metabolites.

PROCEDURE

Karpf et al. (2003) used isolated kidney from rats. Rats are anesthetized with an intraperitoneal injection of 20 mg/kg ketamine plus 60 mg/kg sodium pentobarbitone before surgery. The isolated kidneys are perfused in a recirculating manner in a thermostatically controlled cabinet maintained at 37°C. An erythrocyte-free modified Krebs-Henseleit bicarbonate buffer solution containing bovine serum albumin, glycine, L-cysteine, L-glutamic acid, and glucose in a reservoir is pumped through the kidney via an in-line filter

(8 μm), a membrane oxygenator, glass bubble trap, flow meter, and finally a glass arterial cannula inserted into the renal artery, and returned to the reservoir. Throughout the perfusion, carbogen is supplied to the membrane oxygenator to maintain a constant concentration of oxygen in the perfusate at a value greater than 0.6 mM, and the pH was maintained at 7.4. The viability of the kidney is monitored by measuring glomerular filtration rate (GFR) and the reabsorption of water, sodium, and glucose. The organs are considered viable if the respective values are greater than 0.4 mL/min, 75%, 95%, and 99%, respectively.

Urine is collected over successive 10-min intervals (10–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80, and 80–90 min) from the start of the perfusion; samples of the perfusate are collected from the reservoir at the midpoint times of these intervals.

EVALUATION

Compound levels in samples of the perfusate and urine are measured using appropriate analytical methods for detection of compound levels. The decline in the concentrations of the candidate compound in the perfusate is a measure of the uptake, metabolism, and elimination of the compound by the kidney. The appearance of the candidate compound in the urine is a measure of the renal elimination (glomerular excretion and tubular secretion) of the compound. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the compound in perfusate and urine.

MODIFICATIONS OF THE METHOD

Wang et al. (2004a, b) used isolated kidneys from rats after removal from anesthetized (sodium pentobarbitone (60 mg/kg)) animals. Briefly, a midline laparotomy incision was made from pelvis to sternum on the animal. Next, the right ureter was ligated immediately proximal to the bladder. A solution of mannitol (150 mg) and heparin (100 U) in 1 ml normal saline was injected into the penile vein. The right ureter was then cannulated by the introduction of a cannula consisting of a 200-mm length of tubing (od = 0.61 mm, ID = 0.28 mm, Paton Scientific, Victor Harbour, Australia). The area around the anastomosis of the superior mesenteric artery, renal artery, and aorta was cleared of connective tissue and loose ligatures placed around the superior mesenteric artery and renal artery. After removing the capsule of the kidney, a right-angled glass cannula was inserted into the superior mesenteric artery via a small incision and passed proximally along the superior mesenteric artery, across the aorta and into the renal artery where it was tied in place. Perfusate flow was commenced immediately. The cannulated kidney was then excised from the body of the rat and suspended within a thermostatically controlled cabinet at 34-37°C. The recirculating perfusion medium (160 ml) was pumped at approximately 36 ml/min, which maintained renal artery perfusion pressure at 110 ± 25 mmHg, which was monitored by a manometer, corrected for the intrinsic pressure of the apparatus. The functional viability of each kidney was assessed by the glomerular filtration rate (GFR), which was determined as the renal clearance of [^{14}C]-inulin, urine flow, and the percentage tubular reabsorption of water, glucose, and sodium.

Urine samples were collected in 10-min intervals over the 10- to 130-min period after the bolus. Urine volume was measured gravimetrically in preweighed collection vials. Perfusate samples were collected from the reservoir at the midpoint of each urine collection interval; appropriate amounts of perfusate and of urine were taken for further analysis.

CRITICAL ASSESSMENT OF THE METHOD

The isolated perfused kidney technique has been used for decades to investigate aspects of renal physiology, pharmacology, and pharmacokinetics. The model used most extensively is the isolated perfused rat kidney continuously perfused with medium containing electrolytes, glucose, amino acids, and the oncotic agents, bovine serum albumin (BSA) or dextran. The oncotic agents included in perfusate create "physiological" colloid osmotic pressure, which is one of the key factors to regulate glomerular filtration rate (GFR), tubular reabsorption of water and sodium, and water content in kidney interstitial tissue. BSA is the oncotic agent used most commonly in the isolated perfused rat kidney and affords reasonably stable isolated perfused kidney function. Perfusate BSA concentrations used in the past have varied from 20 to 80 g/l. Generally, the higher the BSA concentration in perfusate, the higher the extent of reabsorption of water and sodium, but at the expense of lower GFR and urine flow rate. Thus, it is necessary for investigators to weigh the balance between GFR and the extent of reabsorption of water and sodium. Practically, 60–65 g/l BSA in perfusate has been used by most investigators employing the isolated perfused kidney.

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Distribution: Across Barriers

36

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Distribution of drugs across barriers plays a predominant role in the processes of absorption (A), distribution (D), and excretion (E) and is thus a major determinant of a drugs pharmacokinetic profile. The barrier at the site of absorption is in most cases built by the enterocytes of the small intestine (see Chap. 29 "Absorption: In Vivo Tests (Radiolabeled)"). Apart from lung, heart, muscle, and brain, the main target organs are kidney and liver which are also the major elimination pathways of drugs. Cell membranes of liver hepatocytes and kidney cells have to be passed in these cases. The brain plays a special role in the distribution of drugs because drugs normally should not enter the central nervous system to avoid severe adverse effects. It is, therefore, protected by the very tight blood–brain barrier. However, when the target is located in the brain, the drug needs to cross this barrier. Meanwhile, there exist several in vitro systems to study drug permeation across this special blood–brain barrier (Sect. 36.1). They cover primary cultures of brain endothelial cells in monoculture or as coculture with astrocytes or pericytes (Sect. 36.1.1), immortalized endothelial cell lines from different species (Sect. 36.1.2), and surrogate models (Sect. 36.1.3).

Over the recent years, many membrane transporters have been cloned, characterized, and localized in cell membranes of different tissues. In numerous in vivo and in vitro studies, membrane transporters mainly expressed in epithelial of the intestine, liver, and kidney and in the endothelium of the blood–brain barrier show clinically relevant interaction with drugs and/or their metabolites. Therefore, they can play an important role in modulating drug absorption, distribution, and elimination.

Membrane transporters can be classified into two major groups: the solute carrier (SLC) transporter family and the ATP-binding cassette (ABC) transporter family (HUGO Gene Nomenclature Committee http://www.gene.ucl.ac.uk/nomenclature/).

The SLC family consists of 51 gene subfamilies with 378 family members, including ion-coupled transporters, facilitated transporters, and exchangers (http://www.bioparadigms.org). SLC transporters mediate the uptake of substrates, including organic anions and cations, peptides, amino acids, bile salts, and nucleosides into the cell. These transporters accept not only physiological or endogenous substrates but also xenobiotics, including drugs and/or their metabolites, and are therefore referred to as drug transporters. Members of the SLC families are the sodium bile salt cotransporters (ASBT, NTCP), oligopeptide transporters (PEPTs), organic anion transporting polypeptides (OATPs) organic anion as well as organic cation transporters (OATs, OCTs, and OCTNs), and multidrug and toxin extrusion proteins (MATEs).

In the ABC-transporter family, 49 genes have been identified and classified into seven subfamilies, including the widely studied P-glycoprotein (MDR1, Pgp), multidrug resistance proteins (MRPs), breast cancer-related protein (BCRP), and the bile-salt export pump (BSEP). ABC transporters mediate the transport of substrates across the cell membrane against their concentration gradient. The energy required for this process is derived from ATP hydrolysis.

Table 36.1 summarizes for in vitro studies the most important drug transporters, their expression profile, well-established probe substrates, and inhibitors. Some of them are defined by the International Transporter Consortium (ITC; *Nature Reviews* Drug Discov. 9, 215–236, from March 2010) as clinically relevant transporters. The ITC members comprising scientists from the industries, Food and Drug Administration (FDA), and universities justify their selection on practical consideration and on clinical evidence that these transporters influence, drug absorption, drug disposition, side effects, and/or drug–drug interactions (DDIs).

In May 2010, the European Medicines Agency (EMA) recommends in their draft guidance that new drug candidates should be evaluated as substrates and inhibitors of drug transporters to assess the potential for clinical drug–drug interactions (Table 36.1).

Therefore, consideration must be given to the role of drug transporters in the absorption (e.g., intestinal Pgp and BCRP), distribution (Pgp in the blood–brain barrier and OATPs for hepatocytes uptake), and excretion (OATs and OCTs for renal elimination) of a new drug candidate in development. Based on the current knowledge, decision trees recommended by the ITC whitepaper can be used for planning and implementing drug transporter studies during the drug development (*Nature Reviews* Drug Discov. 9, 215–236, from March 2010). Examples of clinical transporter study design and in vitro/in vivo correlations for transporters are summarized by Eisenblaetter T. and Weitz D. (Drug discovery and evaluation: *Methods in Clinical Pharmacology* Springer Verlag 2011). Another aspect that demands careful consideration is that by knowing

Table 36.1 Basic characteristics of relevant uptake and efflux transporters

Gene	Transporter	Organ expression	Substrates	Inhibitors
SLC-transporter family				
SLC10A1	NTCP	Liver, pancreas	Estrone-3-sulfate, taurocholate, BSP	Cyclosporine, furosemide
SLC10A2	ASBT	Ileum, kidney	Taurocholate	BSP, cyclosporin A
SLC15A1	PEPT1	Kidney, intestine	Cephalexin, enalapril, cefadroxil, dipeptides, tripeptides	Glycyl-proline
SLC15A2	PEPT2	Kidney, brain, lung	Cephalexin, enalapril, cefadroxil, dipeptides, tripeptides	Zofenopril, fosinopril
SLCO1A2	OATP1A2	Kidney, brain, liver	Statins, estrone-3-sulfate, DHEAS, fexofenadine	Rifampicin, ritonavir
SLCO1B1	OATP1B1[FDA/EMA]	Liver	BSP, statins, estrone-3-sulfate, valsartan	Rifampicin, ritonavir
SLCO1B3	OATP1B3[FDA/EMA]	Liver	BSP, statins, cholecystokinin 8, estradiol-17-β-glucuronide	Rifampicin, ritonavir
SLCO2B1	OATP2B1	Liver, intestine, placenta	BSP, statins, estrone-3-sulfate	Rifampicin, cyclosporine
SLC22A1	OCT1[EMA]	Liver	TEA, MPP, metformin	Quinine, quinidine
SLC22A2	OCT2[FDA/EMA]	Kidney, brain	TEA, MPP, metformin	Tetrabutylammonium, cimetidine
SLC22A3	OCT3	Liver, kidney, heart, placenta, lung, brain, skeletal muscle	TEA, MPP, metformin	Corticosterone, quinidine
SLC22A4	OCTN1	Kidney, liver, brain, intestine, lung, heart, placenta, etc.	TEA, ergothioneine	Verapamil
SLC22A5	OCTN2	Kidney, liver, intestine, lung, heart, skeletal muscle, etc.	Carnitine	Verapamil
SLC22A6	OAT1[FDA/EMA]	Kidney, brain, placenta	PAH, cidofovir, adefovir	Probenecid, glibenclamide
SLC22A7	OAT2	Liver, kidney	cGMP	Indomethacin, bumetanide
SLC22A8	OAT3[FDA/EMA]	Kidney, brain, skeletal muscle	Estrone sulfate, DHEAS, furosemide, bumetanide	Probenecid, indomethacin
SLC22A11	OAT4	Kidney, placenta	Estrone sulfate, DHEAS, methotrexate, bumetanide	BSP, probenecid
SLC47A1	MATE1	Kidney, liver	MPP, TEA	Quinidine, cimetidine
SLC47A2	MATE2	Kidney, intestine, testis	MPP, TEA	Cimitidine, quinidine
ABC-transporter family				
ABCB1	MDR1[FDA/EMA]	Intestine, kidney, liver, brain	Digoxin, loperamide, doxorubicin	Cyclosporine A, quinidine
ABCB4	MDR3	Liver	Digoxin, paclitaxel, vinblastine	Cyclosporine, verapamil
ABCB11	BSEP [EMA]	Liver	Taurocholate, pravastatin	Cyclosporine A, rifampicin
ABCC1	MRP1	Lung, placenta, testis, kidney, skeletal muscle	Estradiol-17-β-glucuronide, estrone-3-sulfate	Cyclosporine A
ABCC2	MRP2	Liver, kidney, intestine	Glutathione, methotrexate, valsartan	Cyclosporine A, delaviridine
ABCC3	MRP3	Liver, intestine	Estradiol-17-β-glucuronide, methotrexate, fexofenadine	Delaviridine, efavirenz
ABCC4	MRP4	Kidney, liver, brain	Adefovir, DHEAS, furosemide, methotrexate, cAMP, cGMP	Celecoxib, diclofenac
ABCG2	BCRP[FDA/EMA]	Intestine, kidney, liver, brain, placenta	Methotrexate, mitoxantrone, statins, imatinib, irinotecan	Fumitremorgin C

BSP bromosulfophthalein, *DHEAS* dehydroepiandrosteron sulfate, *MPP* methylphenylpyridinium, *PAH para*-aminohippurate, *TEA* tetraethylammonium; FDA/EMA indicated transporters are defined as clinically relevant from ITC or EMA

the substrate specificity and the expression profile of each transporter, efficient drug delivery to, or exclusion from, the target organs can be accomplished by appropriately modifying the structure of the drug.

To study these drug transporter interactions, in vitro systems expressing single transporters (Sects. 36.2 and 36.3) or transporter combinations (Sect. 36.4) can successfully be used. Drug uptake mediated by SLC transporters (Sect. 36.2) can be measured by using transfected eukaryotic cells like HEK, CHO (Sect. 36.2.1), or injected oocytes (Sect. 36.2.2). Drug efflux processes mediated by ABC transporters (Sect. 36.3) are investigated using membrane vesicles from either eukaryotic cell lines like HEK, CHO, MDCK (Sect. 36.3.1), or insect cells (Sect. 36.3.2). By using double-transfected MDCK cells, drug uptake and efflux (Sect. 36.4) can be measured in one single setup (Sect. 36.4.1). For liver-specific drug transport, sandwich-cultured hepatocytes can be used (Sect. 36.4.2).

36.1 Blood–Brain Barrier (BBB)

36.1.1 Primary Cultures of Brain Capillary Endothelial Cells

36.1.1.1 Primary Cultures of Porcine Brain Microvascular Endothelial Cells

PURPOSE AND RATIONALE

Franke et al. (1999) have described an in vitro model for screening of drug entry into the brain using primary cultures of porcine brain capillary endothelial cells (PBCECs). By using serum-free culture conditions, transport studies are highly reproducible, and sample analysis (HPLC, LC-MS) is facilitated.

PROCEDURE

Porcine brain microvessel endothelial cells are prepared following a method of Bowman et al. (1983) using modifications described by Tewes et al. (1997) and Franke et al. (2000). Hemispheres of porcine brains of freshly slaughtered animals (female, about 6 months) are collected in ice-cold ethanol (70 % v/v) and stored in PBS (4°C; containing penicillin/streptomycin (200 U/ml each)) for transport and during the preparation procedure. After flaming the hemisphere shortly, the meninges and adhering larger vessels are completely removed. Secretory brain areas are dissected, and the remaining cerebral gray and white matter is homogenized mechanically. The homogenate is supplemented with preparation medium (Earle's Medium M199 containing 0.7 mM L-glutamine, 1% (v/v) penicillin/streptomycin (10 mg/ml) and 1% (v/v) gentamicin (10 mg/ml)) to a final volume of 50 ml per brain hemisphere, and 1% (w/v) dry powdered unspecific protease/dispase II (Sigma) is added for an incubation period of 2 h at 37°C with magnetic stirring. One hundred and fifty milliliters of 18% (w/v) dextran solution per 100 ml of digested homogenate is then added, shaked, and spinned with 6,800 × g for 10 min at 4°C. After dextran density gradient centrifugation, capillary fragments are obtained as pellet and are resuspended in culture medium. This microvessel-fragment suspension is filtered through a nylon mesh (180 µm pore size) and then triturated up and down 3–5 times in a 10-ml pipette with the pipette tip placed directly onto the bottom of a petri dish. For the second digestion, the suspension is incubated with 0.1% (w/v) collagenase/dispase (Boehringer) in 10-ml plating medium (preparation medium + 10% (v/v) ox serum) per brain equivalent for 1 h at 37°C using a flask with hanging stirrer. A discontinuous Percoll gradient is prepared from 15 ml Percoll solution ($\delta = 1.07$ g/ml) and 20 ml Percoll solution ($\delta = 1.03$ g/ml). Ten milliliters of the cell suspension is placed onto the top of the gradient and centrifuged in a swing-out bucket rotor (1,250×g, 10 min, room temperature). Released endothelial cells are collected from the interface of the gradient and washed with an excess of plating medium (110 × g, 10 min, room temperature). The cells are resuspended very gently in plating medium and seeded onto collagen-coated culture surfaces (seeding density: 450 cm^2 per brain). Cultures are kept at 37°C in a humidified atmosphere with 5% CO_2. Twenty-four hours after initial plating, cells are washed with PBS containing Ca^{2+} (0.9 mM) and Mg^{2+} (0.5 mM) and supplied with culture medium (plating medium without gentamicin). For further purification and to obtain a homogenous monolayer, primary cultures of PBCEC are subcultured at second day of culture by gentle trypsination (1:250 trypsin solution in PBS) at room temperature. The displacement of endothelial cells is followed with a reverse phase-contrast microscope and stopped by addition of ox serum when the majority of endothelial cells are removed from the culture surface. The cells are collected and centrifuged with 110×g for 10 min at room temperature and plated in culture

medium with a density of 30,000 cells/cm^2 on rat tail collagen-coated Transwell^{TM} or Transwell-Clear^{TM} filter inserts (24 mm diameter, 0.4 mm pore size, Corning Costar). Medium volumes are 1.5 ml apical and 2.5 ml basolateral. On the second day after passage, the culture medium is replaced by a serum-free assay medium (DME/Ham's F12; 1:1) containing 550 nM hydrocortisone. Within 2–4 days after this medium exchange, the PBCEC have built up a very tight monolayer and are ready for BBB in vitro–transport experiments.

Coating with Rat-Tail Collagen

Collagen fibers are collected as described by Bornstein (1958). Fibers of each tail are incubated in 50 ml 0.1% (v/v) acetic acid at 4°C for 24 h. Centrifugation for 2 h at 5,000 × g separates the fiber residues from solubilized collagen which is aspirated. Total protein content of the collagen solution is determined and adjusted to 0.1 mg/ml. Transwell^{TM} or Transwell-Clear^{TM} filters are coated by adding 100 µl of rat-tail collagen solution per filter and allowed to dry for at least 2 days at 37°C. No further washing steps are occurred (Franke et al. 1999).

Transport Measurements

Directly prior to the experiment, the quality, that means the tightness, of the PBCEC monolayer is assessed by determination of the transendothelial electrical resistance (TEER) of each filter. TEER measurements can be easily performed with the ENDOHM-24^{TM} chamber and the EVOHM^{TM} voltometer (World Precision Instruments, Inc.). The resistance data, expressed in Ω, are multiplied with the filter surface (4.52 cm^2), corrected for blank filter resistance, and expressed in Ω × cm^2.

To measure the transport of drugs across the BBB in vitro, 2.5 µCi of ^{3}H-labeled drug and ^{14}C-sucrose are applied to each Transwell (in case of ^{14}C-labeled substances, permeability studies are performed with ^{3}H-sucrose). This concentration is high enough to ensure sufficient excess to neglect the decrease of tracer in the donor (apical) compartment during the experiments. Volumes of 1.5 ml in the donor (apical) and 2.5 ml in the acceptor (basolateral) compartment avoid hydrostatic pressure. After addition of the radiolabeled compound, samples of 50 µl are taken in duplicate from the basolateral acceptor compartment each 20 min and replaced by 100 µl of fresh assay medium. Cells are kept under culture conditions during the whole transport experiment. Radioactivity is measured after addition of liquid scintillation cocktail in a counter.

Uptake Experiments

To determine the uptake of test substances, filters are gently washed 90 min after drug application with PBS, and cells are solubilized with a solution containing 0.1 M NaOH, 1% (w/v) sodium dodecyl sulfate, and 2% (w/v) Na_2CO_3. Radioactivity of the solubilized cells is counted and compared with the radioactivity of the medium in the donor compartment at 90 min.

Calculation of Permeability Coefficients

Following the majority of publications, permeability coefficients can be calculated according to Pardridge et al. (1990). The permeability coefficient of the endothelial cell layer P_e is calculated as follows:

$$1/P_e = 1/P_t + 1/P_f$$

(P_t permeability coefficient of the total system and P_f permeability coefficient of the cell-free filter)

$$P[\mathrm{cm/s}] = (\mathrm{cpm_t}^a \times \mathrm{volume}^d[\mathrm{cm}^3])/(\Delta t[\mathrm{s}] \times \mathrm{filter\ area}[\mathrm{cm}^2] \times \mathrm{cpm_0}^d)$$

($\mathrm{cpm_t}^a$ total radioactivity in the acceptor compartment after t seconds; $\mathrm{cpm_0}^d$ total radioactivity added at time 0 to the donor compartment; Δt time measured in seconds; filter-area here 4.52 cm^2; volume^d here 1.5 ml)

EVALUATION

Under these culture and assay conditions, PBCEC displayed sucrose permeabilities as low as 2 × 10^{-7} cm/s. This tightness becomes close to in vivo permeabilities of 3 × 10^{-8} cm/s (Ohno et al. 1978) and 1.2 × 10^{-7} cm/s (Levin 1980). Data are expressed as mean±standard deviation (n = 3–6).

CRITICAL ASSESSMENT OF THE METHOD

The absence of serum and presence of hydrocortisone in this in vitro model ensure the appropriate differentiation of brain endothelial cells with a characteristic BBB phenotype including (a) impermeable intercellular tight junctions (Nitz et al. 2001, 2003; Eisenblatter et al. 2001); (b) low pinocytotic activity;

(c) expression of specific transporters like SGLT1 (Elfeber et al. 2004), neutral amino acid transporter, P-glycoprotein (Eisenblatter et al. 2003), ABCG2 (Eisenblatter and Galla 2002), and the transferrin, scavenger, and LDL receptors (Hoheisel et al. 1997); (d) expression of specific enzymes like γ-GT (Mischeck et al. 1989) and alkaline phosphatase (Meyer et al. 1990); and (e) an efficient endothelial barrier function with low paracellular permeability (Hoheisel et al. 1998, Franke et al. 1999, 2000; Nitz et al. 2003).

As described before, TEER measurements can be performed with the ENDOHM-24^{TM} chamber and the EVOHM^{TM} voltometer. Use of the STX-2 "chopstick" electrodes (World Precision Instruments, Inc,) leads to inaccurate data and cannot be recommended for TEER measurements. Very much in favor is a technique called impedance spectroscopy (Wegener et al. 1999).

MODIFICATION OF THE METHOD

Prof. H-J Galla (University of Muenster, Germany) has already established an in vitro BBB model with murine brain capillary endothelial cells based on this procedure (not yet published). Thus, this procedure might easily be adapted to brain endothelial cells of other relevant species in drug discovery like rat, dog, and rabbit.

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EXAMPLES

The industrial application of ranking of CNS penetration was nicely shown by Lohmann et al. (2002). In this work, different in vitro assays were evaluated in order to predict the BBB permeability.

36.1.2 Cocultures of Bovine Brain Microvascular Endothelial Cells and Rat Astrocytes

PURPOSE AND RATIONALE

The passage of substances across the blood–brain barrier (BBB) is regulated in the cerebral capillaries,

which possess certain distinct different morphological and enzymatic properties compared with the capillaries of other organs. To provide an in vitro system with the functional characteristics of a BBB, Cecchelli et al. (1999) developed an in vitro system with cloned bovine brain capillary endothelial cells on one side of a filter and rat astrocytes on the other side which is described in detail below. Recently, Kroll et al. (2009) confirmed in a coculture model of porcine brain cerebral endothelial cells together with astrocytes that if astrocytes are able to contact the endothelial cells, they induce in concert with external factors like glucocorticoids the BBB function in vitro.

PROCEDURE

The cloning of endothelial cell islands is purchased according to Meresse et al. (1989). In brief, microvessels are isolated by mechanical homogenization from one cerebral hemisphere and then seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells. Only capillaries adhere on the extracellular matrix, whereas arterioles and venules can easily be discarded. Five days after seeding, the first endothelial cells migrate out of the capillaries and start to form microcolonies. When the colonies are sufficiently large, the five largest islands are trypsinized and seeded onto 35-mm-diameter gelatin-coated dishes (one clone per dish) in the presence of Dulbecco's modified Eagle's medium supplemented with 15% calf serum, 2 mM glutamine, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and basic fibroblast growth factor (bFGF, 1 ng/ml added every other day). Endothelial cells from one 35-mm diameter dish are then harvested and seeded onto 60-mm diameter gelatin-coated dishes. After 6–8 days, confluent cells are subcultured at the split ratio of 1:20. Cells at the third passage are stored in liquid nitrogen.

For experiments, cells are rapidly thawed at 37°C and seeded onto two 60-mm diameter gelatin-coated dishes. Once they reach confluence, cells are subcultured up to passage eight. The life span of the endothelial cell cultures is about 50 cumulative population doublings. At each passage, the cells are seeded in stock gelatin-coated dishes and on microporous membranes. At this stage, care needs to be given in selecting the matrix on the microporous membrane which will support cell attachment, growth, and differentiation. Different cell culture inserts and coatings have been evaluated by Cecchelli et al. (1999), and they found self-made rat-tail collagen on Millicell-CM inserts (Millipore, 0.4 μm pore size, 30 mm diameter) to give well-organized and confluent monolayers with typical junctions between them.

36.1.2.1 Preparation of Rat Astrocytes

Primary cultures of astrocytes are made from newborn rat cerebral cortex according to Dehouck et al. (1990). The meninges are cleaned off, and the brain tissue is forced gently through a nylon sieve. DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml of gentamicin is used for the dissociation of cerebral tissue and development of astrocytes.

36.1.3 Cocultures of Bovine Brain Capillary Endothelial Cells (BBCECs) and Astrocytes

Astrocytes are plated at a concentration of 2.5×10^5 cells/ml on the bottom side using the filter upside down. After 8 days, filters are properly positioned, and the medium is changed twice a week. Three weeks after seeding, cultures of astrocytes become stabilized. Then, frozen BBCECs are recultured on a 60-mm-diameter gelatin-coated dish. Confluent cells are trypsinized and plated on the upper side of the filters at a concentration of 4×10^5 cells. The medium used for the coculture is DMEM supplemented with 15% calf serum, 2 mM glutamine, 50 μg/ml of gentamicin, and 1 ng/ml of bovine fibroblast growth factor added every other day. Under these conditions, BBCECs form a confluent monolayer in 8 days (Dehouck et al. 1990).

EVALUATION

Cecchelli et al. (1999) compared the extraction ratios in vivo to the permeability of the in vitro model and showed a strong correlation between both values.

CRITICAL ASSESSMENT OF THE METHOD

The relative ease with which such cocultures can be produced in large quantities facilitates the screening of new CNS drugs. This model provides an easier, reproducible, and mass-production method to study the blood–brain barrier in vitro.

Using this model, one has to consider that two different species are used and that cow is a species not typically used in pharmaceutical development. Species differences according to transporter expression and lipid membrane composition have to be taken into account.

Most of the laboratories use enzymatic digestion to isolate endothelial cells (see Sect. 36.1.1.1). Often, cells of capillary, arteriolar, and venular origin as well as pericytes lead to a heterogenous mixture of these different cellular types. The subculture technique enables to circumvent the limitations of primary cultures and to provide large quantities of these monolayers. Endothelial cells can be cultured from passage three after thawing to passage seven, each passage generating at least 75 cocultures.

A complete characterization is summarized by Dehouck et al. (1990).

MODIFICATION OF THE METHOD

Instead of using cocultures with rat astrocytes, some laboratories are also using astrocytic conditioned medium in order to reinduce blood–brain barrier properties (Dehouck et al. 1994). They have found that induction does not require direct cell–cell interactions.

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EXAMPLES

Cocultures of bovine capillary endothelial cells and astrocytes can be used to study the permeability of drugs (Dehouck et al. 1990). Culture plate inserts are set into six-well plates with 2 ml of buffer added to the upper chamber and 2 ml added to the plate coating the inserts. Radiolabeled compounds are added to the upper chamber, and 100 µl is removed from the lower chamber at various times. The radioactivity can be determined, and the permeability can be calculated. Using such techniques, ranking of CNS drugs concerning their permeability into the brain can be achieved.

36.1.4 Cocultures of Porcine Brain Capillary Endothelial Cells and Pericytes

PURPOSE AND RATIONALE

The blood–brain barrier (BBB) comprises the cerebral microvascular endothelium, pericytes, and astrocytes, which are connected by the extracellular matrix. The basis of the barrier function is the formation of complex tight junctions between adjacent endothelial cells which seal the paracellular pathway for various substances from the blood into the brain tissue (Abott et al. 2010). The induction of the BBB phenotype by astrocytes was well described in the past decades and is summarized in chapter "Cocultures of Bovine Brain Mircovascular Endothelial Cells and Rat Astrocytes." Recently, more and more functions of pericytes, like their contribution to angiogenesis and their role in various pathological conditions like brain tumorgenesis, multiple sclerosis, Alzheimer's disease, diabetic retinopathy, and prolonged oxygen deprivation are discovered. Thanabalasundaram et al. (2011a) first described that the impact of pericytes on the brain endothelial barrier integrity depends critically on the pericyte differentiation stage and established a species-consistent coculture model of primary porcine brain capillary endothelial cells (PBCECs) and porcine brain capillary pericytes (PBCPs). The new coculture model is described below and allows the examination of pericytes on pharmacological, transport, migration, and metabolic activity of the BBB (Thanabalasundaram et al. 2011a).

PROCEDURE

36.1.4.1 Preparation and Cultivation of PBCECs and PBCPs

PBCECs are isolated and cultured as described in chapter "Primary Cultures of Porcine Brain Microvascular Endothelial Cells." Briefly, cerebra of freshly slaughtered adult pigs are mechanically homogenized and stepwise digested by two proteases, followed by further purification steps. Twenty-four hours after initial plating, the cells are washed with phosphate-buffered saline (PBS) containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. Puromycin (2.5 μg/ml) is used to remove pericytes according to Perriere et al. (2005). PBCECs are subcultured by fractionated trypsinization 2 days later to reduce contamination by other cells and then cryoconserved as described by Kroll et al. (2009).

PBCPs are obtained from PBCEC preparations which are not treated with puromycin. Since pericytes attach more firmly to the culture substrate than PBCEC, selective trypsinization of PBCEC yields pure PBCP cultures which are grown 14 days in pericyte medium (Dulbecco's modified Eagle's medium Ham's F-12, 2 mM L-glutamine, 100 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin, and 10% (v/v) NCS) before they are cryoconserved (Thanabalasundaram et al. 2011b). Pericytes are characterized by their branched morphology; large size; positive immunostaining for α-smooth muscle actin (α-SMA), desmin, Thy 1.1, and NG-2 proteoglycan; and negative staining for factor-VIII-related antigen and glial fibrillary acidic protein (GFAP).

36.1.4.2 Differentiation Procedure of PBCPs

PBCPs are seeded on collagen G-coated culture flasks (25 cm, 75,000 cells per flask) or on 96-well plates in pericyte medium. After 2 days, the medium is exchanged with serum-free pericyte medium containing 20 ng/ml bFGF (basic fibroblast growth factor) or 4 ng/ml TGFβ (transforming growth factor β). The differentiation medium is renewed with fresh medium 2 days later. The morphological analysis and the Western blot analysis of total protein and cell culture supernatant are carried out after 5 days treatment with the corresponding differentiation medium. Furthermore, it is recommended to verify the relative proliferation of the differentiation stage by a cell proliferation assay like the MTS-based assay.

36.1.4.3 Coculture Model of PBCECs and PBCPs

The pericytes are seeded (5,000 cells/filter) with pericyte medium on the basolateral (bottom) compartment of rat tail collagen (0.54 mg/ml) coated 12-well Transwell® filter inserts (Corning; 1.12 cm growth area, 0.4 μm pore size) (Thanabalasundaram et al. 2011a). The PBCPs are cultivated for 2 days in serum containing pericyte medium. Then, the cells are treated with the corresponding serum-free differentiation medium (20 ng/ml bFGF or 4 ng/ml TGFβ) for 5 days, and the medium is renewed after 2 days. After the differentiation step, the filter inserts are washed twice with serum-free pericyte medium to remove bFGF or TGFβ. Afterward, the PBCECs are seeded in excess (250,000 cells/filter) in plating medium on the apical (upper) compartment. The basolateral compartment contained serum-free pericyte medium. In order to induce the development of the PBCEC barrier integrity, the medium of the apical and basolateral compartment is exchanged 2 days later with fresh serum-free medium. After additional 2 days, the barrier integrity reaches the maximum TEER values, and experiments can be performed. The reference filters without PBCPs are treated according to the coculture procedure first with pericyte medium and then with the corresponding differentiation medium. After washing twice with serum-free pericyte medium, PBCECs are cultured on the apical compartment of the control filter inserts. The basolateral side refers to the brain side where the interaction by secreted signaling molecules of both cell types can be analyzed.

A very detailed description of the new coculture model including supplier information is provided in Thanabalasundaram et al. (2011b).

EVALUATION

Thanabalasundaram et al. (2011a) characterized the differentiation of PBCPs in vitro by analysis of morphological changes, cell proliferation assays, Western blot analysis, immunocytochemistry, and transendothelial electrical resistance (TEER) measurements. They identified two distinct differentiation stages of the primary pericytes in vitro. TGFβ-treated cells developed larger processes and enlarged cell bodies, whereas bFGF-treated pericytes formed shorter processes and smaller cell bodies. α-SMA and actin expression was increased while desmin,

vimentin, and nestin expression was decreased in TGFβ-treated cells when compared to bFGF-treated PBCPs. bFGF-treated PBCPs displayed a higher relative proliferation rate than TGFβ-differentiated PBCPs. In addition, the permeability factors VEGF, MMP-2, and MMP-9 were higher secreted by the α-SMA-positive phenotype, indicating a proangiogenic role of this TGFβ-induced pericyte differentiation stage. The TEER measurement of the barrier integrity of cocultured porcine brain capillary endothelial cells revealed that bFGF-pretreated α-SMA-negative pericytes stabilize the barrier integrity while α-SMA-positive pericytes differentiated by TGFβ decrease the barrier integrity.

CRITICAL ASSESSMENT OF THE METHOD

Due to the fact that pericytes are located next to the endothelial cells and release a large number of endothelial permeability regulating signaling molecules, their impact on the BBB integrity is of great interest. Pericytes are characterized by pluripotent differentiation characteristics, and thus, it remains complicated to analyze their impact on the BBB integrity both in vivo and in vitro. Whereas Nakagawa et al. (2007) revealed a barrier integrity increasing effect of pericytes in a primary rat cell in vitro coculture model, Thanabalasundaram et al. (2010) previously identified that primary porcine pericytes decrease the endothelial barrier integrity by secretion of the permeability factors VEGF, MMP-2, and MMP-9. In their recent assessment, Thanabalasundaram et al. (2011a) could show that different culture conditions can trigger pericyte differentiation in vitro and that the impact on BBB integrity depends on their differentiation status.

MODIFICATION OF THE METHOD

Instead of porcine-derived brain capillary endothelial cells and pericytes, the coculture model might also be established with cells derived from other species like rat (Nakagawa et al. 2007).

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EXAMPLES

Cocultures of porcine capillary endothelial cells and porcine pericytes can be used to study the impact of pericyte-derived extracellular matrix proteins on the endothelial cell monolayer integrity (Thanabalasundaram et al. 2011b) and can provide new insights into the regulation of the BBB integrity by pericytes which will further improve the understanding of the physiological and pathological conditions like angiogenesis, tumorgenesis, multiple sclerosis, and Alzheimer's disease.

36.1.5 Immortalized Cell Lines

36.1.5.1 Immortalized Human Cerebromicrovascular Endothelial Cells

PURPOSE AND RATIONALE

Primary cultures of cerebromicrovascular endothelial cells (CECs) derived from rat, bovine, porcine, mouse, and human can rapidly loose key phenotypic markers of the blood–brain barrier (BBB) and undergo cellular senescence after a limited number of divisions in vitro. Furthermore, expression of BBB markers varies considerably among BCEC obtained from different species. These issues, compounded with the problems associated with very limited availability of human brain biopsies, small initial yield of cells, and short proliferative life span of human cells, greatly restrict

the utility of primary human BCEC as a reliable in vitro BBB model. Therefore, Muruganandam et al. (1997) developed an immortalized human cerebromicrovascular endothelial cell line as an in vitro model of the human blood–brain barrier.

PROCEDURE

Human CECs are isolated by using a modification of the procedure described by Gerhart et al. (1988). Briefly, the brain samples are excised surgically from patients treated with idiopathic epilepsy (Stanimirovic et al. 1996) and cleaned of meninges, surface vessels, and cortical gray matter. The tissue is minced and homogenized and passed once though 350-μm and twice through 112-μm mesh nylon nets. The filtrate is then centrifuged at 1,000×*g* for 10 min, and the pellet is resuspended in 20% dextran (mol wt. 70,000) and again centrifuged at 3,000×*g* for 15 min. The microvessels and capillaries contained in the pellet are collected on a 20-μm nylon mesh, dislodged, and exposed to 1 mg/ml type IV collagenase for 15 min at 37°C. The dissociated microvessels are collected by centrifugation and resuspended in growth media containing 65% medium M199 (Earles's salts, 25 mM HEPES, 4.35 g/l sodium bicarbonate, and 3 mM L-glutamine), 10% fetal bovine serum, 5% human serum, 20% murine melanoma cell-conditioned media (mouse melanoma, Cloudman S91, clone M-3, melanin-producing cells), 5 μg/ml of insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 10 μg/ml endothelial cell growth supplement (ECGS). Dissociated microvessels are seeded in 0.5% gelatin-coated plastic tissue culture dishes and maintained at 37°C in an atmosphere of 5% CO_2 in air. Endothelial cell colonies emerging from attached microvessels at days 4–5 after seeding are isolated using cloning rings; two or three of these cloned colonies are pooled and grown to confluence (10–14 days). The cells are further propagated at 1:3 split ratio every 7–10 days (Muruganandam et al. (1997): human cerebromicrovascular endothelial cells (HCECs).

Transfection of Human CEC with pSV3-Neoplasmid

Human CECs are transfected with the pSV3-neoplasmid encoding for the SV40 large T antigen and the neomycin resistance gene by using a calcium phosphate coprecipitation method (Graham et al. 1973). Transfection solution containing 2.5 M $CaCl_2$ (65 μl), 30 μg of pSV3-neo DNA, 500 μl HBSS buffer (275 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 4.2 mM HEPES, 11 mM dextrose; pH 7.19), and 400 μl water is vortexed briefly and allowed to precipitate for 30 min at room temperature. Human BCECs grown to near confluence in 100 mm dishes are exposed to the transfection solution for 6 h at 37°C. After removing the transfection solution, the cells are subjected to a short (90 s) glycerol shock (20% v/v) and washed with HEPES buffer, pH 7.1. Fresh growth media is added, and the cells are allowed to grow for 48 h at 37°C. Transfected cell cultures are then split (1:3) and seeded in the media containing the neomycin analog G418 (400 μg/ml). Cell colonies that survive 72 h of G418 treatment are isolated using cloning rings and passaged three times in G418-containing medium. The colonies that yielded cultures with cobblestone morphology should be used for initial genotypic and phenotypic analyses. They are called SV-HCEC.

Transport Studies

The barrier-like properties of SV-HCEC are developed in cells grown on a 0.5% gelatin-coated Falcon tissue culture insert (1 μm pore size; 0.83 cm^2 surface area) in 1 ml growth medium. The bottom chamber of the insert assembly contains 2 ml of growth medium either alone or supplemented with the fetal human astrocyte (FHAS)-conditioned medium in a 1:1 (v/v) ratio. The FHAS-conditioned medium is obtained by incubating confluent FHAS in serum-free M199 for 72 h. The transendothelial electrical resistance (TEER) of the endothelial cell monolayers can be measured with the ENDOHM-24^{TM} chamber voltometer (World Precision Instruments, Inc.). Paracellular passage of ^{3}H-sucrose (2 μCi/ml) and ^{14}C-inulin (0.5 μCi/ml) across triplicate SV-HCEC monolayers and across the 0.5% gelatin-coated inserts without cells is determined from clearance values (Pardridge et al. 1990). Samples are collected from the bottom chambers every 15-min over 120-min period. Clearance volume [μl] was calculated as:

$$CL[\mu l] = (C_b \times V_b)/C_a,$$

where C_b is the basolateral tracer concentration, V_b the volume of the basolateral (lower) chamber, and C_a is the initial apical tracer concentration. The slopes of the clearance curves for membranes alone (PS_m) and membranes with cell monolayers (PS_{m+e}) are calculated using linear regression analysis to give

PS [µl/min]. The PS_e value for the SV-HCEC monolayer is calculated from:

$$1/PS_e = 1/PS_{m+e} - 1/PS_m.$$

The permeability coefficient P_e [cm/min] for the endothelial monolayer is derived by dividing PS_e by surface area (0.83 cm^2) of the membranes.

EVALUATION

Permeability coefficients for paracellular diffusion of ^{3}H-sucrose and ^{14}C-inulin across monolayers of HCEC and immortalized SV-HCEC and transendothelial resistances have been compared to human lung microvascular endothelial cells to evaluate the BBB phenotype of the cerebromicrovascular endothelial cell cultures (Muruganandam et al. 1997).

CRITICAL ASSESSMENT OF THE METHOD

Immortalization of especially human cerebromicrovascular endothelial cells is an invaluable tool for identifying specific aspects of the human BBB physiology and to analyze drug penetration into the human CNS. Nevertheless, D. Stanimirovic uses in all her following publications the primary cultures of HCEC and not the immortal cell line SV-HCEC to study transporter and receptor expression at the human BBB and to investigate the complicated brain inflammation processes. Possibly, the phenotype of HCEC is not retained in the Simian virus 40 large tumor antigen-transfected SV-HCEC cell line. One should probably think of a milder immortalization method when developing an immortalized human brain capillary endothelial cell line.

MODIFICATION OF THE METHOD

Rat brain capillary endothelial cells have been immortalized by the entire E1A region of adenovirus 2 by Roux et al. (1994) which was used for a number of cell types without oncogenic transformation. Bovine umbilical vein endothelial cells have been immortalized by transfection with an expression vector containing the human papillomavirus type 16 E6E7 oncogenes (Cajero-Juarez et al. 2002).

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EXAMPLES

Human CEC cultured on filter inserts can be used to measure brain penetration of drugs to make in vitro–in vivo correlations. They can further be used to study, for example, brain inflammation that has been implicated in the development of brain edema and secondary brain damage in ischemia and trauma. Zhang et al. (2000) have studied leukocyte infiltration across the blood–brain barrier and could show the expression of adhesion molecules and leukocyte chemoattractants under ischemia in vitro conditions.

36.1.6 Immortalized Rat Brain Microvascular Endothelial Cells

PURPOSE AND RATIONALE

To overcome the large variability in the starting material and the rapid senescence observed after passages of pure cerebromicrovascular endothelial cells from primary cultures, Roux et al. (1994) produced an immortalized cellular clone displaying a stable, nontransformed phenotype. The aim was to constitute a valuable

in vitro model of the rat blood–brain barrier in order to make mechanistic studies and to avoid pharmaceutical drug screening for CNS penetration in vivo.

PROCEDURE

Primary cultures of rat brain microvessel endothelial cells are prepared according to the method of Bowman et al. (1982) and Roux et al. (1989). Briefly, two brains from 3-month-old Sprague Dawley rats are removed, cleaned of meninges and white matter, minced, and incubated for 1 h at 37°C in 25 ml of minimal essential medium (MEM) containing 1 mg/ml collagenase/dispase, 1% bovine serum albumin (BSA), and 15 mM Hepes, pH 7.4. After enzymatic treatment, brain tissue is suspended in 20 ml medium containing 25% BSA. Microvessels are pelleted by centrifugation at 1,000×*g* for 10 min to eliminate contaminating cells and debris, passed through a 120-μm nylon mesh, and incubated in 10 ml MEM containing 1 mg/ml collagenase/dispase for 3 h. Clumps of cells are layered over 7 ml Percoll gradients prepared by centrifuging 50% isotonic Percoll at 25,000×*g* for 1 h. The band containing mainly endothelial cells is removed and seeded onto extracellular matrix (ECM)-coated dishes (Gospodarowicz et al. 1986). Cultures are maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 10 mM Hepes, 2 mM glutamine, 100 μg/ml gentamicin, 20% heat-inactivated, rat plasma-derived serum, and 1 ng/ml basic fibroblast growth factor, in humidified 5% CO_2/95% air at 37°C. After 3 days, contaminating pericytes surrounding endothelial cell colonies are removed with a modified glass Pasteur pipette. Experiments can be performed on confluent once-passaged 3-week-old cultures (Regina et al. 2001).

36.1.6.1 Transfection of Rat Brain Capillary Endothelial Cells

The plasmid pE1A-neo carries the entire E1A region of adenovirus 2 and the neo gene for resistance to the aminoglycoside G418 (Roux et al. 1994). Transfection is carried out using the calcium phosphate precipitation procedure. Endothelial cells are transfected 24 h after the second passage, at ~50 % confluence, with 1–10 μg plasmid DNA. After 3 h in the presence of DNA, cells are submitted to a 2-min DMSO (10%) shock, then rinsed and refed with complete medium. They are trypsinized 25 h later and seeded at various densities in 6-cm dishes in complete medium supplemented with 300 μg/ml G418. The selective medium is changed every 3–4 days. The clones appear ~2 weeks later; they are individually trypsinized within cloning cylinders and transferred into collagen-coated 24-well plates. For experiments, immortalized rat brain microvessel endothelial cells are plated on rat tail collagen I-coated plates and maintained in a-MEM/Ham's F10 (1:1 v/v) with Glutamax containing 10% heat-inactivated FCS, 300 μg/ml Geneticin (G418), and 1 ng/ml basic fibroblast growth factor (bFGF) in humidified 5% CO_2/95% air at 37°C.

36.1.6.2 Induction of BBB Phenotype by Astrocyte-Conditioned Medium (ACM)

For endothelial cell treatment, the medium is changed to a 1:1 (v/v) mixture of standard endothelial cell culture medium and ACM (Regina et al. 2001). ACM is obtained by incubating 3–5-week-old confluent astrocytes for 48 h in serum-free medium supplemented with 2 mM glutamine and 100 μg/ml gentamicin. Primary cultures of astrocytes are prepared from the cerebral cortex of three newborn rats. After removing meninges, the brain tissue is forced gently through a nylon sieve. Astrocytes are plated at a concentration of 1.2×10^5 cells/ml in 75-cm^2 flasks in αMEM/Ham's F10 (1:1 v/v) with Glutamax™ containing 10% FCS and 100 μg/ml gentamicin. Two weeks after seeding, astrocytes are purified by overnight shaking at 37°C and treated with cytosyl arabinoside and 3-estermethylleucine according to the method of Meyer et al. (1989). Three weeks after seeding, astrocytes are used for preparation of ACM.

EVALUATION

The BBB model system of RBE4 cells (Roux et al. 1994) is used in many publications to study brain transport (carnitine: Mroczkowska et al. 2000; P-glycoprotein and Mrp1: Begley et al. 1996 and Regina et al. 1998) as well as drug-metabolizing enzyme activities (Chat et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

Immortalization of brain capillary endothelial cells is a difficult process, and phenotype characterization must be performed very carefully and always in comparison to primary cultured cells. However, an immortal cell line promises higher reproducibility of results, easier handling, and finally to be less time-, material-, and labor consuming.

MODIFICATION OF THE METHOD

Another immortalized rat brain capillary endothelial cell line is used in some labs, called GPNT. This cell line is obtained from GP8 cell line (Greenwood et al. 1996) by re-transfection with the plasmid pcDNA3-RSV and repeated limiting dilution cloning of the parent line (GPNT: GP8 and the French Company NeuroTech SA). GPNT cells are repeatedly treated with 5 μg/ml puromycin. They are plated on collagen I-coated plates and grown in a-MEM/Ham's F-10 (1:1) containing 10% heat-inactivated fetal calf serum, 0.5 ng/ml basic fibroblast growth factor, 5 μg/ml transferring, 5 μg/ml insulin, 5 ng/ml selenium, and 5 μg/ml puromycin, in humidified 5% CO_2/95% air at 37°C (Theron et al. 2003). P-glycoprotein expression can selectively be increased by repeated puromycin treatment (Demeuse et al. 2004).

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EXAMPLES

Begley et al. (1996) demonstrated the use of RBE4 cells for the assessment of P-glycoprotein activity at the blood–brain barrier. They showed that cellular accumulation of ^{3}H-colchicine and ^{3}H-vinblastine is enhanced in the presence of azidothymidine, chlorpromazine, verapamil, cyclosporine A, and PSC833. Vinblastine uptake was measured in RBE4 cells grown on collagen-coated 24- or 48-well plates. Cells were washed several times with DMEM containing 0.1% BSA and then incubated with 200 nM ^{3}H-vinblastine alone or together with 50 μM of the inhibitors. At several time points (15, 30, 60, and 90 min), the incubation medium was aspirated, and the cells washed with ice-cold medium and then solubilized in 1 N NaOH containing 0.1% SDS. Solubilized cells were pipetted into vials containing scintillation liquid for β-scintillation counting.

36.1.7 A Surrogate BBB Model: MDCK-MDR1 Cells

PURPOSE AND RATIONALE

Early assessment of the ability of a drug candidate to penetrate the CNS is critical during the drug discovery selection process, especially for therapeutic indications that require delivery to a CNS site of action. Equally important is the ability to design drugs for

non-CNS indications that have minimal brain penetration to avoid undesirable CNS side effects. In vitro BBB models using primary and immortalized brain capillary endothelial cells are described in the previous chapters. The Madine Darby canine kidney (MDCK) cell line is increasingly used as a substitute for the more labor-intensive in vitro BBB models in passive permeability and membrane transport studies.

PROCEDURE

Multidrug resistance-transfected MDCK type II (MDR1-MDCKII) cells were firstly described by Evers et al. (1997). They are maintained in minimum essential medium (MEM) containing 10% FBS and 2 mM L-glutamine and are cultured at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells are passaged at 80–90% confluence (every 3–4 days) at a ratio 1:10 using trypsin-EDTA solution according to Irvine et al. (1998). They are grown in the absence of any selection agent to maintain Pgp (MDR1) expression (Mahar Doan et al. 2002). For transport studies, cells are seeded onto polycarbonate Transwell filter membranes at a density of 300,000 cells/cm^2. MDCK monolayers are washed and fed with fresh medium 1 h post-seeding and again 24 h post-seeding, and monolayers are ready for studies 3 days later.

36.1.7.1 Permeability Studies

Drugs are tested at 10 μM concentration and in two directions (apical to basolateral (a-b) and basolateral to apical (b-a)). Monolayer efflux studies are conducted at 37°C in a humidified incubator with shaking (90 rpm) for 60 min. Transendothelial electrical resistance is measured with an Endohm Meter (World Precision Instruments, New Haven, CT). Reference drugs for paracellular transport (^{14}C-mannitol), transcellular transport (^{3}H-propranolol), and Pgp efflux (amprenavir) should be included in each experiment. Concentrations of ^{14}C-mannitol and ^{3}H-propranolol are measured by liquid scintillation counting. Amprenavir is analyzed by cassette LC/MS/MS analysis along with the test drugs.

36.1.7.2 Calculations

The apparent permeability (P_{app}) is calculated with the equation:

$$P_{app}[nm/s] = 1/(S \times C_0) \times (dQ/dt),$$

where S = membrane surface area, C_0 = donor concentration at time 0, and dQ/dt = amount of drug transported per time. A ratio of the b-a and a-b P_{app} value is calculated. Involvement of a Pgp-mediated efflux mechanism is concluded if the b-a/a-b ratio is >1.5 (Mahar Doan et al. 2002).

EVALUATION

Mahar Doan et al. (2002) used the assay to classify 93 CNS and non-CNS drugs. The CNS set of drugs had a sevenfold lower incidence of passive permeability values <150 nm/s compared with the non-CNS set. The majority of drugs (72%) were not Pgp substrates. The CNS drug set had a threefold lower incidence of Pgp-mediated efflux than the non-CNS drug set. For CNS delivery, they concluded that drugs should ideally have an in vitro passive permeability >150 nm/s and not be a good (b-a/a-b ratio <2.5) Pgp substrate.

CRITICAL ASSESSMENT OF THE METHOD

The MDR1-MDCK transport assay was evaluated by Polli et al. (2001). The scientist compared the Pgp transport assay with the calcein-AM and ATPase assays. They tested 66 compounds and yielded two categories of compounds. Category I (n = 35) exhibited concordance across the assays. Category II (n = 31) revealed differences among the assays that related to the apparent permeability (P_{app}) of the compounds. Within category II, two groups are discerned based on the absence (group IIA, nontransported substrates) or presence (group IIB, transported substrates) of monolayer efflux. Detection of efflux (group IIB) is associated with compounds having low/moderate P_{app} values (mean = 16.6 nm/s), whereas inability to detect efflux (group IIA) is associated with compounds having high P_{app} values (mean = 535 nm/s). The calcein-AM and ATPase assays revealed Pgp interactions for highly permeable group IIA compounds but are less responsive than monolayer efflux for low/moderate P_{app} compounds of group IIB. All assays detect substrates across a broad range of P_{app}, but the efflux assay is more prone to fail at high P_{app}, whereas the calcein-AM and ATPase assays are more prone to fail at low P_{app}. When P_{app} is low, efflux is a greater factor in the disposition of Pgp substrates. The MDR1-MDCK assay is more reliable at low/moderate P_{app} and is therefore the method of choice for evaluating drug candidates despite its much lower throughput and more complicated read out.

However, the user needs to take into account that MDCK cells are derived from kidney. Thus, kidney-specific transporters are expressed which do not play any role at the BBB. Furthermore, membrane composition is distinct in epithelial cell type (MDCK) and endothelial cells lining the brain capillaries influencing membrane partitioning of drug candidates.

MODIFICATION OF THE METHOD

To confirm that drugs are Pgp substrates, drugs can also be tested in the presence of a specific Pgp inhibitor, for example, 2 μM GF120918 (Polli et al. 2001). Inclusion of a Pgp inhibitor reduces the b-a/a-b ratio to ~1 for Pgp substrates.

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EXAMPLES

As written earlier, Mahar Doan et al. (2002) demonstrated in a very convincing paper the use of MDCK-MDR1 cell line to classify CNS and non-CNS drugs. They investigated 93 drugs and found that a drug should ideally have an in vitro passive permeability >150 nm/s and not be a good (B-A/A-B ratio < 2.5) Pgp substrate for CNS delivery. Together with physical chemical properties like molecular weight, solute McGowen volume, solute dipolarity/polarizability, hydrogen bond acidity and basiscity, and charges and number of aromatic rings, a good prediction of a drugs permeability into the CNS should be possible. In 2004 (Gombar et al. 2004), they published a first quantitative structure–activity relationship (QSAR) model predicting Pgp substrates that takes into account the ability to partition into membranes, molecular bulk, and the counts and electrotopological values of certain isolated and bonded hydrides. The training set consisted of 95 compounds classified as substrates or nonsubstrates based on the results from MDR1-MDCK efflux assays (Mahar Doan et al. 2002). The model fit the data with sensitivity of 100% and specificity of 91% in the jackknifed cross-validation test. A prediction accuracy of 86% was obtained on a test set of 58 compounds.

36.2 Drug Uptake by SLC Transporters

36.2.1 Drug Transport Mediated by SLC Transporters Using Eukaryotic Cells

PURPOSE AND RATIONALE

Endogenous compounds like bilirubin, bile salts, steroid conjugates, thyroid hormones, prostaglandins, purine metabolites, as well as pharmaceutical drugs are taken up from the blood into the liver and into kidney proximal tubule epithelia in order to be eliminated from the body. In the liver, several compounds are metabolized and excreted into bile or through the sinusoidal membrane of the hepatocytes back into the blood for renal elimination. Hepatic uptake transporters like OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, and NTCP are involved in liver uptake processes, while MATE1 is involved in the canalicular secretion of several substrates. The renal elimination is mediated by the basolateral uptake transporters OAT1, OAT2, OAT3, OCT2, and OATP4C1 and luminal secretion into primary urine by OAT4, MATE1, and MATE2.

Eukaryotic cell lines expressing single uptake transporters at high expression levels are convenient to study uptake of endogenous compounds or pharmaceutical drugs.

PROCEDURE

36.2.1.1 Cell Culture and Cell Lines

HEK293 (human embryonic kidney) cells are cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 95% humidity, and 5% CO_2.

HEK293 cells can be stably transfected by the polybrene (hexadimethrine bromide) method (Brewer 1994) or by using the Flp-In expression system (Invitrogen). Briefly, exponential growing Flp-In HEK293 cells are incubated in a 6-well plate with 0.35 µg expression vector containing the transporter gene, 3 µg pOG44 vector (Ratio 1:9), and with 10 µl Lipofectamine in serum-free medium for 5 h under normal culture conditions. Then, cells are grown overnight in fresh medium and transferred to a 10-cm petri dish before starting selection with G418 or hygromycin, depending on the expression vector. After 2–3 weeks of antibiotic selection, single colonies can be transferred into 96-well plates and cultivated for verification.

36.2.1.2 Uptake Studies in Cell Monolayers

For uptake experiments according to Bakhiya et al. (2006), cells are seeded in 24-well plates (coated with 0.1 mg/ml poly-D-lysine) at a density of 2×10^5 cells/well in cell culture medium. Experiments will be conducted 2–3 days after cell seeding. Before starting the uptake experiments, cells are washed with an appropriate uptake buffer, for example, HBSS solution (130 mM NaCl, 4 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 18 mM glucose, and 20 mM HEPES, pH 7.4). The uptake assay is performed at 37°C and is started by the addition of 200 µl uptake buffer containing ^{3}H- or ^{14}C-labeled substrate to the cells. Unlabeled substrate is added to give the desired final concentration. For inhibition studies, inhibitors are included in the uptake buffer. For termination of the transport, solution is sucked off, and 0.5 ml ice-cold PBS buffer is immediately added into the wells. Cells are subsequently washed three times with PBS buffer, lysed with 0.5 ml of 1 N NaOH, and neutralized with 0.5 ml of 1 N HCl. The ^{3}H or ^{14}C content is assayed by liquid scintillation counting. The net uptake rates are calculated by subtracting values obtained with HEK cells from those obtained with transporter transfected HEK cells.

EVALUATION

36.2.1.3 Verification of the Test System

- Stably transfected cell line: Expression should be verified by immunoblotting and/or immunofluorescence microscopy and/or on mRNA level by real time PCR.
- At least 1.5-fold uptake of a substrate in stably transfected compared to empty vector cells.
- Time dependency experiments with time points between 0.5 and 30 min help to find the optimal time point, which is in the linear range of the time curve.
- Determination of K_m values of well-known substrates.
- Inhibition studies with known inhibitors (IC_{50} values).
- Protein concentrations are determined by using standard protocols like the Bradford method.

36.2.1.4 Procedure for Analyses of a Test Compound

See Fig. 36.1.

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- The eukaryotic cell lines expressing single uptake transporters at high expression levels.
- Low background activity.
- High-throughput screening.
- Radioactive-labeled substrates are preferred for uptake assays because of the high sensitivity and easy quantification.
- Opportunity for multiple transfection of cells (see Sect. 36.4.1).

Disadvantages

- Direct comparison to in vivo data is difficult.

MODIFICATION OF THE METHOD

- Uptake experiments are also performed in other plate formats, for example, 96-well or 6-well plates. The seeding density is depending on the plate format and the time point of experiment (1–3 days after seeding).
- Alternatively, CHO (Chinese hamster ovarian) cells can be used instead of HEK293 cells.
- Another possibility with lower sensitivity and variety of substrates is the measurement of fluorescent substrates like 6-carboxyfluorescein (which is a substrate for OATs) or the time-consuming analyses by LC/MS.
- Services for interaction studies with uptake transporters are offered by PortaCellTec biosciences (www.portacelltec.de).

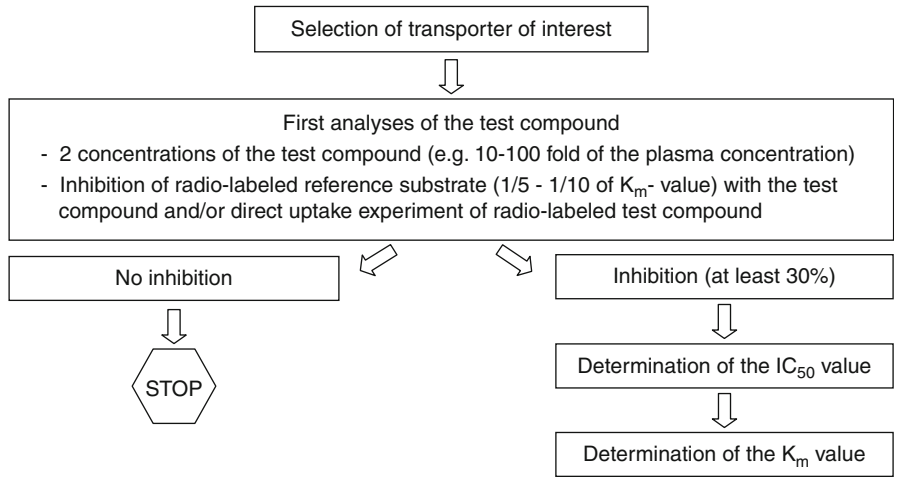

Fig. 36.1 Procedure for analysis of a test compound

36.2.1.5 Uptake in Suspension Cells

Due to high background binding of some radiolabeled substrates (e.g., ^{3}H-bilirubin) to poly-D-lysine-coated plastic dishes, uptake of these compounds into transfected cells is measured in cell suspension (Cui et al. 2001). For uptake assays, cells are detached from culture flasks by knocking, washed twice with uptake buffer, and resuspended in uptake buffer at a density of 3×10^6 cells/ml. ^{3}H-Bilirubin is diluted with human serum albumin in uptake buffer (75,000–100,000 dpm/ml). Unlabeled bilirubin is added to give the desired final concentrations. Uptake is started by mixing 1 ml of cell suspension with 1 ml bilirubin/albumin solution to give a final radioactivity of 37,500–50,000 dm/ml and stopped at different time points by centrifugation of the mixture at 13,000 rpm for 10 s. Cell pellets are washed twice with 1 ml of uptake buffer containing HSA and lysed in 2 ml of 0.2% SDS in water. Aliquots (300 µl) of the lysate are counted for radioactivity. To determine the nonspecific binding of ^{3}H-bilirubin, cells are incubated with ^{3}H-bilirubin in the presence of HSA for 1 min at 4°C. Cell-associated radioactivity measured under these conditions is used as a blank and subtracted from all other values.

36.2.1.6 Uptake in Hepatocytes by Silicon Oil Technique

According to Sugiyama 2008 for uptake assays, hepatocytes are diluted with ice-cold Krebs-Henseleit buffer at a concentration of 1–2 $\times$ 10^6 viable cells/ml.

Glass round-bottom centrifuge tubes are prechilled at 4°C, and 160 µl of cell suspension is added to each tube. The cell suspension is warmed in a water bath at 37°C for 3 min. To initiate cellular uptake, 160 µl substrate solution (37°C) is added to the cell suspension. When cells are sampled at 30 s, 2 min, and 5 min, an aliquot of 80 µl of the hepatocyte suspension is obtained at each time point and loaded into a sampling tube containing 100 µl of the oil mixture (46.8 g silicon oil (Sigma-Aldrich) and 8.6 g mineral oil (Sigma); final density: 1.015) and 50 µl 2 N NaOH to separate the hepatocytes from the incubation medium. The sampling tube is then centrifuged at 10,000$\times g$ for 10 s using a benchtop centrifuge. During the process, hepatocytes pass through the oil layer into alkaline solution, and the incubation medium remains above the oil layer. After centrifugation, the sample tube is incubated overnight at room temperature to enable complete lyses of the cells in NaOH phase. The sampling tube is (alternatively, after freezing) cut at the middle of the oil layer, and the upper and lower phases are transferred in two separate scintillation vials. The lower phase containing the lysed cells are neutralized with 50 µl of 2 N HCl. Radioactivity is measured using liquid scintillation counting.

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EXAMPLES

36.2.2 Example for OATs

The OATs play an important role in the secretion of several loop diuretics (e.g., torasemide). Torasemide and its metabolite are secreted through the sinusoidal membrane of the hepatocytes into the blood. The renal elimination is mediated by active uptake from the blood by OAT1 and OAT3 into proximal tubule cells followed by an OAT4-mediated efflux into the primary urine. In stably transfected HEK293 cells, Hagos et al. (2007) determined K_i values for torasemide and its metabolites using [^{3}H] PAH and [^{3}H] estrone sulfate as substrates. For example, the K_i values for torasemide were determined by Dixon plot analyses with 55 μM for OAT1, 90 μM for OAT3, and 47 μM for OAT4.

36.2.3 Example for OATPs

Coadministration of statins and fibrates is associated with an increased risk for myopathy, which may be due in part to a pharmacokinetic interaction. In case of the drug–drug interaction between cerivastatin and gemfibrozil, involvement of both metabolism and transporters is discussed. Shitara et al. (2004) have presented the interaction of gemfibrozil and gemfibrozil-glucuronide on the ^{14}C-cerivastatin uptake by the liver-specific OATP1B1 (OATP2) uptake transporter. Using transfected MDCK cells, they showed that gemfibrozil and gemfibrozil-glucuronide significantly inhibited the OATP1B1-mediated uptake of cerivastatin with IC_{50} values of 72 and 24 μM, respectively. These IC_{50} values of gemfibrozil and gemfibrozil-glucuronide were lower than their total plasma concentrations. However, because of the high plasma protein binding, the unbound concentrations of gemfibrozil and gemfibrozil-glucuronide were at most 0.97 and 2.3 μM, respectively, that is, less than the IC_{50} values obtained in the presented study. Because only unbound drugs interact with transporters, these results suggest that it is unlikely to cause the reported serious drug–drug interaction between cerivastatin and gemfibrozil. However, it is likely that gemfibrozil or its glucuronide inhibits the metabolism of cerivastatin in the liver because they are actively transported to the liver and accumulate there (Sallustio et al. 1996). The estimated unbound concentration in the liver of gemfibrozil-glucuronide is about 90 μM (Shitara et al. 2004).

36.2.4 Drug Transport Mediated by SLC Transporters Using *Xenopus laevis* Oocytes

PURPOSE AND RATIONALE

Transporters of the SLC family are responsible for the uptake of endogenous compounds or pharmaceutical drugs into the liver and kidney. To study liver- or kidney-specific uptake of such compounds, oocytes expressing single uptake transporters at a very high expression level are a widely used technology. The large size (~1 mm) of the oocytes facilitates the injection of cRNA, which is translated efficiently and results in a high number of transporters in the plasma membrane.

PROCEDURE

Xenopus laevis frogs can be purchased from different suppliers, for example, Nasco (www.enasco.com) or Xenopus Express (www.xenopus.com).

The procedure described is based on the references Vavricka et al. (2002), Hagenbuch et al. (1990), and Kullak-Ublick et al. (1995). In vitro synthesis of SLC transporter complementary RNA (cRNA) can be performed according to Kullak-Ublick et al. (1995) using T7 RNA polymerase (alternatively: T3 or SP6;

Promega Corp, Madison, WI). *Xenopus laevis* oocytes are prepared according to Hagenbuch et al. (1990): Frogs are anesthetized by immersion for 15 min in a 0.17% solution of ethyl m-aminobenzoate (MS-222). Oocytes are removed (Colman 1984) and incubated at room temperature for 3 h in Ca^{2+}-free OR-2 solution (82 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 2 mg/ml collagenase (Sigma type I) and 10 units/ml penicillin and 10 µg/ml streptomycin. They are then washed in modified Barth's solution consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES-NaOH (pH 7.6), 0.3 mM $Ca(NO_3)_2 \times 4H_2O$, 0.41 mM $CaCl_2 \times 6H_2O$, and 0.82 mM $MgSO_4 \times 7H_2O$ and containing 10 units/ml penicillin and 10 µg/ml streptomycin. Stage V and VI oocytes are selected. After an overnight incubation at 18°C modified Barth's solution, healthy oocytes are injected with 5 up to 23 ng of cRNA, cultured for 3 days in a medium containing 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.05 mg/ml gentamicin, and 15 mM HEPES (pH 7.6) and washed once with sodium-free buffer consisting of 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.5), trace amounts of radiolabeled substrate ^{3}H-BSP or ^{3}H-rifampicin, and appropriated substrate and inhibitor concentrations. Water-injected oocytes are used as controls for unspecific substrate uptake. After an incubation period of 20 min, the uptake experiment is stopped by adding 6 ml of ice-cold solution consisting of sodium-free choline buffer and 1 mM unlabeled substrate. Oocytes are washed twice with 6 ml ice-cold stop solution. Each oocyte is dissolved in 0.5 ml of 10% sodium dodecyl sulfate and 5 ml scintillation fluid, and the oocyte associated radioactivity is determined.

EVALUATION

Determination of K_m values of radiolabeled compounds is possible by using nonlinear regression (Vavricka et al. 2002). Inhibition constants (K_i) are estimated by Dixon plot analysis and linear regression using ordinary least squares.

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- High expression levels.
- Low background activities of the oocytes.
- Injection of several cRNAs at the same time is possible.

Disadvantages

- Susceptible system, for example, to fluctuating temperature or transportation.
- Low reproducibility because of high variability of the oocytes.
- Fluctuating gene expression.
- Time-consuming and therefore suitable only for low throughput.
- Correlation to in vivo data is difficult.

MODIFICATION OF THE METHOD

- Alternatively, pre-injected Xenopus oocytes are commercially available by BD Biosciences, Woburn, MA.

36.2.4.1 Electrophysiological Studies

Xenopus laevis oocytes are also suitable for electrophysiological studies on electrogenic transporters. The method has been described by Wolff et al. (2007). Briefly, studies are carried out 3 days after cRNA injection at room temperature. Current recordings are made in ORI-2 without gentamicin and sodium pyruvate using the two-electrode voltage clamp technique with a commercial amplifier (OC725, Warner, Hambden, CT, USA). Borosilicate glass microelectrodes are filled with 3 M KCl and have resistances of ~1 M. The resting membrane potential of the oocytes ranged between −28 and −46 mV and holding currents to achieve a potential of −60 mV were in the range of −10 to −40 nA. Steady-state currents are obtained during 5 s voltage pulses from −60 mV to potentials between −90 and 0 mV in 10-mV steps. The current–voltage (I–V) relationships for substrate-induced currents, ΔI, are determined by subtraction of the steady-state currents in the absence of substrate from currents in the presence of substrate, respectively.

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EXAMPLES

The antibiotics rifamycin SV and rifampicin reduce sulfobromophthalein (BSP) elimination in humans. Using injected oocytes, Vavricka et al. (2002) have demonstrated that rifampicin is transported by OATP1B1 (OATP-C) and OATP1B3 (OATP8) and that both rifampicin and rifamycin SV inhibit OATP1B1-, OATP1B3-, OATP2B1-, and OATP1A2-mediated BSP uptakes. These results show that rifamycin SV and rifampicin interact with OATP-mediated substrate transport to different extents. Inhibition of human liver OATPs can explain the previously observed effects of rifamycin SV and rifampicin on hepatic organic anion elimination.

36.3 Drug Efflux by ABC Transporters

36.3.1 Drug Transport Mediated by ABC Transporters Using Eukaryotic Membrane Vesicles

PURPOSE AND RATIONALE

Transporters expressed in organs such as intestine, liver, and kidney may significantly affect uptake, distribution, and elimination of drugs. Efflux transporters are often accounted for the transport of endogenous compounds and pharmaceutical drugs out of the body into bile, urine, or gut in order to protect the cell. The efflux transporters of the ATP-binding cassette (ABC) proteins use the energy of ATP hydrolysis to mediate the transport of the substrate against a concentration gradient.

In vitro studies on the function of efflux transporters are mainly performed using cell systems or vesicle preparations from various sources such as tissue, primary cells, or cell lines transfected with specific transport proteins.

In whole-cell systems, the drug efflux can be measured directly only after the cells are loaded with the study compound. Inside-out vesicles can be perfectly used to study single efflux processes because preloading of cells is not necessary and because of well-defined study conditions.

PROCEDURE

36.3.1.1 Preparation of Plasma Membrane Vesicles from Eukaryotic Cell Lines

Selection of the cell line or tissue type influences not only the technique for membrane vesicle preparation but also the resulting percentage of inside-out-oriented plasma membrane vesicles. A sufficient amount of inside-out-oriented vesicles is essential since only this fraction, with the ATP-binding domains oriented to the outer surface, mediates ATP-dependent transport of a labeled substrate into the vesicle.

The procedure described is based on references Jedlitschky et al. (1994, 1996), Leier et al. (1994a, b), and Keppler et al. (1998).

Eukaryotic cells expressing ABC transporters (e.g., HEK-MRP1) (about 3×10^9) are harvested by centrifugation (1,200×*g*, 10 min, 4°C) and washed twice in ice-cold phosphate-buffered saline (PBS: 0.15 M NaCl, 50 mM KH_2PO_4, pH 7.4). Hypotonic lysis of the cell pellet (about 5 ml) is induced by 40-fold dilution with hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EDTA) supplemented with proteinase inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.8 μM E64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane], 1 μM leupeptin, and 0.3 μM aprotinin]. After gentle stirring on ice for 1.5 h, the cell lysate is centrifuged at 100,000×*g* for 40 min at 4°C. The pellet is then resuspended in 20 ml of hypotonic buffer and homogenized with a Potter-Elvehjem homogenizer (500 rpm, 2 strokes/min, 30 strokes, 4°C).

The homogenate is diluted with incubation buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4) and centrifuged for 10 min at 12,000×*g* at 4°C. The resulting postnuclear supernatant is stored on ice. The corresponding pellet is resuspended in 20 ml of

incubation buffer supplemented with proteinase inhibitors and homogenized and centrifuged again as described earlier. Both postnuclear supernatants are combined and centrifuged at 100,000×*g* for 40 min at 4°C. The pellet is resuspended in 20 ml of incubation buffer and homogenized manually by 50 strokes with a tightly fitting Dounce B (glass/glass) homogenizer (Fisher Scientific, Pittsburgh, PA) on ice.

The homogenate is diluted by addition of 10 ml of incubation buffer to give a crude membrane fraction which is then layered on top of a 38% (w/v) sucrose solution in 5 mM HEPES-KOH, pH 7.4 and centrifuged at 280,000×*g* for 2 h at 4°C in a swing-out rotor. The turbid layer at the interface is collected and diluted in 20 ml of incubation buffer, resuspended and homogenized by 20 strokes with a Dounce B homogenizer on ice, and finally washed by centrifugation at 100,000×*g* for 40 min at 4°C. The resulting pellet is diluted in 1 ml of incubation buffer. Vesicles are formed by passing the suspension 20 times through a 27-gauge needle with a syringe. Aliquots of the membrane vesicle suspension are frozen and stored in liquid nitrogen.

36.3.1.2 Measurement of ATP-Dependent Transport of Leukotriene C_4 (LTC4) by Rapid Filtration Through Nitrocellulose Filters

Various labeled substrates for the conjugate export pumps encoded by MRP1 and MRP2 genes may serve to assess the transport rate in membrane vesicle preparations (e.g., leukotriene C4, 17β-glucuronosyl estradiol, 6α-glucuronosylhyodeoxycholate, 3α-sulfatolithocholyltaurine, glucuronosyl-etoposide, doxorbicin, daunorubicin, vinblastine). Because of its high affinity and specificity for MRP1 and MPR2 and commercial availability, ^{3}H-LTC4 is a preferred substrate for transport measurements. This method is based on references Bohme et al. (1993), Buchler et al. (1994), and Leier et al. (1994a).

Using LTC_4 as substrate, nitrocellulose filters (0.2 μm pore size, 25 mm diameter) are soaked in incubation buffer (0.25 mM sucrose, 10 mM Tris–HCl, pH 7.4). A rapid filtration apparatus from Millipore (Bedford, MA) is prepared. A final volume of 110 μl of transport assay mixture (4 mM ATP (potassium salt), 10 mM creatine phosphate (Tris salt), 10 mM $MgCl_2$, 10 mM Tris/HCl (pH 7.4), 5 mM glutathione (reduced), 0.25 mM sucrose, 100 μg/ml creatine phosphokinase (2 units/110 μl), 50 nM ^{3}H-LTC_4 (50 nCi/110 μl); pH 7.4) is preincubated at 37°C for 1 min. Blanks are prepared by replacing ATP by 5′-AMP.

Membrane vesicle suspension should be thawed quickly at 37°C and stored on ice for about 40 min before use. The transport assay is initiated at 37°C by addition of membrane suspension (30 μg protein) to the transport assay mixture (110 μl). Twenty microliter aliquots are removed after 30- or 60-s intervals, immediately diluted with 1 ml of ice-cold incubation buffer, and immediately filtered through nitrocellulose membrane using the vacuum of the filtration apparatus (200 mbar). Filter membranes are rinsed twice with 5 ml of cold incubation buffer, dried, and dissolved in 10 ml scintillation fluid to count for radioactivity.

The transport rate of ^{3}H-LTC_4 into the membrane vesicle is calculated by subtracting the corresponding values in the presence of 5′-AMP (blank sample) from those in the presence of ATP.

EVALUATION

The protein concentration of the membrane vesicle preparation is determined by standard protocols like the Lowry method. The measurement of the Na^+, K^+-ATPase activity may serve to assess the enrichment of plasma membranes relative to the original homogenate (Scharschmidt et al. 1979). This enrichment should be 15- to 30-fold. The sidedness of the membrane vesicle preparation may be estimated by the activity of an ectoenzyme, nucleotide pyrophosphatase (EC 3.6.1.9), in the presence or absence of Triton X-100 for solubilization of the membrane vesicle (Bohme et al. 1994). The percentage of inside-out-oriented plasma membrane vesicles from HL60 cells (Jedlitschky et al. 1994), mastocytoma cells (Leier et al. 1994b), or HeLa (Leier et al. 1994a) cells should range from 30% to 40%.

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Vesicles can be made by a various number of sources.
- Vesicles can be stored at −80°C for a month.
- Membrane vesicles are useful in assessing the mechanism of transport (driving force) and identifying substrates and inhibitors of efflux transporters.
- This assay is less time and labor-intensive compared to cellular Transwell assays.

– Drug transport by a specific transport protein can be characterized without interference from other transport processes.
– In contrast to the cellular system, the drug interacts directly with the efflux transporters in the vesicle model without preloading the cells with the drug.
– For inhibition studies, passive permeability of the potential inhibitor is not relevant, and compared to cell-based assays, the concentration of the inhibitor is known.

Disadvantages
– Preparation of membrane vesicles is a time-consuming and lab-intensive process.
– Major disadvantages in identifying substrates are that compounds with a high passive permeability can penetrate the vesicles in both directions passively much faster than by the efflux transporter.

MODIFICATION OF THE METHOD

36.3.1.3 Preparation of Plasma Membrane Vesicles

The preparation of plasma membrane vesicles from liver canalicular membrane is highly enriched with the canalicular (apical) isoform MRP2 (Buchler et al. 1996). Methods for the isolation of hepatocyte canalicular membranes from liver tissue have been described in detail (Bohme et al. 1994; Boyer and Meier 1990). The percentage of inside-out-oriented vesicles in these preparations amounts to 32%. Alternatively, transfected HEK and MDCK cells are often used to study ATP-dependent transport into inside-out vesicles (Cui et al. 1999; Leier et al. 2000).

36.3.1.4 Measurement of ATP-Dependent Transport of Leukotriene C_4 (LTC4) by Centrifugation of the Vesicles Through a Gel Matrix

For substrates more hydrophobic than LTC_4 which binds strongly to nitrocellulose filters (e.g., glucuronosyl-etoposide, glutathione S-conjugates of melphalan and chlorambucil), small Sephadex G-50 columns should be employed (Jedlitschky et al. 1996, Bohme et al. 1993, and Buchler et al. 1994). For this procedure, NICK spin columns (Pharmacia, Uppsala, Sweden) (0.2 g Sephadex G-50/3.3 ml) are prepared, rinsed with incubation buffer, and centrifuged at $400\times g$ for 4 min at 4°C. Aliquots of the incubations are diluted in 80 µl of ice-cold incubation buffer and loaded immediately onto the columns. After rinsing with 100 µl of incubation buffer, the columns are centrifuged at $400\times g$ for 4 min 4°C. The effluents are collected and counted for radioactivity.

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EXAMPLES

Para-aminohippurate (PAH) is widely used as model substrate for organic anion transport in proximal tubule epithelia (kidney). Leier et al. (2000) investigated PAH as a substrate for the apical multidrug resistance protein MRP2 (ABCC2) which is expressed in kidney, as well as in liver and small intestine. Membrane vesicles from HEK-MRP2 cells and from control cells were incubated with various concentrations of ^{3}H-PAH, and the net ATP-dependent transport into inside-out vesicles was determined. Transport rates at 10 μM PAH were 22 pmol/mg protein/min and 1.6 pmol/mg protein/min with membrane vesicles from HEK-MRP2 and HEK-control cells, respectively. The K_m value for PAH was 880 μM. MRP2 substrate leukotriene C4 and the MRP1-inhibitor MK571 inhibited MRP2-mediated PAH (100 nM) transport with IC_{50} values of 3.3 and 4.0 μM, respectively. The nephrotoxic mycotoxin ochratoxin A inhibited MRP2-mediated PAH transport with an IC_{50} value of 58 μM. Ochratoxin A itself was a substrate of MRP2 (Leier et al. 2000).

36.3.2 Drug Transport Mediated by ABC Transporters Using Membrane Vesicles from Insect Cells

PURPOSE AND RATIONALE

Multidrug resistance proteins (MRPs) have been suggested to play an important role in the transport and detoxification of a wide range of endogenous compounds and xenobiotics. They are predominantly expressed at the apical membrane of the small intestine, proximal tubules of the kidney, and canalicular membrane of hepatocytes being involved in intestinal, renal, and hepatobiliary excretion of compounds.

To obtain a relatively high expression of transporter protein expression, baculovirus-infected Sf9 insect cells can be used.

Vesicles can be used to study substrate specificity of transporters, transport of xenobiotics and endogenous substances, transporter-mediated drug–drug interaction, hepatotoxicity, pharmacological efficacy, and transporter-metabolism interplay in drug disposition.

PROCEDURE

36.3.2.1 Preparation of Plasma Membrane Vesicles

According to Chu et al. (2004, 1997), *Spodoptera frugiperda* (Sf9) cells in suspension are grown in Sf-900 II SFM medium in the absence of serum (Invitrogen, Carlsbad, CA). About 4×10^7 Sf9 cells are seeded in 175 cm^2 tissue culture flasks. After the cells become attached, the medium is removed, and 3 ml of medium and 3 ml of virus stock containing, for example, MRP1, MRP2, or MRP3 (about 5–8 $\times$ 10^7 recombinant baculovirus/ml) are added to infect the cells. One hour after addition of the virus to the cells, cell culture medium is added up to a final volume of 30 ml. After incubation for 72 h at 26°C, the cells are harvested and washed twice in ice-cold washing buffer (50 mM Tris/HCl, 300 mM mannitol, 0.5 mM PMSF, pH 7.0) and centrifuged at 800×*g* for 5 min at 4°C. The cell pellet is resuspended in ice-cold TMEP buffer (50 mM Tris, 50 mM mannitol, 2 mM EGTA-Tris, 2 mM DTT, aprotinin (8 μg/ml), leupeptin (10 μg/ml), PMSF (50 μg/ml), pH 7.0) and homogenized for 10 min on ice using a tight-fitting Dounce homogenizer. After centrifugation at 800 × g for 10 min at 4°C, the supernatant is collected and centrifuged at 100,000×*g* for 1 h at 4°C. The pellet is resuspended in TMEP buffer and passed 20 times through a 27-gauge needle. The vesicles are dispensed in aliquots, frozen in liquid nitrogen, and stored at −80°C until use.

36.3.2.2 Vesicular Uptake Studies by Rapid Filtration Technique

As reported by Chu et al. 1997, the transport medium contained the radiolabeled substrate (e.g., ^{3}H-ethinylestradiol-3-*O*-sulfate or –3-*O*-glucuronide, ^{3}H-estradiol-17β-D-glucuronide, ^{14}C-ethacrynic acid glutathione), 250 mM sucrose, 10 mM Tis/HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM adenosine 5′ triphosphate (ATP) or 5 mM adenosine monophosphate (AMP), and an ATP-regenerating system (10 mM creatinine phosphate and 100 μg/ml creatine phosphokinase). The uptake study is performed at 37°C. After preincubation for 3 min at 37°C, the uptake study is started by the addition of vesicle suspension (10 μg protein). The final incubation volume is 20 μl. In the inhibition study, the inhibitors are dissolved in transport buffer and preincubated with radiolabeled substrates for 3 min. At designated time points, transport is terminated by

adding 1 ml of ice-cold stop solution containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose, and 100 mM NaCl. The stopped reaction mixture is filtered through 0.45 μm HA Millipore filters (Millipore Corporation, Bedford, MA) and subsequently washed twice with 5 ml ice-cold stop solution. For the uptake study with ^{3}H-estrone-3-sulfate, glass fiber (type A/E) filters (Gelman Sciences, Dorval, Quebec, Canada) are used to minimize nonspecific binding to membrane filters. The radioactivity retained on the filter and in the reaction mixture is measured in a liquid scintillation counter. ATP-dependent uptake is determined as the difference in uptake in the presence and absence of ATP.

36.3.2.3 ATPase Measurements

ATPase activity of the isolated Sf9 cell membranes is estimated by measuring inorganic phosphate liberation as described by Sarkadi et al. (1992). The differences between ATPase activities measured in the absence and presence of vanadate (100 μM) can be plotted.

EVALUATION

36.3.2.4 Membrane Preparations

The membrane protein concentrations of membrane vesicles are determined as described by Sarkadi et al. (1992).

36.3.2.5 Data Analysis

Kinetic parameters for the ATP-dependent uptake are obtained by fitting the data to the following equation:

$$V_c = V_{max} \times S/(K_m + S), \qquad (36.1)$$

where V_c is the initial uptake rate of substrate (pmol/min/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM), and V_{max} is the maximum uptake rate (pmol/min/mg protein).

The inhibition constant (K_i) values for evaluating the inhibitory effect of, for example, ethinylestradiol-3-*O*-glucuronide on the uptake of ^{14}C-ethacrynic acid glutathione by MRP2 is obtained by fitting the following equation to the data as described by Chu et al. (1997):

$$V_{(+I)}/V_{(-I)} = 1/[1 + (I/K_i)], \qquad (36.2)$$

where $V_{(+I)}$ and $V_{(-I)}$ represent the transport velocity in the presence and absence of inhibitor, respectively, and I is the inhibitor concentration. This equation is derived based on the assumptions that firstly inhibition is competitive or noncompetitive and secondly that the radiolabeled substrate concentration used (e.g., ^{14}C-ethacrynic acid glutathione (2 μM)) is much lower than its K_m value (15 μM for MRP2).

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Vesicles can be stored frozen for a long period.
- High-throughput screening.
- Both uptake and efflux transporters can be studied.
- Compared to mammalian cell lines, Sf9 cells have a reduced transporter background and higher expression levels.

Disadvantages

- Lipid membrane composition and glycosylation mechanism are different in insect cells compared to eukaryotic cells.
- The reduced cholesterol content of Sf9 can influence the activity of efflux transporters.

MODIFICATION OF THE METHOD

Ready to use membrane vesicles for the ABC transporters MDR1, MRP1, MRP2, MRP3, and MXR are commercially available from Solvo Biotechnology (Hungary, Budapest; www.solvo.com) and can be used for uptake measurements. Membranes and protocols for ATPase measurements are also available.

Recombinant baculoviruses can be prepared as described by Bakos et al. (1998) by using the BaculoGold Transfection Kit according to the manufacturer's recommendations (Pharmingen, San Diego, CA) or by using the pAcUW21 plasmid (Invitrogen, San Diego, CA).

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EXAMPLES

Bakos et al. (2000) studied the interactions of the human multidrug resistance proteins MRP1 and MRP2 with the organic anions probenecid, furosemide, sulfinpyrazone, penicillin G, and indomethacin. Firstly, they determined the concentrations at half-maximum uptake rates ($K_{1/2}$) for N-ethylmaleimide glutathione (NEM-GS) with $K_{1/2}$ 200 μM for MRP1 and 2.5 mM for MRP2. The effect of organic anions on the relative rate of NEM-GS uptake by MRP1 and MRP2 was then analyzed: Sulfinpyrazone and probenecid efficiently inhibited ATP-dependent active vesicular NEM-GS uptake by MRP1. In case of indomethacin, low concentrations produced a significant stimulation, and only indomethacin concentrations of more than 100 μM inhibited the NEM-GS uptake by MRP1. For MRP2, probenecid inhibited NEM-GS uptake, whereas low concentrations of sulfinpyrazone and indomethacin strongly stimulated the transport of this GS conjugate. Higher sulfinpyrazone and indomethacin concentrations were inhibitory again. The addition of methotrexate caused a slight inhibition of the NEM-GS uptake in both MRP1 and MRP2. Penicillin G caused a major stimulation of MRP2 transport, whereas benzbromarone or furosemide was inhibitory. These tracer uptake experiments clearly suggested that various organic anions acted differently on the two transporters.

36.4 Drug Uptake and Efflux

36.4.1 Drug Transport Mediated by SLC and ABC Transporters Using Double-Transfected Cells

PURPOSE AND RATIONALE

The uptake of substances into hepatocytes and the subsequent export into bile can be viewed as a vectorial transport process. This vectorial transport in human liver can be mimicked in an experimental model system using MDCK cells, which grow in polarized fashion with distinct apical and basolateral membrane domains when cultured on an appropriate filter membrane support (Kopplow et al. 2005). Prof. Keppler (DKFZ, Heidelberg, Germany) established a double-transfected Madin-Darby canine kidney (MDCK) cell line expressing the human hepatocyte basolateral uptake transporter hOATP1B3 (OATP8) and the apical conjugate export pump MRP2 (Cui et al. 2001). Also quadruple-transfected cells are generated with three basolateral transporters hOATP1B1, hOATP1B3, and hOATP1A2 and the apical transporter MRP2 (Kopplow et al. 2005).

To study the transcellular transport via basolateral and apical transporters of renal epithelial cells, Sato et al. (2008) established double-transfected MDCK cells expressing human organic cation transporter hOCT2 and hMATE1.

Double-transfected cells can give a better understanding of the synergistic role of uptake and efflux transporters and therefore facilitate the comparison to in vivo data.

PROCEDURE

36.4.1.1 Cell Culture and Cell Lines

MDCKII cells are cultured according to Cui et al. (2001). In brief, cells are cultured in minimum essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 95% humidity, 5% CO_2 at 37°C. For selection, antibiotics are added (1 mg/ml Zeocin, 800 μg/ml G418 disulfate, 500 μg/ml hygromycin B). Cells are passaged every 3–4 days at 1:10 to 1:15 ratios.

Double-transfected MDCKII cells expressing the human uptake transporter OATP1B3 and the apical export pump MRP2 can be generated by stepwise transfection of MDCK cells. Transfectants expressing recombinant OATP1B3 are selected with hygromycin. The clone with the highest OATP1B3 expression is further transfected with the vector construct pcDNA3.1-MRP2 with the full-length human MRP2 cDNA. After selection with 950 μM hygromycin and 800 μM G418 disulfate for 3 weeks, the transfectants are screened for both MRP2 and OATP1B3 expression by immunoblot analyses (Cui et al. 2001).

36.4.1.2 Transport Measurements

According to Cui et al. (1999, 2001), for transport assays, MDCKII cells are grown on Transwell™ Clear polyester membrane inserts (pore size 0.4 μm; Corning Costar #3450, Cambridge, MA) for 8 days in culture medium without antibiotics. Medium is replaced twice. Butyrate, at a final concentration of 10 mM, is added to the medium 24 h before use of the cells. Cells are first washed with transport buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). Subsequently, ^{3}H-labeled substrates (^{3}H-BSP, 50–100 nM, 12.3 Ci/mmol) or nonlabeled drug candidates (50 μM) are added in transport buffer either to the apical compartments (1.5 ml) or to the basolateral compartments (2.5 ml). After 90 min, samples from the opposite compartments are taken and analyzed either by liquid scintillation spectrometry or by LC-MS/MS.

The transcellular leakage is determined by incubating cells with 50 μM ^{14}C-inulin carboxylic acid in the basolateral compartments for 30 min and measuring the radioactivity in the apical compartments. The transcellular leakage should be less than 1%.

EVALUATION

Expression and localization of OATP1B3 and MRP2 in MDCK cells was demonstrated by immunoblotting and confocal laser scanning microscopy (Cui et al. 2001). ^{3}H-labeled sulfobromophthalein (BSP) was a substrate for both transport proteins and was transferred from the basolateral to the apical compartment at a rate at least six times faster by double-transfected MDCK-OATP1B3/MRP2 cells than by single-transfected MDCK-OATP1B3 or MDCK-MRP2 cells.

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Opportunity to study transcellular/vectorial transport of a compound
- Study of efflux transporters without preloading of cells or preparation of membrane vesicles
- Facilitates the comparison to in vivo data

Disadvantages

- No complete human tissue model, just providing information on partial function.
- For drugs, which are highly membrane permeable, the permeation rate across the cell monolayer on the Transwell membrane insert might overcompensate the active transport of the drug. In these cases, the transport rate does not differ between double-transfected, single-transfected, and wild-type cells.
- Only MDCK cells are suitable, HEK293 or CHO cells are not growing tight enough and therefore show too much transcellular leakage.
- The evaluation and quantification of the results is more difficult by using multiple-transfected cells.

MODIFICATION OF THE METHOD

For inhibition studies, the inhibitors are preincubated in both apical and basolateral compartments for one hour and then added simultaneously with ^{3}H-BSP into the basolateral compartment and without ^{3}H-BSP into the apical compartment. After 90 min, the radioactivity is measured in the apical compartment. Using different inhibitor concentrations, the K_i (IC_{50}) value can be calculated. This technique can also be used for drug–drug interaction studies on the level of transporters.

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EXAMPLES

Sasaki et al. (2002) established the double-transfected MDCK cell line, which expresses both OATP1B1 (OATP2) and MRP2 on basal and apical membranes. They measured the basolateral-to-apical transport of the lipid-lowering drug pravastatin. The K_m value of pravastatin was 24.3 +/− 10.4 μM, which was in the same magnitude to the K_m of estradiol-17β-glucuronide and comparable with the K_m reported for OATP1B1 only (33.7 μM, Hsiang et al. 1999). Because pravastatin is a low-affinity substrate of MRP2 (220 μM, Yamazaki et al. 1997), the rate-determining step in the transcellular transport of this compound is the uptake mediated by OATP1B1. This hypothesis is consistent with the previous observations that uptake is the rate-determining step for the biliary excretion of pravastatin in rats (Yamazaki et al. 1997).

Double-transfected MDCK cell are established within the members of SLC transporter family only, as shown for OCT1 or OCT2 in combination with MATE1 (Konig et al. 2011). The double-transfected MDCK-OCT1-MATE1 cells and MDCK-OCT2-MATE1 cells demonstrated a significantly higher transcellular transport of metformin (by 50 μM) compared to control cells or single (OCT1, OCT2, or MATE1)-transfected cell. The total metformin transcellular transport and the net-transcellular transport (from the basal into apical compartment) were 6- to 12-fold. The vectorial transport was not saturated up to 2.5 mM concentration of metformin. These in vitro experiments underline the coordinated hepatic elimination of drugs by OCT1 and MATE1 as well as the renal elimination of metformin in proximal tubule cells by OCT2 and MATE1.

36.4.2 Liver-Specific Drug Transport in Sandwich-Cultured Hepatocytes

PURPOSE AND RATIONALE

The knowledge regarding hepatic drug uptake and efflux of compounds in humans is an essential part of the drug development process.

Several human SLC uptake transporters (OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, NTCP) are expressed on the basolateral (sinusoidal) membrane of human hepatocytes and mediate the hepatic uptake of hydrophilic charged compounds. The apical (canicular) efflux from hepatocytes to bile is driven by ATP-dependent ABC transporters comprising MDR1, BSEP, BCRP, and MRP2. Basolateral excretion of bile acids from hepatocytes into blood is mediated by MRP3 and MRP4.

Primary hepatocyte culture is a reliable in vitro model to study transporter-mediated substrate uptake and efflux in the liver and to evaluate the cooperation of these different drug transporters. Compared to monolayer hepatocytes, freshly or cryopreserved hepatocytes cultured between two layers of gelled collagen (SCH = sandwich-cultured hepatocytes) retain more in vivo–like properties, including formation of intact canalicular network and polarized excretory function. Therefore, SCH are utilized for long-term studies.

In addition, this SCH in vitro model system can also be used to study species differences in drug transport, transport regulation, drug–drug interactions, drug–transporter–enzyme interaction, and hepatotoxicity.

PROCEDURE

36.4.2.1 Hepatocyte Isolation and Culture

Male Wistar rats (250–280 g) can be obtained from Charles River. They are allowed to free access to food (laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water and are housed in a constant alternating 12-h light (6:00 a.m. to 6:00 p.m.) and dark cycle.

According to Liu et al. (1999b), hepatocytes are isolated with a two-step perfusion method, and according to LeCluyse et al. (1996), Anneart et al. (2001) and Chandra et al. (2001), after the rat is anesthetized (60 mg/kg ketamine + 12 mg/kg xylazine intraperitoneal), the portal vein is cannulated, and the liver is perfused with a Ca^{2+}-free buffer (118.1 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 1 mM EGTA, 4.7 mM KCl, 1.2 mM KH_2PO_4, pH 7.4) equilibrated with 95% O_2/5% CO_2 at a rate of 30 ml/min. The inferior vena cava is cannulated to establish a recirculating system (100 ml Ca^{2+}-free buffer). After 10 min of perfusion, 0.05–0.075 g of collagenase and 4 mg of soybean trypsin inhibitor are added to the

perfusate reservoir to obtain a final collagenase concentration of ~200 U/ml; 1 min later, 1 ml of $CaCl_2$ is added. The liver is perfused for ~10 min with the collagenase buffer, removed from the rat, and immersed in ice-cold medium (DMEM containing 5% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 4 mg/l insulin, and 1 μM dexamethasone). The capsule surrounding the liver is torn gently in order to release the hepatocytes. The cells are filtered through a 70-μm mesh filter and then centrifuged ($50\times g$) for 2 min at 4°C. The pellet is resuspended in equal parts of medium and isotonic Percoll and centrifuged ($70\times g$) for 5 min at 4°C to separate out the nonviable cells. The pellet is resuspended in medium and centrifuged ($50\times g$) for 2 min at 4°C. Hepatocytes are counted in a hemocytometer, and viability is determined using the trypan blue exclusion method. Viability is always >90% with a yield of 2–3 × 10^8 cells. Cells are resuspended in medium and diluted to a final concentration of 1 × 10^6 cells/ml.

36.4.2.2 Preparation of Sandwich-Cultured Hepatocytes (SCH)

According to Abe et al. 2008, hepatocytes are seeded at a density of 1.75 million cells per well on 6-well BioCoat™ plated (BD Biosciences Discovery Labware) in 1.5 ml Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS, 10 mM insulin, 1 μM dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units penicillin G sodium, and 100 μg streptomycin sulfate. Cells are incubated at 37°C in a humidified incubator with 95% O_2/5% CO_2 and allowed to attach for 2 h, at which time the media is aspired to remove unattached cells and fresh media is added. Twenty-four hours later, cells are overlaid with BD Matrigel™ basement membrane matrix (BD Biosciences Discovery Labware) at a concentration of 0.25 mg/ml in 2 ml ice-cold DMEM containing ITS™ (insulin/transferring/selenium culture supplements from BD), 0,1 μM dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units penicillin G sodium, and 100 μg streptomycin sulfate. Hepatocytes are cultured for 4 days; media is changed daily.

36.4.2.3 Accumulation in Sandwich-Cultured Hepatocytes

According to Abe et al. 2008, on day 4, hepatocytes are rinsed twice and then preincubated for 10 min at 37°C with 2 ml of warmed Hank's balanced salt solution (HBSS) containing Ca^{2+} (standard; cells + bile) or Ca^{2+} -free HBSS (cells), in order to maintain or disrupt the tight junctions sealing bile canalicular network, respectively. Subsequently, hepatocytes are incubated with test compound (0.5–30 μM for ARB or statin; 1 μM for [^{3}H]taurocholate or [^{3}H] E2-17ßG) in standard HBSS for 5–20 min (ARB or statin) or 10 min ([^{3}H]taurocholate or [^{3}H]E2-17ßG) at 37°C. For uptake inhibition studies, BSP (a known inhibitor of organic anion transporters and Na^+ taurocholate-cotransporting polypeptide) is added simultaneously with test compound to the hepatocytes. After incubation, the dosing solution is aspirated from the cells, and uptake is stopped by washing the cells three times with ice-cold standard HBSS. For radiolabeled compounds, cells are lysed with 1 ml of 0.5% Triton X-100 in phosphate-buffered saline. For ARBs and statins, cells are lysed with 1 ml of 70% (v/v) methanol and sonicated for 20 s with a sonic dismembrator (model 100; Thermo Fisher Scientific, Waltham, MA) and stored at <−70°C until analysis. The samples are analyzed for drug concentrations by liquid scintillation counting or by liquid chromatography with tandem mass spectrometry.

36.4.2.4 Efflux Studies in Sandwich-Cultured Hepatocytes

Compared to the accumulation protocol, basolateral and canalicular efflux studies in SCH are defined by the reversal of substrate incubation and tight junction disruption steps. According to Liu et al. (1999a, b), hepatocytes cultured in a collagen-sandwich configuration are incubated in 3 ml of standard buffer (Hanks' balanced salt solution: 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 136.9 mM NaCl, and 5.6 mM D-glucose) at 37°C for 10 min. Each dish received 3 ml of standard buffer containing 1 μM ^{3}H-taurocholate or 3.6 μM ^{14}C-salicylate followed by incubation at 37°C for 10 min. Subsequently, the incubation buffer is removed, and cultures are washed four times with 3 ml ice-cold standard buffer to quench the transport processes and remove extracellular substrate. Efflux is initiated by addition of 3 ml of standard buffer or Ca^{2+}-free buffer to each dish. Aliquots of efflux buffer (0.1 ml) are removed at designated times and analyzed by liquid scintillation spectrometry.

EVALUATION

- Taurocholate, a model bile acid, has been used to evaluate the potential for drugs to inhibit bile acid transport in the SCH.
- Time and concentration should be within the linear range of uptake, preferably within the first 10–15 min.
- Substrate accumulations are corrected for nonspecific binding by using Matrixgel-precoated dishes without cells.
- Determination of protein levels of transporter in freshly isolated hepatocytes day 0 and SCRH and on day 4 of culture by immunoblot analysis.
- Efflux or uptake data is normalized to the total solubilized protein content. Bio-Rad DC protein assay kit (Bio-Rad Laboratories) can be used to determine the protein concentration in the culture extracts using BSA as standard. Triton X-100 (1%) does not interfere with this assay.

36.4.2.5 Data Calculation

Hepatic uptake clearance of substrates may be quantified based on the total accumulation of substrate in standard HBSS in the linear range of uptake according the following equation:

$$\text{In vitro } Cl_{\text{uptake}} = \frac{\text{Accumulation (cells + bile)}}{AUC_{0-T}}.$$

The accumulation (pmol/mg protein), biliary excretion index (BEI;%), and in vitro intrinsic Cl_{biliary} (ml/min/kg) are calculated using B-Clear® technology (Qualyst, Inc, Raleigh, NC) based on following equations (Liu et al. 1999a):

$$\text{BEI (\%)} = \frac{\text{Accumulation (cells + bile)} - \text{Accumulation (cells)}}{\text{Accumulation (cells + bile)}} \times 100.$$

The in vitro biliary clearance may be quantified based on the total accumulation of substrate in the bile canalicular network divided by the area under the concentration curve of the dosing medium using the following equation:

$$\text{In vitro } Cl_{\text{biliary}} = \frac{\text{Accumulation (cells + bile)} - \text{Accumulation (cells)}}{AUC_{0-T}}.$$

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Investigate hepatic uptake and efflux kinetics of substrates in in vivo–like properties.
- Most hepatic transporters and drug-enzymes are expressed.
- Compared to freshly isolated hepatocytes, SCH can be used for long-term studies.
- Good in vitro and in vivo correlation.
- Cryopreserved hepatocytes are a good alternative compared to freshly isolated hepatocytes.

Disadvantages

- Isolation of hepatocytes is an intensive method that has to be optimized for each species.
- SCH is a static model with no bile and blood flow.
- Using primary cultures of hepatocytes, the retention of transporter expression and activity needs to be evaluated after each preparation.
- The extent of bile canalicular network formation and optimal levels of metabolizing enzymes and transport proteins are important factors to consider when determining the appropriate time in culture to conduct functional studies.

MODIFICATION OF THE METHOD

An exclusive sandwich-cultured hepatocyte system for hepatobiliary disposition is also commercially available by Qualyst, Inc. (www.qualyst.com), named B-CLEAR™.

36.4.2.6 Cryopreserved Hepatocytes as SCH

Hepatocytes cryopreserved is an enabling technology with the advantages over freshly isolated cells including long-term storage, ease of experimental scheduling, choice of precharacterized lots for experiments, and repeat experiments with hepatocytes from the same donors. Application of this model has been extended to include hepatocytes from other species (Rose et al. 2006 and Swift and Brouwer 2010).

Cryopreserved human hepatocytes are also commercial available from Invitrogen life technologies (http://www.invitrogen.com), BD Biosciences (http://www.bdbiosciences.com/eu/index.jsp), and Celsis (http://www.celsis.com/), and the use has been successfully established by Li et al. (2010) and Maeda et al. (2010).

De Bruyn et al. (2011) determined transporter-mediated substrate uptake activities in individual and pooled batches of cryopreserved human hepatocytes and showed accumulation values that were comparable with the mean of the individual batches.

Houle et al. (2003) compared the transporter activities in cryopreserved and freshly isolated hepatocytes and found no significant difference in the transport rates of ^{14}C-taurocholate, ^{3}H-estrone sulfate, and ^{3}H-estradiol-17β-D-glucuronide.

36.4.2.7 Accumulation Studies with Fluorescence Substrates

Another unique tool that has been utilized with the SCH model system is incubation with fluorescence probes to study transport function. For MDR1-mediated in vitro biliary excretion, rhodamine 123 and ^{3}H-digoxin can be used in accumulation studies according to Annaert et al. (2001). Cells are rinsed twice with 2 ml of warm standard HBSS and incubated in 3 ml of the same buffer for 10 min at 37°C for experiments in which the effect of a Pgp modulator (e.g., GF120918; GlaxoSmithKline) is investigated, cells are preincubated for 60 min with the appropriate concentration of the Pgp modulator. Subsequently, cells are incubated in 3 ml of 1 μM substrate dissolved either in standard or Ca^{2+}-free HBSS for 1–30 min and subsequently rinsed for times with 3 ml of ice-cold HBSS. For rinsing of ^{3}H-digoxin-treated hepatocytes, 10 μM unlabeled digoxin is added to the rinsing buffer to reduce nonspecific binding. Hepatocytes are lysed with 3 ml of 0.5% Triton X-100 solution by placing plates on a rotator for 20 min at room temperature. Cell lysates are analyzed by fluorescence spectroscopy for Rh123 analysis and by liquid scintillation spectroscopy for ^{3}H-digoxin.

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EXAMPLES

In vitro $Cl_{biliary}$ values have been shown to correlate well with in vivo intrinsic $Cl_{biliary}$ values in rats and humans for a number of compounds.

Abe et al. (2008) studied in vitro the biliary clearance in sandwich-cultured human hepatocytes of angiotensin II receptor blockers and HMG-CoA reductase inhibitors that undergo limited metabolism and compared the predicted in vitro values with estimated in vivo hepatic clearance data in rats.

The average biliary excretion index and in vitro intrinsic $Cl_{biliary}$ values of olmesartan, valsartan, pravastatin, rosuvastatin, and pitavastatin were 35%, 23%, 31%, 25%, and 16%, respectively, and 0.943, 1.20, 0.484, 3.39, and 5.48 ml/min/kg, respectively. $Cl_{biliary}$ values predicted from sandwich-cultured human hepatocytes correlated with estimated in vivo hepatic clearance values based on published data (no in vivo data in humans was available for pitavastatin), and the rank order was also consistent. BSP, an OATP inhibitor, inhibited the uptake of all drugs. BEI and $Cl_{biliary}$ values of olmesartan, valsartan, pravastatin, and rosuvastatin, known Mrp2 substrates, were reduced in SCH from Mrp2-deficient (TR^-) compared to wild-type (WT) rats.

Thus, in vitro $Cl_{biliary}$ determined in rat SCH can be used to estimate and compare in vivo $Cl_{biliary}$ of compounds in rat and to characterize transport proteins responsible for their hepatic uptake and excretion.

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Distribution In Vivo

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Manfred Zimmer

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37.1 Distribution In Vivo QWBA

PURPOSE AND RATIONALE

Distribution studies with radiolabeled test substances in animals are an important part of the drug development process. Traditional routine methods used for these studies are quantitative tissue distribution studies (QTD) and alternatively whole-body autoradiography (WBA) with detection of the radioactivity in whole-body sections on X-ray film (Baker 1989). WBA is a qualitative detection method with a very high local resolution which includes all organs and many small substructures.

Radioluminography (RLG) is an alternative method of radiation detection based on the phosphorus imaging technique. RLG is much more sensitive than the WBA technique, its exposure time is much shorter, and it has a much wider linear measure range. Because of the latter property, RLG enables a quantification of drug concentrations in whole-body sections.

Quantitative whole-body autoradiography (QWBA) is based on the RLG technique and the use of standards obtained from dilution series containing known concentrations of radioactivity. Isotopes used in QWBA are mainly ^{14}C and ^{3}H. These standards were cut together with the whole-body sections to ensure an identical thickness and used for the internal calibration. The information of the calibration curve allows the determination of the concentrations in the organs and tissues of interest which can be derived from the measured area and the section thickness.

The results of distribution studies form the principal basis for the assessment of exposure and the elimination of residues in human organs and tissues. Direct

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determinations of exposure in man are generally limited to measurements in blood and plasma, which is easily accessible. Distribution patterns are determined in animals, usually rats, instead. Apart from the standard study design described below, all kinds of animals can be used, up to the size of rabbits. The results obtained with QWBA provide pharmacokinetic data on the test substance and/or its metabolites and evidence for interpretations regarding potential toxicological and pharmacological target organs.

Additionally, before the first study with radiolabeled test substance in man can be started, a risk assessment of a human radiokinetic study is mandatory. The estimation of the radiation exposure in humans given a radiolabeled dose is based on exposure data obtained typically from QWBA studies in animals.

PROCEDURE

Male pigmented and nonpigmented rats, weighing approx. 200 g, are used. At pre-selected time points (e.g., 0.25, 1, 2, 4, 8, 24, 72, 168 h) after administration of the radiolabeled test compound, the animals are sacrificed by CO_2 overdose, fixed on a piece of cardboard, embedded in sodium carboxymethyl cellulose, and immediately deep-frozen using liquid nitrogen (−197°C). In these frozen blocks, a certain number of holes are drilled and filled with radioactive standards (with definite concentrations of radioactivity obtained from dilution series). These blocks are put into a Cryotome (cooled microtome at a temperature of approx. −18°C, e.g., Leica CM3600), and sagittal whole-body sections are cut at different section levels (eye, brain, adrenal, kidney, thyroid; other sections as well). Before sectioning, adhesive tape is placed on top of the frozen surface to enable easy handling of the sections. The thickness of the section amounts to 25 µm. The sections are dehydrated for at least 12–16 h in the chamber of the Cryotome (freeze-drying). Then the sections are cut out of the tape, fixed on a cardboard, and covered with a special plastic foil (e.g., Leica, 2-µm thickness) to prevent the sections from sticking on the detection medium. Subsequently, the cardboard with the section is placed on the photosensitive surface of an imaging plate (IP), and both are put into a black plastic bag for exposure. The bag is sealed using the vacuum-technique (Kloss 1973) to enable an intimate contact with the photosensitive medium. Exposure time is at least 48 h in a shielding box at room temperature. Subsequently, the IPs are scanned with an image reader (e.g., BAS5000, raytest), and digitized autoradiograms are obtained (Fig. 37.1).

In parallel to these investigations, the biostability of the labeling position in the radiolabeled test compound is examined. For this purpose, the volatile radioactivity in the cage air after administration is monitored over 24 h.

In addition to this standard procedure, the following special issues should be mentioned when autoradiography is also used: drug penetration of blood-brain barrier, placental transfer, drug excretion via milk in dams, more detailed distribution in organs and tissues (e.g., distribution pattern in the brain: sequential coronal sectioning in anteroposterior direction; drugs being developed for treatment of, e.g., osteoarthritis: extent of penetration of drug in cartilage of, e.g., the knee joint), drug (e.g., herbicides) localization in plants, drug distribution at cellular and subcellular level, and receptor microscopic autoradiography (Stumpf 2003).

EVALUATION

The digitized autoradiograms are analyzed with appropriate software (e.g., AIDA, raytest); regions around the organs/tissues of interest can be drawn electronically (Fig. 37.2). The radioactive standards, cut together with the whole-body sections, are used as internal calibration. The information of the calibration curve provides the link between counts/area and radioactivity/area and allows the determination of the concentrations in the organs and tissues of interest which can be derived from the measured area and the section thickness. Considering the background counts of the IP, the specific radioactivity of the labeled test compound, and the area size of the region of interest, the corrected counts can be transferred into (amount of drug equivalent/g tissue) (due to the fact that only radioactivity can be measured, the value is defined as "equivalent"). The concentrations in the organs/tissues at different time points after administration are listed in tables and can be depicted as concentration-time profiles, bar charts, etc. (Fig. 37.3).

CRITICAL ASSESSMENT OF THE METHOD

Pros:

Distribution of compound and/or metabolites detected.

In situ situation in the body reflected.

Different subunits of organs can be regarded separately.

Rapid, impressive, accepted method (Fig. 37.4).

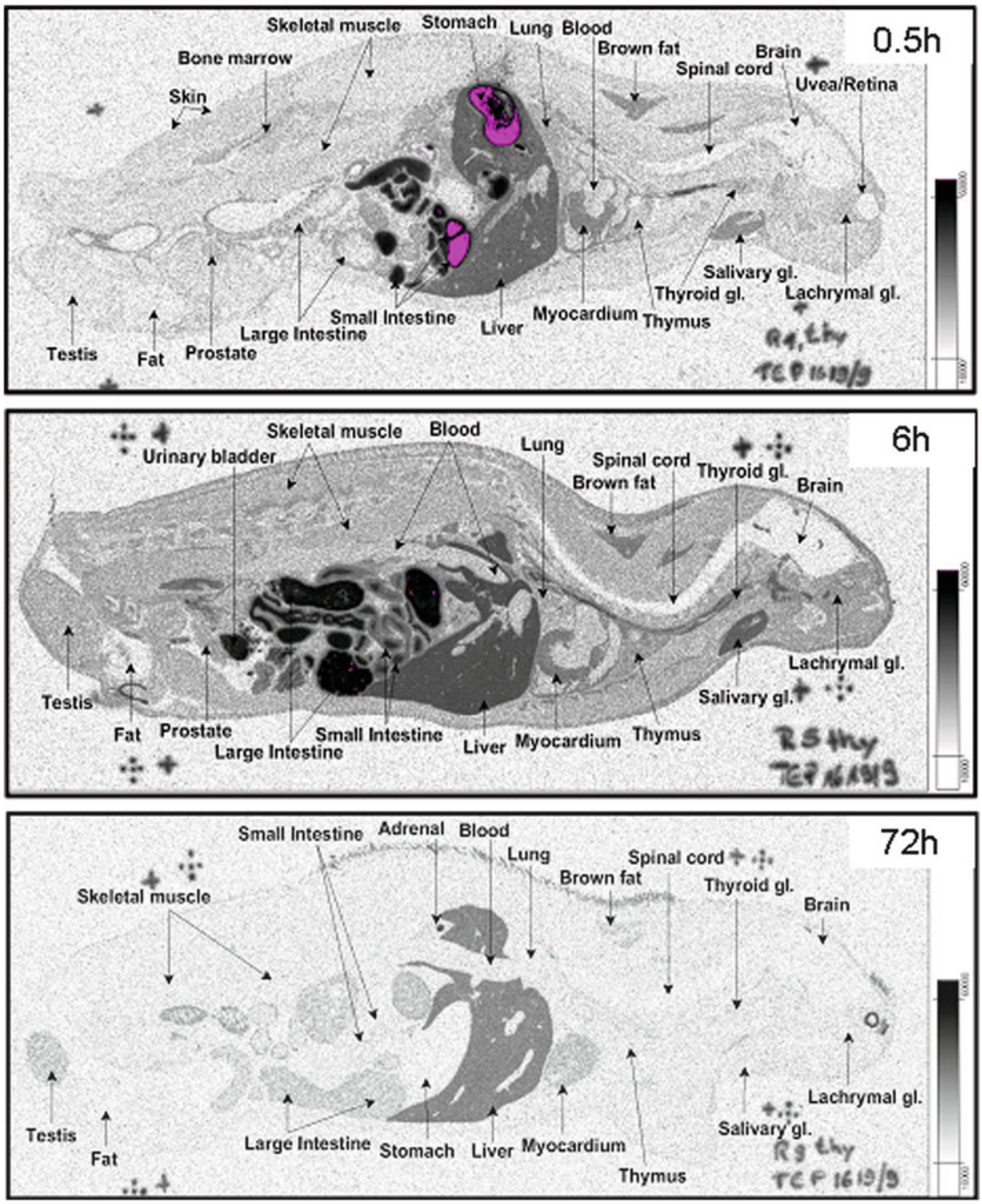
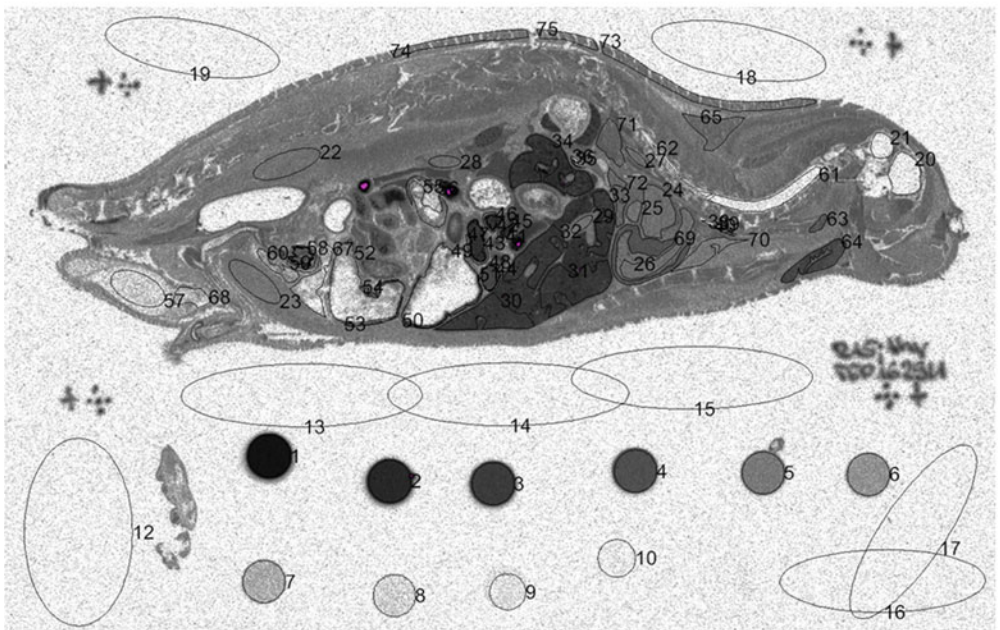


Fig. 37.1 Three autoradiograms at different time points (0.5, 6, 72 h) after oral administration of the radiolabeled test compound. The different radioactivity concentrations are depicted as shades of *gray*, from dark (high concentrations) to *white* (low concentrations)



File Edit Evaluation Options View Window Help

No	Name	Area [mm²]	Intensity [PSL]	Intensity-Bkg [PSL]	Intensity/Area [PSL/mm²]	Intensity/Area-Bkg [PSL/mm²]	Recalc. Conc. [Bq/g]
63	Thyroid_gl	5.2	454.3	450.0	87.3148	86.4887	4872
70	Thymus	25.2	744.7	723.9	29.4965	28.6704	1622
57	Testis	46.6	154.1	115.6	3.3076	2.4815	142
62	Spinal_cord	5.5	10.7	6.1	1.9328	1.1067	63
61	Spinal_cord	40.3	73.3	40.0	1.8188	0.9927	57
48	Small_I_wa	2.6	125.4	123.3	49.1436	48.3176	2728
47	Small_I_wa	4.4	292.8	289.1	66.3977	65.5716	3698
46	Small_I_wa	2.9	203.2	200.7	68.9025	68.0764	3838
45	Small_I_wa	1.7	121.7	120.2	70.0469	69.2208	3902
44	Small_I_co	10.9	1647.7	1638.7	151.6357	150.8096	8475
43	Small_I_co	16.0	5081.6	5068.4	317.8594	317.0333	17763
42	Small_I_co	4.5	1422.2	1418.5	318.1183	317.2922	17777
41	Small_I_co	2.9	1135.5	1133.2	393.6886	392.8625	21992
75	Skin	17.2	459.3	445.0	26.6935	25.8674	1464
74	Skin	36.7	950.6	920.2	25.8751	25.0490	1418
73	Skin	66.3	2083.4	2028.7	31.4463	30.6202	1732
23	Skeletal_mu	39.1	1349.7	1317.4	34.5031	33.6770	1904
22	Skeletal_mu	43.2	1622.3	1586.6	37.5448	36.7187	2075
64	Salivary_gl	32.5	4814.2	4787.3	148.1261	147.3000	8279
60	Prostate	13.4	203.0	192.0	15.1585	14.3324	813
40	Oesoph_wa	0.6	26.2	25.7	44.3687	43.5426	2459
39	Oesoph_wa	0.6	18.0	17.5	29.3322	28.5061	1613
36	Oesoph_wa	0.5	15.7	15.3	32.8611	32.0350	1812
38	Oesoph_co	2.3	15.0	13.1	6.4680	5.6420	321
35	Oesoph_co	1.9	12.3	10.7	6.3465	5.5204	314
69	Myocardium	44.5	4550.6	4513.8	102.2048	101.3787	5707
72	Lung	14.3	791.2	779.4	55.1514	54.3253	3066
71	Lung	26.2	1698.3	1676.7	64.8768	64.0507	3612
34	Liver	78.6	24875.3	24810.4	316.6029	315.7768	17693
33	Liver	32.3	8745.2	8718.5	270.4089	269.5828	15114
32	Liver	50.5	13407.0	13365.3	265.2853	264.4592	14828
31	Liver	73.8	20845.9	20785.0	282.5342	281.7081	15791
30	Liver	55.7	14172.3	14126.3	254.3035	253.4774	14215
56	Large_I_wa	4.2	163.5	160.1	39.3872	38.5611	2179
54	Large_I_wa	2.5	84.8	82.8	33.8185	32.9924	1866
53	Large_I_wa	2.6	82.2	80.0	31.4261	30.6000	1731
51	Large_I_wa	2.0	101.3	99.6	49.6175	48.7914	2755
50	Large_I_wa	2.1	109.5	107.7	52.0238	51.1977	2890
55	Large_I_co	25.8	639.9	618.6	24.8368	24.0107	1359

Fig. 37.2 Analysis of a digitized autoradiogram. Regions of interest are marked with thin *lines*. Oval areas around the autoradiogram were used for subtraction of the background, and the *circles* below with different shades of *gray* correspond to the radioactive standards used for calibration

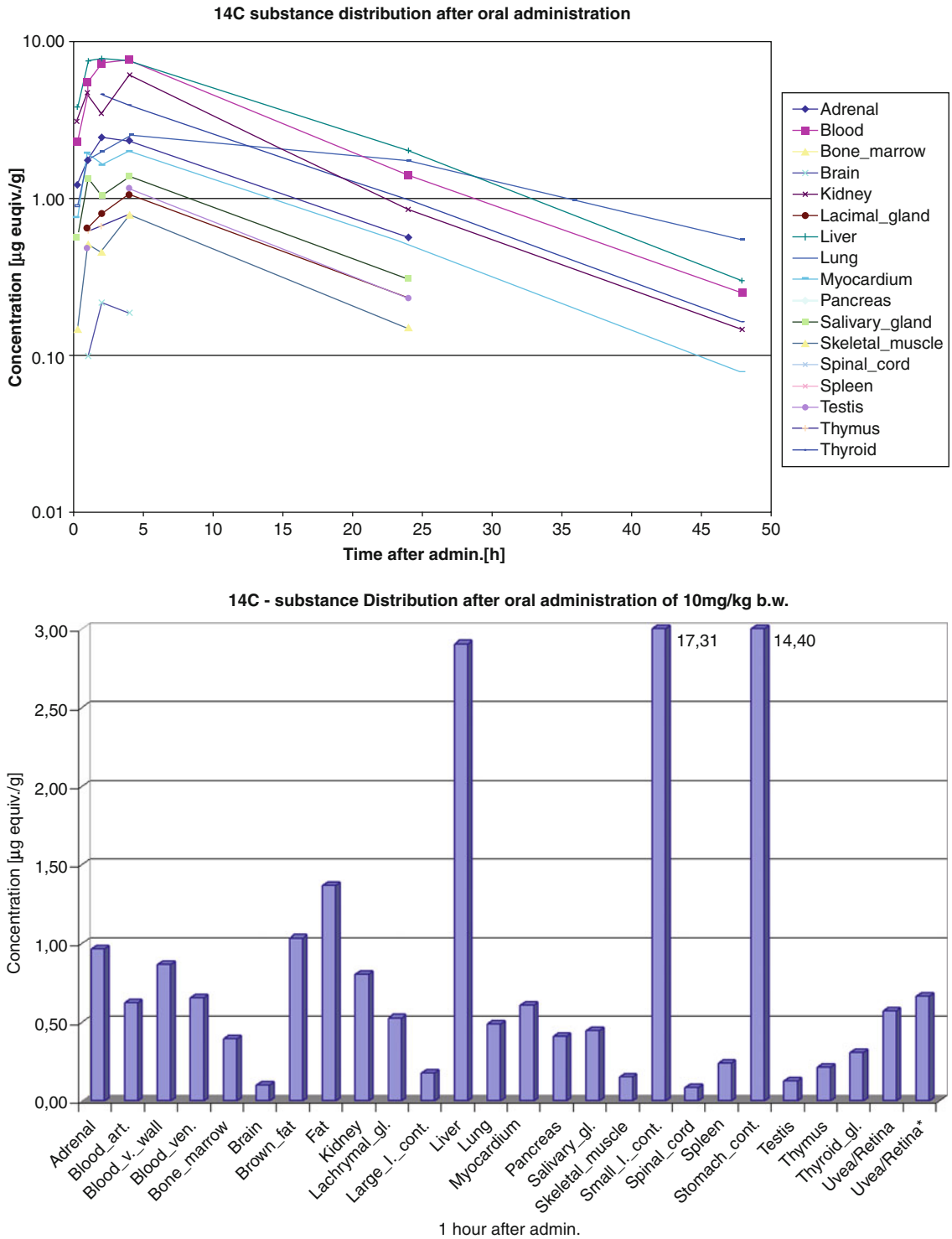


Fig. 37.3 Quantitative analysis of organs and tissues. The concentrations (expressed as drug equivalents/g) were calculated by dividing the measured radioactivity concentrations by the specific radioactivity of the administered compound. *Upper plot* shows the concentration-time profile; *lower plot* shows the concentrations at the time of Cmax

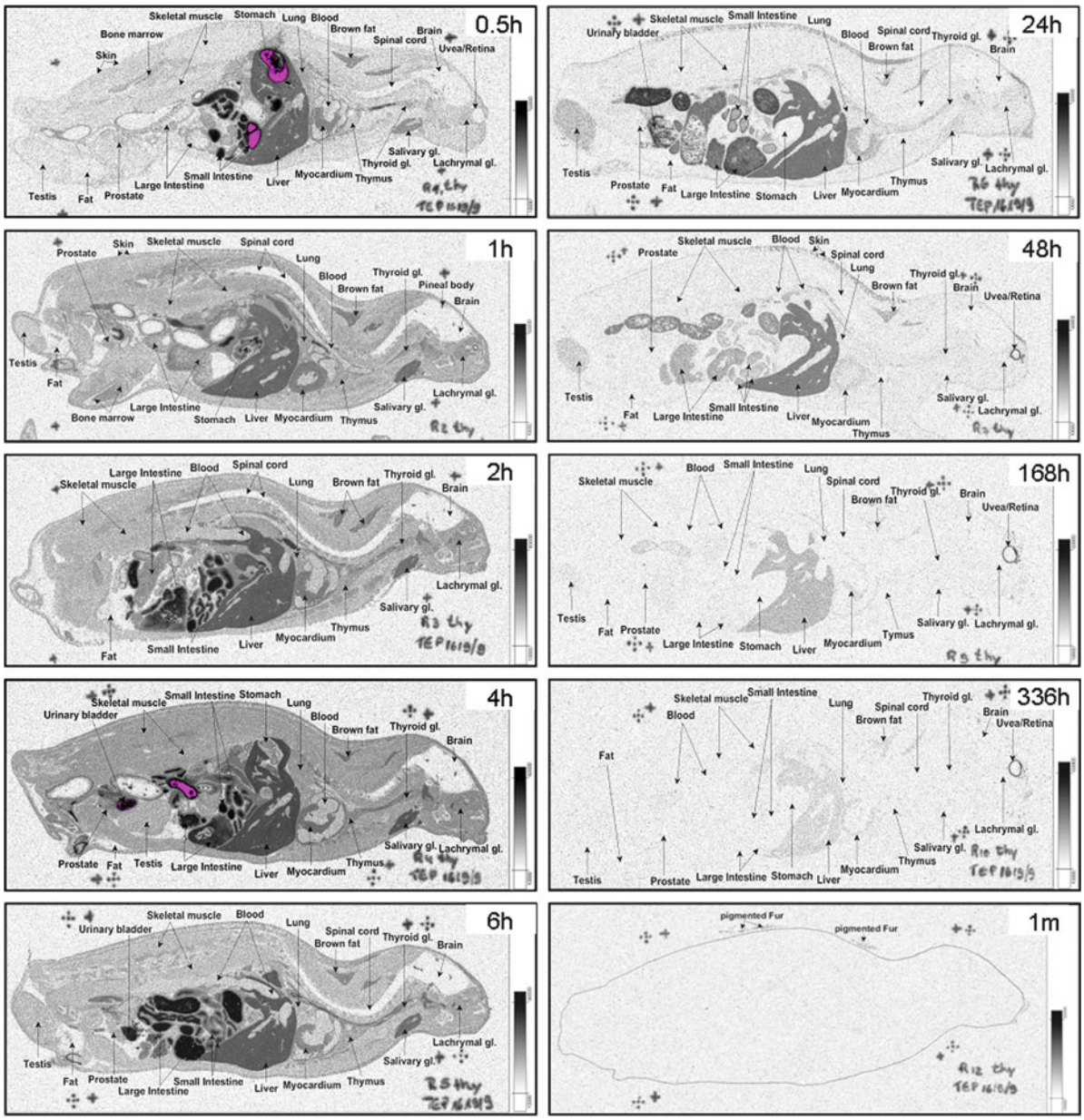


Fig. 37.4 Digitized autoradiograms at different time points (0.5 h–1 month) after oral administration of the radiolabeled test compound. The different radioactivity concentrations are depicted as shades of *gray*, from *dark* (high concentrations) to *white* (low concentrations)

Accepted as basis for human mass balance studies (dosimetry calculations).

Cons:

Radiolabeled compound required.

Total radioactivity detected, not parent compound or any metabolites.

Blood content of the organs and tissues not exactly known.

It is not possible to differentiate parent compound from conjugated metabolites or other radiolabeled fragments of the original molecule.

MODIFICATION OF THE METHOD

Although WBA is a relatively old method having its origin in the beginning of the twentieth century, this technique was undergoing some considerable modifications during the last 20 years and is now used widely in the chemical and pharmaceutical industries. The first whole-body autoradiogram (WBA) of a frog which had been exposed to radium and was placed on a photographic plate was published by E.H. London (1904). The original WBA technique was invented by Swen Ullberg (1954). He did research in the field of antibiotics and wanted to investigate whether the concentrations in the different infected organs or tissues in the body were high enough for therapeutic effects. There was no sufficient method available, so he developed a technique to get the appropriate information in situ. Mice were injected with radiolabeled penicillin and snap frozen in liquid air. Whole-body sections were cut on a hand-driven sledge microtome in a freezer room at −15°C. Frozen and dehydrated sections were exposed to X-ray film. Using different section levels, a qualitative estimation of the distribution in organs and tissues could be obtained.

The evolution from qualitative to quantitative WBA occurred during the last 20 years: from X-ray films and introduction of tritium film (increased sensitivity and removal of the antiscratch layer) to the semiquantitative analysis in the 1980s using densitometry to the quantification in the 1990s when digitized autoradiograms and radioluminography (RLG) were developed as new methods of radiation detection (Mori and Hamaoka 1994; Steinke et al. 2000).

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37.2 Distribution In Vivo QTD

PURPOSE AND RATIONALE

Distribution studies with radiolabeled test substances in animals are an important part of the drug development process. Routine methods used for these studies are quantitative whole-body autoradiography (QWBA) and alternatively quantitative tissue distribution studies (QTD) with dissection of the animals and measurement of radioactivity in preselected organs and tissues using a liquid scintillation counter.

The results of these studies form the principal basis for the assessment of exposure and the elimination of residues in human organs and tissues, in which direct determination is of course impossible. Direct determinations of exposure in man are generally limited to measurements in blood or plasma, which is easily accessible. Distribution patterns and pharmacokinetic parameters (e.g., elimination half-lives, time of maximum concentrations in organs and tissues) are determined in animals, usually rats, instead. Radiolabeled test substance is administered to ensure, as far as possible, determination of the whole of the parent compound and its metabolites. The results provide pharmacokinetic data on the distribution of the total radioactivity, information on any accumulation or specific affinities of the test substance or its metabolites, and evidence for interpretations regarding potential toxicological and pharmacological target organs (Steinke et al. 2000).

Additionally, before the first study with radiolabeled test substance in man can be started, a risk assessment of a human radiokinetic study is mandatory. The estimation of the radiation exposure in humans given a radiolabeled dose is based on exposure data obtained typically from QTD—or QWBA—studies in animals.

PROCEDURE

Male rats, weighing approx. 200 g, are used. At preselected time points (e.g., 0.25, 1, 2, 8, 24, 96 h; 3 animals per group) after administration of the radiolabeled test compound, the animals are sacrificed

(CO_2 overdose, exsanguination) and immediately dissected. Organs and tissues are removed in the following dissection order (from expected low to high concentrations to avoid cross contaminations): blood, plasma, eyes, brain, skin (without hair and subcutaneous fat), subcutaneous fat, skeletal muscle, bone (femur), bone marrow, thyroid, lungs, thymus, heart, testis, prostate, retroperitoneal fat, spleen, kidney, adrenal, urinary bladder, liver, pancreas, mesenteric lymph nodes, stomach contents, small intestine contents, large intestine contents, stomach wall, small intestine wall, and large intestine wall.

Blood and plasma samples are taken on Combusto-Cones (e.g., Canberra-Packard), weighed, dried at room temperature, and combusted in a sample oxidizer (e.g., Canberra-Packard GmbH, Model Oximate 80/307, Frankfurt am Main, FRG), and the $^{14}CO_2$ formed is absorbed with Carbo-Sorb® (e.g., Canberra-Packard). The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

After removal, larger organs and tissues are homogenized with ULTRA-TURRAX® appliances (e.g., IKA, Staufen, Germany) after addition of deionized water, the amount of which depended on the consistency of the tissue. Smaller tissues are finely cut. The specimens are dissolved in volumetric flasks at 60–70°C in a mixture of Digestin® (e.g., Merck, Darmstadt, Germany) and water, ethanol is added if required to prevent foam formation, addition of approx. 0.3 ml Perhydrol® (e.g., Riedel de Haën, Seelze, FRG) is sufficient to remove discolorations, and measurements are then performed after addition of the scintillator.

Radioactivity measurements are carried out by the liquid scintillation counting procedure in β-spectrometers (e.g., Canberra-Packard 4530), using an external standard device which permitted the counting efficiency to be determined by the channel ratio method (Dyer 1980).

The scintillators Permafluor E+, recommended by Canberra-Packard for their automatic combusters, and Rotiszint eco plus (e.g., Roth, Karlsruhe, Germany) for dissolved samples are used, and blank values are concurrently measured in the studies and deducted from those measured. These blank values originate from material of untreated animals.

EVALUATION

Considering the count conditions of the analytic device, the specific radioactivity of the labeled test compound, and the weights of the analyzed specimen, the corrected counts can be transferred into (amount of drug equivalent/g tissue) (due to the fact that only radioactivity can be measured, the value is defined as "equivalent").

The concentrations are expressed in μg equivalents drug/g tissue and in % of administered radioactivity, i.e., they represent the sum of the original compound and/or radioactively labeled metabolites. To obtain a unit independent of metabolization, the test preparation concentrations in μg equivalents/g can be converted to nmol using the molecular weight:

1 μg of the free compound is equivalent to (1,000/MW) nmol.

The pharmokinetic parameters in the blood, plasma, and excreta and the kinetics in organs and tissues are calculated using an appropriate pharmacokinetics software (e.g., WinNonlin).

The detection limit is determined using the individual blank values for the corresponding biological material by the following formula:

$$\text{Detection limit} = \text{background (Bq)}/(\text{initial weight (g)}^{*} \text{specific radioactivity(Bq/μg)}).$$

At least the double blank value (Bq) is required for evidence of a significant concentration value (μg equivalents per g or ml).

The concentrations in the organs/tissues at different time points after administration are listed in tables and can be depicted as concentration-time profiles, bar charts, etc.

CRITICAL ASSESSMENT OF THE METHOD

QTD was the "golden standard" when the method of WBA (Ullberg 1954) was not yet able to quantify the distribution.

The results of the two methods complemented one another. Whole-body autoradiography is a qualitative detection method with a very high local resolution which includes all organs and many small substructures, whereas measurement after dissection yields quantitative concentration data for a limited, preselected number of organs.

A vast change occurred when a new technology allowed the quantification of WBA in the 1990s. Digitized autoradiograms and radioluminography (RLG) were developed as new methods of radiation detection (Mori and Hamaoka 1994).

Since the organs and tissues are not destroyed during a QWBA study (in contrast to the

Organ/Tissue	0.5h	1h	2h	4h	6h	24h	48h	72h	168h	336h	lm[5]	6m[6]	0.083h[7]
Adrenal	1.83	6.33	9.20	22.06	18.34	7.42	3.33	1.10	0.24	0.07	**BLQ**	**BLQ**	4.02
Adrenal_cortex	0.40	1.15	1.87	2.58	1.59	0.33	0.23	0.15	**BLQ**	**BLQ**	**BLQ**	**BLQ**	1.83
Adrenal_medulla	6.64	26.64	37.39	79.62	82.63	43.63	19.47	11.12	1.36	0.44	0.21	**BLQ**	13.87
Bladder_cont.	10.05	36.00	68.78	216.63	49.80	6.34	0.15	0.35	0.05	0.03	**BLQ**	**BLQ**	0.04
Bladder_wall	0.17	0.85	0.78	ND	0.78	ND	0.04	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.24
Blood_art.	-	-	-	-	-	-	-	-	-	-	-	-	1.49
Blood_ven.	-	-	-	-	-	-	-	-	-	-	-	-	3.88
Blood_mean	0.19	0.39	0.42	0.43	0.28	0.09	0.08	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	2.68
Bone	0.30	0.77	0.69	0.85	0.40	0.07	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.68
Bone_marrow	0.44	1.14	0.98	1.27	0.58	0.09	0.07	0.03	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.88
Brain	**BLQ**	**BLQ**	**BLQ**	0.02	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.03
Brown_Fat	1.01	3.11	2.43	3.15	2.35	0.51	0.44	0.18	0.15	0.14	0.07	0.04	0.62
Fat	0.03	0.05	0.04	0.05	0.05	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.02
Glandular_stomach	ND	3.11	3.64	3.07	2.15	0.14	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	1.05
Kidney	0.86	2.02	1.72	2.14	1.27	0.33	0.15	0.19	0.07	0.04	**BLQ**	**BLQ**	4.29
Lachrymal_gl.	0.32	1.10	1.49	1.54	0.92	0.08	0.04	0.03	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.68
Large_I._cont.	0.10	0.55	19.83	79.89	128.69	8.03	0.91	0.22	0.08	0.03	**BLQ**	**BLQ**	0.04
Large_I._wall	0.65	1.69	2.30	2.99	11.22	0.48	0.10	0.09	0.04	**BLQ**	**BLQ**	**BLQ**	1.17
Liquor	**BLQ**	**BLQ**	**BLQ**	0.05	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**
Liver	3.94	5.45	8.81	11.44	9.61	3.62	2.47	1.70	0.40	0.13	**BLQ**	**BLQ**	1.27
Lung	0.51	1.08	1.23	1.07	0.68	0.14	0.08	0.04	**BLQ**	**BLQ**	**BLQ**	**BLQ**	2.61
Myocardium	1.40	2.70	3.25	3.33	2.18	0.49	0.20	0.29	0.14	0.10	0.07	0.04	4.46
Oesoph._cont.	157.95	0.59	19.83	0.80	0.90	**BLQ**	0.06	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	2.16
Oesoph._wall	ND	6.00	3.09	2.93	3.43	0.21	0.11	0.03	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.85
Pancreas	0.72	2.19	2.32	3.03	1.49	0.09	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	1.27
Pineal_body	0.88	1.75	2.73	2.46	1.47	0.14	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	2.91
Pituitary_gl.	0.53	2.83	2.95	4.00	2.16	0.32	0.10	0.06	0.06	**BLQ**	**BLQ**	**BLQ**	1.63
Plexus_choroideus	0.50	1.99	2.87	4.05	2.75	0.31	0.07	0.02	**BLQ**	**BLQ**	**BLQ**	**BLQ**	1.31
Prostate	0.24	0.78	0.66	1.10	0.66	0.11	0.03	**BLQ**	**BLQ**	**BLQ**	**BLQ**	**BLQ**	0.24
Renal_cortex	0.52	0.98	0.94	1.05	0.78	0.29	0.18	0.17	0.10	0.07	**BLQ**	**BLQ**	3.83
Renal_medulla	1.24	2.96	2.52	2.89	1.61	0.36	0.11	0.22	0.05	**BLQ**	**BLQ**	**BLQ**	3.96

Fig. 37.5 QWBA. Concentrations in organs and tissues after oral (0.5 h–6 m) or after intravenous (0.083 h) administration of the radiolabeled drug. The concentrations (expressed as drug equivalents/g) were calculated by dividing the measured radioactivity concentrations by the specific radioactivity of the administered drug. The limit of quantification (LOQ) in this study amounted to 0.014 μgEq/g; concentrations below were marked as BLQ

homogenization of the organs and tissues for LSC measurements), substructures can be distinguished clearly (e.g., adrenal medulla and adrenal cortex often show very different concentrations of radioactivity) (Fig. 37.5).

To save animals and time, the quantitative tissue distribution study is more and more replaced by the quantitative whole-body autoradiography.

MODIFICATION OF THE METHOD

As the procedure of the animal study part of this method is well established, modifications occur mainly in the technological part by improvement of the performance of liquid scintillation counters and the appropriate software. As this method belongs no longer to the "cutting edge" skills in the drug development process, modification activities are moderate.

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Distribution In Vivo: Other Methods

38

Yves Archimbaud

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38.1 Positron Emission Tomography

PURPOSE AND RATIONALE

Distribution in vivo could be studied by positron emission tomography (PET). Positron emission tomography is a sensitive and specific functional noninvasive three-dimensional imaging method that permits rapidly and directly measurement of the total radioactivity from a drug labeled with a positron-emitting radionuclide (Gupta et al. 2002).

PROCEDURE

The drug should be labeled with positron-emitting isotopes such as carbon-11, oxygen-15, nitrogen-13, and fluorine-18. The radionuclides are predominantly produced by charged particle nuclear reactions using a cyclotron. Radiotracers are produced after replacement of a normal constituent in a biologically active compound of interest with a radionuclide without modifying their pharmaceutical, biological, or biochemical properties.

The optimal isotopes should be selected depending of the pharmacokinetic of the drug and their half-life values (20.4, 2.03, 10, and 109.8 min for ^{11}C, ^{15}O, ^{13}N, and ^{18}F, respectively).

The labeled compounds are generally administered by intravenous infusion to avoid local radiation damage. The amounts of radioactivity injected varied between 200 and 800 MBq (Bergström et al. 2003). Just after administration, the camera is activated to start the acquisition. When a positron-emitting radionuclide is decaying, it emits a positron that will interact with an electron in an annihilation event whereby these particles jointly are converted to two oppositely directed gamma rays of 511 keV energy. The gamma

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rays readily penetrate the tissue and a fraction of them emerge out of the body to be recorded by the PET camera with rings of external nuclear detectors, which has high resolution and sensitivity. Computer processing of data generates sets of tomographic images with a spatial resolution of 0.5–5 mm depending of the scanner type (0.5–1 mm on animal scanners and 2–5 mm on clinical scanners) after correction for attenuation and detector efficiency. An image is acquired for a predefined time length and at a predefined time after administration. Timing of PET imaging should be well selected depending of the radionuclide and the drug. The data depict the spatial and temporal distribution of total radioactivity. Images are displayed on a computer monitor for inspection. Quantitative values on the drugs/tracers radioactivity concentrations in different organs are determined. For this, regions of interest are manually outlined in the images to represent targeted regions for analysis. Within these regions, the radioactivity concentration is determined for each image in the sequence, resulting in a time-radioactivity data set. The radioactivity is rapidly decaying according to the half-life of the radionuclide, and therefore, a correction is applied to compensate for this. Integral methods or compartmental analysis are used to analyze PET data. The most widely used integral method is the standardized uptake value SUV: SUV = local radioactivity concentration (administered radioactivity/body weight). Arterial blood and tissue combined data are needed in compartmental modeling in order to define exchange parameters and assess steady-state distribution volumes of the drug in organs.

EVALUATION

Quantitative values on the drugs/tracers radioactivity concentration-time profile in different organs or subregions of organs are determined.

CRITICAL ASSESSMENT OF THE METHOD

PET has a number of advantages. The entire time course of the distribution can be determined quantitatively in a living animal or human with a temporal resolution of seconds to minutes. Each subject can be used as its own control. PET is highly sensitive with the capacity to detect subnanomolar concentrations of radiotracers.

PET has a number of disadvantages: tracers with short half-lives limit the time during which they can be studied. For example, drugs labeled with ^{11}C may be too rapidly metabolized relative to the 20.4 min half-life and thus meaningful information may be limited. The logistics is challenging with the need of a cyclotron. PET can only measure radioactivity and not discern the chemical form of it; it is not possible in a PET study to be certain that the radioactivity signal is related to the original compound or including metabolites thereof. In order to make it likely that the proper conclusion is drawn, it is recommended that metabolism studies be performed in which the fraction of radioactivity constituted by intact tracer is determined in plasma and in the target organ. Additionally, the plasma profile of radioactive metabolites should be assessed in the PET study. Timing of PET imaging is not so easy to determine.

MODIFICATIONS OF THE METHOD

New materials with higher sensitivity or resolution have been designed: A three-dimensional PET scanner using gadolinium oxyorthosilicate (GSO) crystals (Surti and Karp 2004).

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38.1.1 Example

PET has already been applied to a wide number of drugs to demonstrate activity in vivo from standard chemotherapy such as 5-fluorouracil (5-FU). The pharmacokinetics of 5-FU has been successfully

investigated using radiotracers and is the most common anticancer drug imaged with PET (Kissel et al. 1997). This is due to the ease of chemical synthesis of ^{18}F-fluorouracil (5–[^{18}F] FU). A pharmacokinetic quantitative model to quantify the intracellular 5–[^{18}F] FU concentration in liver metastases of patients with colon cancer revealed extrahepatic and intrahepatic clearances of 0.66 ± 0.33 and 0.52 ± 0.25 L/min, respectively. A pharmacodynamic relationship between tumor uptake of 5–FU and response, first seen in mice, has also been demonstrated in humans by PET methodology (Moehler et al. 1998). Studies showed that colorectal liver metastases with a higher uptake of 5–FU at 2 h, as measured by a standard uptake value (SUV), had a negative growth rate, whereas those with a low SUV had disease progression. Therefore, PET helps to differentiate between responding and nonresponding metastatic sites.

38.2 Microdialysis

PURPOSE AND RATIONALE

Distribution in vivo could be studied by microdialysis. Microdialysis is an in vivo technique that permits measurement of unbound drug or metabolite concentrations in extracellular fluid of specific tissue location. The unbound drug concentrations have been shown to be responsible for the pharmacological effects. The basic principle is to mimic the function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue with a physiological liquid (Ungerstedt 1991).

PROCEDURE

A microdialysis probe is inserted in a selected tissue or fluids (like brain, muscle, skin, liver, tumor, blood, and bile (Elmquist and Sawchuk 1997)) of an animal (mouse, rat, rabbit, dog, and monkey) or a human. The probe consists of a small semipermeable hollow fiber membrane, connected to an inlet and outlet tubing with a small diameter. The probe is continuously perfused with a physiological solution such as a Krebs-Ringer solution (Na^+ 148 mM, K^+ 4.0 mM, Ca^{2+} 2.3 mM, and Cl^- 156 mM) (Benveniste and Huttemeier 1990). The perfusate should have an ionic composition comparable to the extracellular fluid surrounding the probe and should be at ambient temperature. After a 24-h recovery, the drug is administered by a relevant route (orally, intravenously) to an animal or a human and the perfusate is infused at a flow rate of 0.1–5 μl/min. The drug able to pass the semipermeable membrane will diffuse over the membrane down their concentration gradient into or out of perfusate. The solution that exits the probe, the dialysate, can be collected during interval periods. The concentrations of the drug in the dialysate reflect the concentrations in the extracellular fluid around the semipermeable part of the probe. However, as the dialysis procedure is not performed under equilibrium conditions, the concentration in the dialysate will be less than that in the extracellular fluid. The in vivo recovery, used to describe this relationship, is determined by the use of an internal standard by retrodialysis (Larsson 1991). The concentrations of the drug are determined by a relevant and sensitive analytical method and then are corrected for in vivo recovery.

EVALUATION

Quantitative values on the unbound drug or metabolite concentration-time profile in different tissues or subregions of tissues are determined.

CRITICAL ASSESSMENT OF THE METHOD

Microdialysis has a number of advantages: concentration profiles of drug could be obtained without fluid loss from freely moving individual subjects in specific subregions of tissues.

Microdialysis has also a number of disadvantages: the probe could elicit tissue trauma after implantation, the determination of in vivo recovery is time consuming, and sensitive analytical methods are needed due to the diluting effect (De Lange et al. 2000).

MODIFICATIONS OF THE METHOD

New probes (Evrard et al. 1996) and methods for analysis and for recovery are developed. The optimal conditions for composition of the perfusion solution, the flow rate, and the postsurgery interval are searched.

Microdialysis probes are now commercially available in various sizes, designs, and materials. Microdialysis probes can be flexible for soft peripheral tissues and fluids or rigid for brain. Four probe geometries are available: linear, loop, side-by-side, and concentric. The semipermeable membrane is generally chosen as long as possible, typically between 1 and 10 mm. The probe radius is generally chosen as small

as possible, typically between 200 and 400 μm O.D. to cause minimal disturbances within the tissue. Dialysis probes are made of various materials (e.g., celluloses and copolymers like polyacetonitrile/sodium methallyl sulfonate and polycarbonate/ether). The molecular mass cutoff (5–50 kDa), inertness, and permeability to solutes of the probes could be different.

Perfusion solution used in microdialysis experiments vary widely in composition and pH. Ideally, the composition, ion strength, osmotic value, and pH of the perfusion solution should be as close as possible to those of the extracellular fluid of the dialyzed tissue. Perfusion fluids should be at body temperature.

Perfusion flow rates range between 0.1 and 5 μl/min. The tendency is to use lower flow rates as this may increase recovery, provided that an analytical technique is available to deal with the smaller-sized samples.

Experiments should be executed between 24 and 48 h after implantation, after recovery from early tissue reactions and before the start of long-term reactions.

Sensitive analytical methods should be used such as liquid chromatography combined with mass spectroscopy, microbore liquid chromatography, and capillary electrophoreses.

Different methods to determine in vivo recovery could be used: extrapolation to zero-flow rate (Jacobson et al. 1985), no-net-flux (Lonnroth et al. 1987), dynamic-no-net flux (Olson and Justice 1993), and retrodialysis using an internal standard (Larsson 1991). The zero-flow rate method is based on the principle that recovery is a function of flow rate. The dialysate concentrations are plotted as a function of flow rate and by extrapolating to zero-flow rate where the dialysate is in equilibrium with the extracellular fluid; the dialysate concentration found at zero-flow rate should equal the actual in vivo concentration. The no-net-flux method is based on the principle that drug transport across the membrane is a function of perfusate concentration while maintaining the extracellular concentration of the analyte at steady state. Different concentrations are perfused through the membrane. The dynamic-no-net-flux method, an extended version of the no-net-flux method, allowed the estimation of the recovery as a function of time. Several animals are continuously perfused with one perfusion concentration. The retrodialysis method is based on the principle that an internal standard with membrane diffusion characteristics close to the analyte of interest is added to the perfusate and its relative loss by retrodialysis is calculated. The assumption is that in vivo retrodialysis of the internal standard is the same as in vivo dialysis of the analyte. An advantage of this method is that fluctuations in recovery of the probe during the experiment are taken into account by the continuous retrodialysis of the marker during the entire experiment.

Recovery of lipophilic compounds binding to components of the dialysis equipment could be increased by including albumin or a lipid emulsion to the dialysate (Carneheim and Ståhle 1991).

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38.2.1 Example

Microdialysis was used to assess morphine 6-beta-D-glucuronide (M6G) and morphine brain distribution in extracellular fluid after systemic administration in rats (Stain-Texier et al. 1999). M6G penetrated into the brain was distributed and trapped preferentially than

morphine in the extracellular fluid and therefore was available to bind at opioid receptors, explaining how M6G induces more potent central analgesia than morphine.

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Drug–Drug Interaction: Enzyme Induction 39

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PURPOSE AND RATIONALE

Enzyme induction can be considered a natural defense mechanism against xenobiotics such as environmental agents (e.g., dioxins), tobacco smoke, ethanol, nutritional constituents in grilled meat or cruciferous vegetables, herbal medicines such as St. John's wort, and therapeutic drugs. Following ingestion, the first barriers for xenobiotics are metabolizing enzymes in the intestinal epithelium and efflux transporter proteins, most notably P-glycoprotein, that transport substances from the epithelial cells back into the intestinal lumen. The remaining xenobiotics pass via the portal circulation into the liver – the organ specialized in xenobiotic metabolism and the major site of enzyme induction. Thus, as a defense mechanism, the metabolizing enzymes and P-glycoprotein can be induced, enhancing xenobiotics elimination in rate and capacity.

For therapeutic drugs, induction of drug-metabolizing enzymes and drug transporters is defined as an increase in activity levels in response to multiple exposures to the drug. The major consequences of enzyme induction are pharmacokinetic drug–drug interactions leading to a faster metabolic elimination of coadministered drugs by the induced enzyme. As a consequence, plasma concentrations may remain subtherapeutic resulting in loss of efficacy. Alternatively, the induced metabolic pathway could lead to increased formation of an active metabolite from an inactive prodrug or to increased formation of toxic metabolites, resulting in adverse effects.

Some well-known human enzyme inducers are listed in Table 39.1. Carbamazepine and phenobarbital induce metabolizing pathways that metabolize these inducers themselves, a process called autoinduction.

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Table 39.1 Examples of in vivo human enzyme inducers and the induced drug-metabolizing enzymes

Inducer	Major enzymes induced
Carbamazepine	CYP3A4, UGT
Omeprazole	CYP1A2
Phenobarbital	CYP2B6, CYP3A4, UGT
Phenytoin	CYP2B6, CYP3A4, UGT
Rifampicin	CYP2B6, CYP3A4, UGT
St. John's wort	CYP3A4
Troglitazone	CYP2B6, CYP3A4

Table 39.2 Nuclear receptors (NR) for inducers of drug-metabolizing enzymes and transporter proteins. The receptors listed are the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), and glucocorticoid receptor (GR). Major enzyme isoforms regulated by the corresponding receptors are shown in bold

NR	Enzymes induced	Examples of inducers
AhR	**CYP1A1**, **CYP1A2**, CYP1B1, UGT1A1, SULT1A1, GST-A2, ALDH, MDR1	Aryl hydrocarbons Dioxins
CAR	**CYP2B6**, UGT1A1, CYP2C8, CYP2C9, CYP3A4, CYP2A6, SULT1A1, OATPs, MRP3, MRP2	Phenobarbital
PXR	**CYP3A4**, CYP2B6, CYP2C8, CYP2C9, UGT1A1, GST-A2, MDR1, MRP2, OATP2, OCT1	Rifampicin
GR	**CYP3A4**, other PXR-induced enzymes	Dexamethasone

Enzyme induction, with some exceptions, is initiated via the binding of the inducers to nuclear receptors leading to increased gene expression and protein synthesis (Waxman 1999; Honkakoski and Negishi 2000; Jones et al. 2000; Sueyoshi and Negishi 2001; Kewley et al. 2004). Differences in induction potential result from different affinities of inducers to the nuclear receptors, which also explains the observed species differences in induction. The key nuclear receptors for enzyme inducers are listed in Table 39.2.

- *Aryl hydrocarbon receptor (AhR)*: AhR is a basic helix-loop-helix protein belonging to the PER-ARNT-SIM family of transcription factors (Kewley et al. 2004). AhR is the key receptor for CYP1A gene activation by aryl hydrocarbons (e.g., 3-methylcholanthrene) and dioxins. AhR resides in the cytoplasm and, upon ligand binding, translocates to the nucleus. In the nucleus, AhR forms a heterodimer with the AhR nuclear translocator (ARNT) which then binds to the dioxin-response elements of the cytochrome P450 1A (CYP1A) and other responsive genes. This process is limited by a negative feedback mechanism: AhR activates its own repressor (AhR repressor). Evidence for AhR induction of CYP1A includes that AhR knockout mice are resistant to dioxin-elicited CYP1A1 induction.
- *Constitutive androstane receptor (CAR)*: CAR is also known as constitutively active receptor; it is called "constitutive" because it transactivates retinoid acid response element without binding to any ligand. CAR induces CYP2B6 and, to a lesser extent, CYP3A4. Responding to phenobarbital exposure in liver, CAR translocates to the nucleus, forms a dimer with the retinoid X receptor (RXR), and activates the phenobarbital-responsive enhancer module by binding to DR-4 motifs (Sueyoshi and Negishi 2001). The mechanism of phenobarbital activation of CYP2B6 is unknown. Phenobarbital is not a ligand to CAR but may affect CAR via a kinase pathway. CAR translocation and activation are inhibited by protein phosphatase and kinase inhibitors.

  Pregnane X receptor (PXR): PXR, also called SXR, is responsible for CYP3A4 induction (Lehmann et al. 1998) as well as CYP2C8 and CYP2C9 induction. Activators of PXR include pharmaceuticals such as rifampicin, phenobarbital, nifedipine, clotrimazole, mifepristone and metyrapone. Many PXR ligands also activate CAR. The mechanism of the PXR response to inducers consists in ligand binding, formation of a heterodimer with the retinoid X receptor-α, RXR-α, and transactivation of the ER6 (everted repeat with a six base pair spacer) elements upstream of the cytochrome P450 genes. PXR and RXR-α are induced by activation of the glucocorticoid receptor (GR); glucocorticoids, e.g., dexamethasone, activate GR and lead to the induction of PXR and RXR, resulting in CYP3A4 induction. Downregulation of PXR and CAR is mediated by cytokines such as interleukin-6 and results in repression of several cytochrome P450 isoforms (Lee et al. 2006). Species differences in the affinity of PXR for the inducers (Jones et al. 2000) account for the dramatic rodent–human difference in response to rifampicin (LeClyse 2001). Rifampicin is a potent inducer of CYP3A4 in humans but a poor CYP3A2 inducer in rodents (Lu and Li 2001).

Stronger induction in rodents than in humans is known for dexamethasone inducing CYP3A2 in rats.

- *Glucocorticoid receptor (GR)*: Glucocorticoids, e.g., dexamethasone, bind to GR and induce PXR and RXR expression. As a result, the induction effects of PXR ligands are enhanced in general and, in particular, CYP3A4 induction in human hepatocytes. The mechanism is protein–protein interplay (cross-talk) between GR and other receptors rather than direct binding of GR to cytochrome P450 genes.
- *Nonreceptor-related mechanisms*: Enzyme induction may occur via nonreceptor-related mechanisms. CYP2A6 induction by phenobarbital and rifampicin, although known to be mediated via receptor-dependent mechanisms, may involve mRNA stabilization. Likewise, the CYP2E1 induction mechanism includes stabilization of mRNA and protein.

Enzyme induction in laboratory animals may be observed in preclinical toxicity studies in which drugs are administered repeatedly in high doses. The induction response is rapid; increases in microsomal enzymes may be detected as early as 1 day after treatment. Peak induction responses may require 4–7 days or more, depending on the inducer, the dose, and the enzyme. The dose–response curve for enzyme induction is generally steep and shows saturation; the induction response is reversible upon discontinuation of treatment. If metabolizing pathways are induced that metabolize the inducer itself (autoinduction), this may lead to a decreased exposure in the animals and a reduced safety margin.

In rats, induction of drug-metabolizing enzymes may affect the microsomal cytochrome P450 enzymes, epoxide hydrolase, and glucuronyl transferases, among other enzymes. The profile of induced enzymes differs between the genders. Microsomal enzyme induction in rats is generally associated with proliferation of smooth endoplasmic reticulum and liver hypertrophy which is evident from increases in liver weight and histological changes (Amacher et al. 1998). Occasionally, mild increases in serum alanine transaminase may be observed. In conclusion, rat microsomal enzyme induction, as well as hepatomegaly, is generally not accompanied by adverse histological effects on the liver or substantial clinical chemistry changes in serum. Enzyme induction is viewed as a benign, adaptive change in response to xenobiotics that stimulate the hepatic drug-metabolizing enzyme system.

However, particular sensitivity to enzyme induction in rats is seen in thyroid hormone homeostasis. Mechanisms involved are increased glucuronidation and increased hepatic uptake of thyroid hormones (Cunha and van Ravenzwaay 2005). The alterations in thyroid hormone clearance are accompanied by an increase in serum levels of thyroid-stimulating hormone and, after long-term treatment, follicular cell proliferation of the thyroid gland that promotes thyroid tumor formation.

In dog toxicity studies, microsomal enzyme activities may be significantly elevated without any indication of hepatocellular or hepatobiliary damage like increased liver weight, histological changes, or serum chemistry changes (Amacher et al. 2001). In conclusion, the hepatic enzyme induction in dogs is seen as a benign adaptive response. The cytochrome P450 enzyme induction profile in dogs resembles the profile observed in humans more than the profile in rats (Graham et al. 2002).

In humans, the possible pharmacokinetic consequences of enzyme induction include decreased or absent bioavailability for orally administered drugs, increased hepatic clearance, or accelerated formation of active or toxic metabolites. Most of the effects of inducers are not detected against the background of interindividual variability of exposure in humans. However, the addition of a potent inducer to, or its withdrawal from, an existing drug regimen may cause pronounced changes in the plasma levels of coadministered drugs and may cause severe drug–drug interactions. Alternatively, if metabolizing pathways are induced that metabolize the inducer itself (e.g., carbamazepine, phenobarbital), this may result in subtherapeutic plasma concentrations and consequently loss of efficacy.

Toxicological consequences of enzyme induction in humans are rare and appear to be limited to hepatotoxicity in ethanol-type induction of CYP2E1. Chronic ethanol intake induces CYP2E1 which increases the transformation of paracetamol to the hepatotoxic metabolite N-acetyl-p-benzoquinone imine (Raucy et al. 1989). Increased risk of paracetamol-induced hepatotoxicity in human following regular ethanol consumption has been conjectured (Chandok and Watt 2010). The likely mechanism of increased

toxicity is that the first step of paracetamol activation mediated by CYP2E1 is accelerated by long-term ethanol exposure, whereas inactivating enzymes are unchanged or even expressed in decreased amounts if alcoholic liver disease is already present.

Clinically relevant drug–drug interactions may emerge when a potent inducer, in particular an inducer of CYP3A4, is coadministered with a drug that is a substrate of CYP3A4. For example, anticonvulsants and antibiotics are known to cause failure of contraception and breakthrough bleeding. Such drugs include phenytoin, carbamazepine, phenobarbital, rifampicin, and griseofulvin which induce cytochrome P450 enzymes, including CYP3A4 (Li et al. 1999; Wang et al. 2004). At a dose of 300 mg daily, rifampicin was found to reduce the plasma levels of ethinylestradiol and norethindrone by about 60% (LeBel et al. 1998).

An even more pronounced interaction of rifampicin was observed with verapamil. Long-term treatment with rifampicin reduced the oral bioavailability of verapamil by 96% (Fromm et al. 1996). In contrast, after intravenous administration of verapamil, the plasma AUC was reduced by only 20%. The difference in plasma exposure after oral and intravenous dosing demonstrates the strong effect of enzyme induction on first-pass metabolism.

Cytochrome P450 enzyme induction can affect the pharmacokinetics of a drug molecule upon multiple dosing and can result in pharmacokinetic drug–drug interactions with coadministered drugs causing potential therapeutic failure. Therefore, it is important that investigational drugs are explored for possible enzyme induction. Optimally, a sequence of enzyme induction studies is planned, moving from in vitro studies to in vivo human studies.

Initial in vitro evaluation of an investigational drug can focus on CYP1A2, CYP2B6, and CYP3A4 induction, reflecting the three important mechanisms of cytochrome P450 induction mediated by the AhR, CAR, and PXR pathways. The mechanistic basis of drug–drug interactions enables more general approaches. If it is confirmed that the investigational drug does not induce CYP3A4, it can be concluded that it is also not an inducer of CYP2C8 or CYP2C9. When an in vitro evaluation cannot rule out the possibility that an investigational drug is an inducer of CYP3A4, an in vivo evaluation can be conducted using the most sensitive CYP3A4 substrate, e.g., oral midazolam. Negative results in a clinical interaction study with midazolam rule out that the investigational drug induces CYP3A4, rendering further clinical investigations of CYP3A4 induction unnecessary. In case of positive results, less sensitive but clinically relevant CYP3A4 substrates, e.g., oral contraceptives, may need to be tested for clinically relevant interaction.

The "gold standard" accepted for in vitro enzyme induction assays are freshly isolated human hepatocytes. However, for routine application in the drug discovery setting, the availability of such cells poses severe limitations, and new in vitro approaches to evaluate enzyme induction have been developed (McGinnity et al. 2009).

- *Reporter cell lines*: Reporter cell lines are suitable for early screening of enzyme inducers that act through AhR and PXR that control the expression of drug-metabolizing enzymes and drug transporters. Cell lines with AhR or PXR and their respective responsive gene elements linked to a reporter gene, e.g., luciferase (Raucy and Lasker 2010), can be engineered by transient or stable transfection. The results with reporter cell lines are in general similar to those of freshly isolated human hepatocytes. However, reporter cell lines may lack some drug-metabolizing enzymes and drug transporters that are present in hepatocytes, and this may explain some incidences of significantly different results. In addition, with CAR, no unequivocal results were obtained from such transactivation assays.
- *Ligand binding assays*: These assays assess the binding of ligands to nuclear receptors (Raucy and Lasker 2010). Results have shown good correlation with cell-based transactivation assays. Examples are the scintillation proximity assay, fluorescent polarization, or fluorescence resonance energy transfer assays. Compounds are tested for their ability to compete with a labeled ligand for the ligand-binding domain of the nuclear receptors. However, both agonists and antagonists are detected in this type of competitive binding assay and, in addition, some other disadvantages are inherent to this type of assay. A further type of binding assay is based on the yeast two-hybrid system; these assays also constitute a powerful screening tool.

- *Immortalized hepatocytes*: Immortalized hepatocytes allow the expansion of hepatocytes in culture and thus overcome the major drawback of freshly isolated human hepatocytes. Cell lines may be derived from human hepatomas, e.g., HEPG2 or HepaRG, or obtained by transfection of human hepatocytes such as the immortalized Fa2N-4 cell line (McGinnity et al. 2009). Not all cell lines reflect freshly isolated human hepatocytes in all respects, however. HepG2 cells carry the embryonic cytochrome P450 isoforms CYP1A1 and CYP3A7 rather than CYP1A2 and CYP3A4 found in the adult human liver. Fa2N-4 cells do not carry functional CAR; they are suitable for AhR-mediated CYP1A2 and PXR-mediated CYP3A4 induction, but not CAR-mediated CYP2B6 induction. HepaRG cells are very promising for the assessment of induction potential of test compounds. They show differentiated hepatocyte morphology and can express phase I and phase II drug-metabolizing enzymes as well as drug transporters normally found in the liver. In addition, HepaRG express AhR, CAR, and PXR at levels comparable to cultured human hepatocytes. If it is confirmed that CAR-mediated induction is present in this cell line, HepaRG cells will offer clear advantages for use in drug discovery.
- *Cryopreserved human hepatocytes*: Attachable cryopreserved human hepatocytes allow planning of experiments and therefore are more convenient than freshly isolated human hepatocytes for enzyme induction studies. Cryopreserved human hepatocytes are a reliable test system for the evaluation of enzyme induction. Catalytic activities and mRNA levels of CYP1A2, 2B6, 2C9, 2E1, and 3A4 remain inducible by the standard cytochrome P450 enzyme inducers after cryopreservation (Roymans et al. 2005). It has been shown that human hepatocytes cultured after cryopreservation are responsive to CYP1A2, CYP2B6, and CYP3A4 inducers, but they have significantly lower basal (uninduced) levels of these enzymes than freshly isolated human hepatocytes.

The above-mentioned experimental systems are more convenient than freshly isolated human hepatocytes. However, freshly isolated human hepatocytes are universally accepted as the "gold standard," as the known mechanisms of enzyme induction, including induction of gene expression, mRNA stabilization, and protein stabilization, can be adequately studied. The following procedure describes a study design and methods for the evaluation of the in vitro induction potential of an investigational drug (test compound) in cultures of freshly isolated human hepatocytes (Koose and Bünning 2010). The induction potential is assessed by determining CYP1A2, CYP2B6, and CYP3A4 enzyme activities and mRNA levels. This protocol is based on various publications (Madan et al. 2003; LeClyse et al. 2005; Hewitt et al. 2007b, 2007c).

39.1 Procedure for In Vitro Induction Studies in Freshly Isolated Human Hepatocytes

39.1.1 Human Hepatocytes

The induction potential of a test compound is evaluated in cultures of freshly isolated human hepatocytes from at least three donors. Hepatocyte cultures are obtained from commercial vendors. The donor demographic information includes age, gender, ethnicity, and pathology. Inclusion criteria are that donors are aged 18 or older and tested negative for HIV, hepatitis B, and hepatitis C infection. The hepatocytes are plated in 6-well collagen-coated plates at a cell density of 2.1×10^5 cells/cm^2 (2×10^6 cells/well). To be acceptable for an enzyme induction study, the viability of the hepatocytes has to be >80% after isolation, and the cultures have to be confluent after overnight incubation. Cultures are shipped at 37°C; a short transportation time is advantageous. Hepatocyte cultures are received approximately 20 h after isolation and are placed in a humidified 5% CO_2 incubator at 37°C for about 20 h. Hepatocyte treatment starts about 40 h after isolation of the hepatocytes.

39.1.2 Study Design

Hepatocyte treatment is started by replacing the culture medium with 2,400 µL/well of hepatocyte incubation medium. The hepatocyte incubation medium consists of Williams Medium E containing GlutaMax I (equivalent to 4 mM L-glutamine),

penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL), insulin (40 μg/mL), and dexamethasone (100 nM). For each treatment, six wells are designated for enzyme activity evaluation and three wells for mRNA determination. The following treatments are performed:

- 0.1% DMSO (vehicle control)

  Attention should be paid to the effects of organic solvents and their concentrations (Easterbrook et al. 2001; Iwase et al. 2006).
- 10, 25, and 60 μM omeprazole in 0.1% DMSO.
- 500, 750, and 1,000 μM phenobarbital in 0.1% DMSO.
- 5, 10, and 30 μM rifampicin in 0.1% DMSO.

  This protocol includes three concentrations of the reference inducers to determine the maximum induction effect of the reference inducers which varies from batch to batch of human hepatocytes.
- At least four concentrations of test compound in 0.1% DMSO.

  For selection of test compound concentrations, the solubility of the test compound is determined by incubation in hepatocyte incubation medium with 0.1% DMSO, at 37°C for 24 h. Test compound concentrations span two to three orders of magnitude, or if plasma levels from clinical studies are known, test compound concentrations will span plasma levels up to a concentration 10-fold to 100-fold above the highest plasma level.

The incubation medium is replaced with fresh incubation medium containing the relevant compound every 24 h up to a total treatment period of 72 h. The medium removed after 24-, 48-, and 72-h treatment periods is tested for lactate dehydrogenase (LDH) activity in a cytotoxicity assay.

39.1.3 Morphology

The cell morphology of the hepatocyte cultures is evaluated by phase-contrast microscopy upon receipt of the cells and at the start and the end of the 72-h treatment period.

39.1.4 Cytotoxicity

Cytotoxicity is assessed on the base of LDH release from human hepatocytes after 24, 48, and 72 h of treatment. Vehicle-treated (0.1% DMSO) and triton X100-treated (0.5% triton X100) hepatocyte cultures are included as references, and tamoxifen-treated (10, 20, 30, 40, 60, and 100 μM tamoxifen) hepatocyte cultures are included as positive control. Colorimetric determinations of LDH activity of four replicates per well with 100 μL culture medium are carried out in 96-well plates using a cytotoxicity detection kit (LDH). The absorbance of the samples is measured at 492 nm in a microplate reader. Cellular viability is calculated by:

$$\text{Cellular Viability } (\%) = \frac{\text{Absorbance (Treatment Sample)} - \text{Mean Absorbance (Triton} \times 100)}{\text{Mean Absorbance (Vehicle)} - \text{Mean Absorbance (Triton} \times 100)} \times 100$$

Noncytotoxic concentrations of the test article should be used. Cytotoxicity may lead to an erroneous conclusion of no induction, as cytochrome P450 enzyme activities may be decreased due to the lower number of viable cells or compromised cell metabolism.

39.1.5 Evaluation of CYP1A2, CYP2B6, and CYP3A4 Activities

Preparation of hepatocyte homogenates: After 72-h treatment with vehicle, reference inducers, and test compound, the hepatocyte cultures are harvested. From the six identically treated wells, designated for enzyme activity evaluation, the hepatocytes from two wells are combined to give triplicate samples for each treatment. Each well is treated by the following procedure. The hepatocyte incubation medium is removed from the well and replaced with 1 mL ice-cold phosphate-buffered saline (PBS); the cells are scraped and transferred to a 50-mL centrifugation tube. The well is washed three times with 3 mL ice-cold PBS buffer which is added to the centrifugation tube. The hepatocytes from two wells are combined in the centrifugation tube and are centrifuged at 863×*g* for 10 min at 4°C.

Table 39.3 Sample dilution and LC-MS/MS injection volume

CYP enzyme	Marker metabolite (analyte)	Sample dilution	LC-MS/MS injection volume
CYP1A2	4-Acetamidophenol	20 µL + 80 µL gradient solution A	10 µL
CYP2B6	Hydroxybupropion	10 µL + 90 µL gradient solution A	10 µL
CYP3A4	6β-Hydroxytestosterone	32 µL + 77 µL gradient solution A	20 µL

The supernatant is removed, and the hepatocyte pellet is suspended in 250 µL ice-cold PBS buffer. The hepatocyte suspensions are kept at 4°C while they are homogenized by ultrasonic disruption in a beaker resonator with 3 rounds of 12 strokes, each stroke with 70% of the maximal energy for 1 s. The homogenate is immediately shock frozen and stored at −80°C until used for preparation of S9 fraction and for determination of enzyme activity and protein concentration.

Incubations of S9 fractions with probe substrates: As selective probe substrates, 100 µM phenacetin for CYP1A2, 500 µM bupropion for CYP2B6, and 200 µM testosterone for CYP3A4 assays are used. Phenacetin and testosterone are dissolved in acetonitrile and bupropion in acetonitrile/water 1:1 (v/v) at their 1,000-fold final concentrations. The stock solutions are diluted 1:500 (v/v) in 100 mM potassium phosphate buffer, pH 7.4, and are finally diluted 1:1 (v/v) into the reaction mixtures. Hepatocyte homogenates are thawed and centrifuged at 9,000×*g* for 30 min at 4°C. The supernatant S9 fractions (20 µL) are added to reaction mixtures, containing 70 µL water, 30 µL of 40 mM magnesium chloride, and 150 µL probe substrate in 100 mM potassium phosphate buffer, pH 7.4. The reaction mixtures are brought to 37°C for 10 min before the enzyme reactions are initiated by addition of 30 µL of 10 mM NADPH. After a 15-min incubation period, 100 µL reaction mixture is removed and added to 300 µL acetonitrile containing internal LC-MS/MS standard. Incubations are performed on triplicate samples for each treatment. Samples are kept frozen at −20°C until LC-MS/MS analysis.

Analytical LC-MS/MS methods: The amounts of marker metabolites formed in the reaction mixtures are quantified by LC-MS/MS analysis (CTC HTS PAL autosampler, Agilent 1100 HPLC system coupled to an Applied Biosystems API4000 with turbo ion spray). [D_4]-4-Acetamidophenol is used as internal standard for CYP1A2, [$^{13}C_6$]-hydroxybupropion for CYP2B6, and [D_4]-6β-hydroxytestosterone for CYP3A4 assays. Calibration ranges are 1–1,000 ng/mL for 4-acetamidophenol, 0.3–300 ng/mL for hydroxybupropion, and 4–4000 ng/mL for 6β-hydroxytestosterone. Frozen incubation samples, calibration standards, and quality control samples are thawed, centrifuged for 5 min at 14,800×*g* and diluted, and an aliquot is applied to LC-MS/MS analysis (Tables 39.3–39.6).

Table 39.4 HPLC systems

Column	Luna C18(2), 3 µ, 100 × 2 mm, Phenomenex
Guard column	C18 (ODS), 4 × 2 mm, Phenomenex
Temperature	40°C +/− 1°C (4-acetamidophenol and 6β-hydroxytestosterone)
	22°C +/− 2°C (hydroxybupropion)
Gradient (mobile phase)	A: 0.1% (v/v) formic acid/water
	B: 0.1% (v/v) formic acid/acetonitrile

Analytical data treatment: The software Analyst version 1.4.2 (Applied Biosystems) is used for data acquisition, integration, and generation of standard curves and calculation of product concentrations. The peak area ratios (analyte/internal standard) for calibration standards are plotted against nominal concentrations to construct calibration curves using weighted ($1/x^2$) linear regression parameters. The acceptance criteria of the quality control and calibration standard results, expressed as the absolute percent difference, are set at 20% for the low calibration standard and low level quality control and at 15% for other calibration standards and quality controls. Analytical runs are accepted if at least 2/3 of all the quality control samples and at least 1/2 of the quality control samples at each quality control level meet the acceptance criteria. The calibration range is adjusted in case a rejected standard falls at either end. Data below limit of quantification (LOQ) of the assay are not used for calculation.

Determination of protein concentration: Protein concentrations of hepatocyte S9 fractions are determined with a bicinchoninic acid protein assay kit.

Table 39.5 HPLC gradients for LC-MS/MS analysis

CYP enzyme	Marker metabolite (analyte)	Time (min)	%A	%B	Flow rate (μL/min)
CYP1A2	4-Acetamidophenol	0–1.0	98	2	350
		1.0–3.0	98 → 10	2 → 90	
		3.0–6.5	10	90	
		6.5–7.0	10 → 98	90 → 2	
		7.0–12.0	98	2	
CYP2B6	Hydroxybupropion	0–1.0	85	15	350
		1.0–3.0	85 → 55	15 → 45	
		3.0–3.5	55 → 10	45 → 90	
		3.5–6.5	10	90	
		6.5–7.0	10 → 85	90 → 15	
		7.0–11.0	85	15	
CYP3A4	6β-Hydroxytestosterone	0–3.0	80	20	350
		3.0–7.2	80 → 58	20 → 42	
		7.2–8.2	58 → 10	42 → 90	
		8.2–12.5	10	90	
		12.5–13.0	10 → 80	90 → 20	
		13.0–16.0	80	20	

Table 39.6 Mass spectrometer quantitation method parameters

CYP enzyme	Marker metabolite (analyte)/internal standard	Polarity	Transition (m/z)	Collision energy (eV)
CYP1A2	4-Acetamidophenol	Positive	152.02→110.14	23
	$[D_4]$-4-Acetamidophenol	Positive	156.12→114.14	23
CYP2B6	Hydroxybupropion	Positive	256.19→139.02	37
	$[^{13}C_6]$-Hydroxybupropion	Positive	262.18→145.11	37
CYP3A4	6β-Hydroxytestosterone	Positive	305.19→269.13	21
	$[D_4]$-6β-Hydroxytestosterone	Positive	309.19→273.18	21

Colorimetric measurements in two dilutions are carried out using the microplate procedure with a sample/working reagent ratio of 1:20 and bovine serum albumin as standard. The absorbance is measured at 570 nm using a microplate reader. Protein concentrations are used to normalize enzyme activities.

Calculation of enzyme activities: Enzyme activities, E, are expressed in pmol × min^{-1} × mg^{-1} as the formation of marker metabolite relative to protein. From incubations of the triplicate samples for each treatment, the arithmetic mean and arithmetic standard deviation of the enzyme activities are calculated. If the triplicates of the enzyme activities differ by more than 30% (coefficient of variation), one value is excluded from calculations of the arithmetic mean and arithmetic standard deviation.

39.1.6 Determination of CYP1A2, CYP2B6, and CYP3A4 mRNA Levels

Total RNA preparation: After 72 h of treatment, the hepatocyte incubation medium is removed from the cultures, and 600 μL of lysis buffer from the MagNA Pure Compact RNA Isolation Kit is added to each of the wells designated for mRNA analysis. The plates are stored frozen at −80°C until RNA preparation. Extraction of total RNA from cell lysates is performed using the MagNA Pure Compact RNA Isolation Kit on the MagNA Pure Compact instrument; isolated RNA is stored at $< -60°C$.

cDNA synthesis and quantitative real-time PCR: mRNA is reverse transcribed into single-stranded cDNA using oligo-$p(dT)_{15}$ primers and AMV reverse transcriptase of a first-strand cDNA synthesis kit for

Table 39.7 LightCycler parameters

Denaturation	95°C for 10 min	
Amplification	35 Cycles	
	Segment 1	95°C for 10 s
	Segment 2	68°C (decreasing 0.5°C/cycle until reaching 58°C) for 10 s
	Segment 3	72°C for 16s

RT-PCR (AMV). Reverse transcription is performed in a final volume of 30 µL which contains 12 µL of the isolated RNA. After the reverse transcription reaction has been stopped, the reaction mixtures are diluted 1:10 with RNase-free water and stored in aliquots at < -60°C. Real-time PCR reactions are performed in capillaries using a LightCycler 1.5 instrument. For analysis, 10 µL of cDNA is amplified using the LightCycler fast start DNA Master^{PLUS} SYBR Green I Kit and the LightCycler primer sets obtained from Search-LC (Search-LC 2010). "Touchdown PCR" is performed in a final volume of 20 µL. Quantification is performed using calibration standards supplied with the LightCycler primer sets. LightCycler data are analyzed using LightCycler software version 3.5. The fluorescence signal readings are analyzed using the fit points method. The copy numbers for both the genes of interest – human CYP1A2, CYP2B6, and CYP3A4 – and the housekeeping genes – human β-actin, cyclophilin B, and glyceraldehyde-3-phosphate dehydrogenase – are determined out of the same cDNA sample in triplicates. LightCycler primer sets are designed such that all PCR reactions use the same temperature profile (Table 39.7).

Analytical data treatment: Transcript copy numbers are obtained from PCR reactions of triplicate samples for each treatment. The transcript copy number of each gene of interest (GOI) – CYP1A2, CYP2B6, and CYP3A4 – is normalized to the transcript copy number of each of the three housekeeping genes (HKG) – β-actin, cyclophilin B, and glyceraldehyde-3-phosphate dehydrogenase . Multiple normalization of target gene expression by using multiple housekeeping genes (e.g., β-actin, cyclophilin B, and glyceraldehyde-3-phosphate dehydrogenase expression) has been shown to be superior to normalization by a single housekeeping gene (Marker 2007). For normalization, the transcript copy number (transcripts/10 µL 1:10 diluted cDNA) of the gene of interest (C_{GOI}) is divided by the transcript copy numbers of each of the three housekeeping genes (C_{HKG}) of the same sample. The arithmetic mean of C_{GOI}/C_{HKG} is formed for each of the three HKG (HKG, $_{1–3}$) from the transcript copy numbers obtained from the PCR reactions of the triplicate samples. The arithmetic mean, E, and the arithmetic standard deviations of the normalizations to the three housekeeping genes are formed.

$$\bar{x}_1 = \frac{1}{n}\sum_{n=1}^{3}\frac{C_{GOI}}{C_{HKG,1}} \quad \bar{x}_2 = \frac{1}{n}\sum_{n=1}^{3}\frac{C_{GOI}}{C_{HKG,2}} \quad \bar{x}_3 = \frac{1}{n}\sum_{n=1}^{3}\frac{C_{GOI}}{C_{HKG,3}}$$

$$E = \frac{1}{3}\sum \bar{x}_1 + \bar{x}_2 + \bar{x}_3$$

39.2 Evaluation of Enzyme Induction

Enzyme activity represents the most relevant endpoint for drug–drug interaction evaluations. However, there are inducers which are also enzyme inhibitors; quantification of enzyme activity, as well as either mRNA (e.g., quantitative RT-PCR) or total protein (e.g., using Western blotting), will allow the evaluation of such compounds. An example is ritonavir; it is an inducer and also a potent inhibitor of CYP3A4 (Foisy et al. 2008). The induction effect of ritonavir does not show up in enzyme activity but becomes apparent in gene expression (quantification of CYP3A4 mRNA).

Solvent control: The study includes a solvent sample as negative control to determine the level of induction (see below).

Positive control: The study includes positive controls with known inducers (e.g., omeprazole, phenobarbital, and rifampicin for CYP1A2, CYP2B6, and CYP3A4, respectively). The positive controls are necessary to demonstrate that the experimental conditions are adequate for the evaluation of induction. A study is considered valid only if the positive controls are found to significantly induce the corresponding cytochrome P450 isoforms. In the current protocol, the maximum induction effect of the reference inducers is assessed by including three concentrations of the reference inducers to accommodate batch-to-batch variations of human hepatocytes. The maximal induction of the reference inducers is compared to the induction caused by the test compound.

Criteria for in vitro induction: Enzyme activity represents the most relevant endpoint for drug–drug interaction evaluations. For subsequent prediction of clinical enzyme induction, the following aspects are to be considered (Hewitt et al. 2007a; Chu et al. 2009; FDA 2012).

- A drug that produces a change that is equal to or greater than 40% of the positive control can be considered as an enzyme inducer in vitro, and in vivo evaluation is warranted.
- An alternative endpoint is the use of an EC_{50} value which represents a potency that can be used to compare the potency of different compounds.
- If induction studies with a test compound confirm that it is not an inducer of CYP3A4, then it can be concluded that the test compound is also not an inducer of CYP2C8 or CYP2C9.

Calculation of % positive control: The induction, expressed as percent of positive control, resulting from treatment with test compound is calculated for enzyme activity and mRNA expression by:

$$\%\ \text{positve control} = \frac{E_{\text{test compound}} - E_{\text{vehicle control}}}{E_{\text{positive control}} - E_{\text{vehicle control}}} \times 100$$

$E_{compound}$: enzyme activity or normalized mRNA expression with test compound treatment

$E_{positive\ control}$: enzyme activity or normalized mRNA expression with reference inducer treatment (of the three concentrations of reference inducer, the concentration is chosen as the positive control which shows the largest induction)

$E_{vehicle\ control}$: enzyme activity or normalized mRNA expression with vehicle treatment

Calculation of fold induction: The induction, expressed as fold induction over vehicle control, resulting from treatment with test compound is calculated for enzyme activity and mRNA expression by:

Fold induction over vehicle control = $E_{compound}/E_{vehicle\ control}$

$E_{compound}$: enzyme activity or normalized mRNA expression with test compound treatment

$E_{vehicle\ control}$: enzyme activity or normalized mRNA expression with vehicle treatment

In vitro–in vivo correlation of CYP3A4 induction: Recently, predictive models have been developed to facilitate extrapolation of CYP3A4 induction from in vitro systems such as freshly isolated human hepatocytes and cryopreserved human hepatocytes to clinical CYP3A4 induction (Shou et al. 2008; Kaneko et al. 2010). Shou et al. include the following parameters into the prediction of the drug–drug interaction from freshly isolated human hepatocytes: EC_{50} and E_{max} of CYP3A4 induction in vitro, fractions unbound of the inducer in human plasma ($f_{u,\ p}$), and hepatocytes ($f_{u,\ hep}$), relevant clinical in vivo plasma concentrations of the inducer ($[Ind]_{max,\ ss}$), and fractions of the affected drugs cleared by CYP3A4 ($f_{m,\ CYP3A4}$). The model has the potential to be used for classifying an inducer in vitro as none, weak, moderate, and strong, corresponding to in vivo induction based on the AUC in the induced state compared to the control and ranked as none ($\geq$75%), weak (50–75%), moderate (20–50%), or strong ($\leq$20%). Kaneko et al. predict clinical CYP3A4 induction from in vitro data using cryopreserved human hepatocytes. The parameters of the model include the free human plasma concentration at steady state ($C_{ss,\ u}$) and the relative factor (RF) determined from the in vitro ratio of the concentration of an inducer to the reference standard. Inducers are classified by the $C_{ss,u}$/RF values into categories of no, low, medium, and high potency inducers correlating to the predicted degree of CYP3A4 induction in vivo in humans.

EXAMPLE

The induction profile of the reference inducers of human cytochrome P450 enzymes omeprazole, phenobarbital, and rifampicin – each at three different concentrations – was studied in freshly isolated human hepatocytes from three donors. Both the induction of CYP1A2, CYP2B6, and CYP3A4 enzyme activities (Table 39.8) and mRNA levels (Table 39.9) were investigated. The induction responses are reported relative to the maximal observed induction – set as 100%, and the fold induction relative to the solvent control is given in parenthesis.

Regarding the enzyme activity, the largest induction responses for CYP1A2 were observed at a concentration of 25 μM omeprazole for all three donors with an 8.9- to 11-fold induction. For CYP2B6, 1,000 μM phenobarbital showed the maximum response with a 12- to 23-fold induction. For CYP3A4, the maximum response was observed at 10 μM rifampicin for one donor and at 30 μM rifampicin for two donors with a 9.1- to 19-fold induction.

Regarding the mRNA level, CYP1A2 maximum expression levels were observed at 60 μM omeprazole for all donors with a 19- to 46-fold induction. Highest

Table 39.8 In vitro cytochrome P450 induction of enzyme activity by reference inducers

Enzyme activity – % positive control (fold induction)										
Treatment		CYP1A (phenacetin O-deethylase)			CYP2B6 (bupropion-hydroxylase)			CYP3A (testosterone 6β-hydroxylase)		
		Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
Solvent (0.1% DMSO)	0.1%	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
Omeprazole	10 μM	75 (8.8)	43 (5.3)	72 (6.8)	16 (4.5)	16 (2.8)	17 (2.8)	13 (3.0)	24 (3.0)	3 (1.5)
	25 μM	100 (11)[a]	100 (11)[a]	100 (9.1)[a]	32 (7.9)	41 (5.6)	22 (3.3)	22 (4.4)	26 (3.1)	2 (1.4)
	60 μM	75 (8.9)	76 (8.7)	74 (7.0)	50 (12)	50 (6.7)	39 (5.1)	11 (2.7)	17 (2.4)	0 (1.1)
Phenobarbital	500 μM	7 (1.7)	8 (1.9)	4 (1.3)	71 (17)	90 (11)	51 (6.4)	80 (13)	106 (9.6)	53 (10)
	750 μM	8 (1.9)	11 (2.1)	7 (1.6)	83 (19)	98 (12)	64 (7.7)	78 (13)	83 (7.7)	63 (12)
	1,000 μM	13 (2.3)	11 (2.1)	18 (2.4)	100 (23)[a]	100 (12)[a]	100 (12)[a]	95 (16)	90 (8.2)	114 (21)
Rifampicin	5 μM	3 (1.3)	2 (1.2)	3 (1.2)	37 (9.1)	30 (4.4)	33 (4.5)	83 (14)	88 (8.1)	79 (15)
	10 μM	3 (1.3)	2 (1.2)	1 (1.1)	38 (9.4)	33 (4.7)	31 (4.2)	80 (13)	83 (7.7)	100 (19)[a]
	30 μM	0 (1.0)	1 (1.1)	<0 (0.8)	39 (9.5)	31 (4.5)	23 (3.4)	100 (16)[a]	100 (9.1)[a]	82 (16)

[a]Reference positive control

Expression of the results: % positive control $= \frac{E_{compound} - E_{vehicle\ control}}{E_{positive\ control} - E_{vehicle\ control}} \times 100\%$ fold induction $= E_{compound}/E_{vehicle\ control}$

$E_{compound}$: enzyme activity with test compound treatment

$E_{positive\ control}$: enzyme activity with reference inducer treatment

$E_{vehicle\ control}$: enzyme activity with vehicle treatment

Table 39.9 In vitro cytochrome P450 induction of mRNA expression by reference inducers

mRNA level – % positive control (fold induction)										
Treatment		CYP1A2			CYP2B6			CYP3A4		
		Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
Solvent (0.1% DMSO)	0.1%	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
Omeprazole	10 μM	43 (8.7)	29 (6.6)	66 (31)	16 (3.0)	12 (1.9)	26 (5.0)	25 (3.4)	32 (2.5)	6 (7.2)
	25 μM	61 (12)	61 (13)	54 (25)	27 (4.3)	26 (2.9)	21 (4.3)	32 (4.0)	29 (2.4)	6 (7.3)
	60 μM	100 (19)[a]	100 (20)[a]	100 (46)[a]	55 (7.7)	32 (3.4)	73 (12)	24 (3.3)	9 (1.4)	21 (22)
Phenobarbital	500 μM	<0 (0.82)	2 (1.4)	1 (1.3)	100 (13)	79 (6.9)	80 (13)	85 (9.1)	87 (5.2)	53 (54)
	750 μM	<0 (0.76)	1 (1.3)	2 (1.8)	75 (10)	91 (7.8)	78 (13)	112 (12)	95 (5.5)	60 (61)
	1,000 μM	0 (0.99)	3 (1.5)	3 (2.2)	96 (13)[a]	100 (8.5)[a]	100 (16)[a]	124 (13)	90 (5.3)	73 (74)
Rifampicin	5 μM	<0 (0.79)	2 (1.4)	0 (1.0)	42 (6.2)	31 (3.4)	25 (4.9)	99 (10)	92 (5.4)	57 (57)
	10 μM	<0 (0.72)	1 (1.3)	0 (1.1)	45 (6.5)	31 (3.3)	34 (6.2)	100 (10)[a]	99 (5.7)	65 (66)
	30 μM	<0 (0.55)	3 (1.5)	3 (2.2)	41 (6.0)	27 (3.0)	42 (7.4)	98 (10)	100 (5.8)[a]	100 (10)[a]

[a]Reference positive control

Expression of the results: % positive control $= \frac{E_{compound} - E_{vehicle\ control}}{E_{positive\ control} - E_{vehicle\ control}} \times 100\%$ fold induction $= E_{compound}/E_{vehicle\ control}$

$E_{compound}$: normalized mRNA expression with test compound treatment

$E_{positive\ control}$: normalized mRNA expression with reference inducer treatment

$E_{vehicle\ control}$: normalized mRNA expression with vehicle treatment

CYP2B6 mRNA expression levels were observed at 1,000 μM phenobarbital for all three donors with an 8.5- to 16-fold induction. For CYP3A4, 10 μM rifampicin showed the maximum response for one donor and 30 μM rifampicin for two donors with a 5.8- to 100-fold induction.

These results demonstrate the degree of variability in the induction response by human hepatocyte cultures from various donors.

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Drug–Drug Interaction: Enzyme Inhibition 40

Angela Dudda and Gert Ulrich Kuerzel

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40.1 Introduction

Inhibitory drug interactions have received considerable attention in the 1990s because some prominent drugs (e.g., terfenadine) caused life-threatening adverse effects when prescribed with other commonly used comedications (e.g., antibiotics). At about the same time, in vitro technologies were developed to study drug interactions with individual human P450 enzymes by using either enzyme-specific substrates or recombinant P450 isoenzymes. Along with guidance documents issued by the US FDA and European Agency for the Evaluation of Medicinal Products, the evaluation of in vitro drug interactions and the subsequent prediction of in vivo drug–drug interactions from in vitro data have become an integral part of the drug development process (revised draft guidance FDA 2006; EMEA 2010).

The following discussions and assay descriptions are related to cytochrome P450 inhibition. Although most drug interaction studies are related to P450 isoenzymes, other enzyme systems may also contribute to significant drug interactions such as phase II enzymes (e.g., Dietmann and Stork 1976; Kumar et al. 1996; Zucker et al. 2001; Rayer et al. 2001), cytosolic enzymes (Obach et al. 2004), or transporters (e.g., Floren et al. 1997; Abel et al. 2001). Additional assays related to phase II, cytosolic enzyme, or P-glycoprotein interactions are published in literature (e.g., Obach et al. 2004; bdbiosciences.com; Polli et al. 2001; Schwab et al. 2003; Schinkel and Jonker 2003).

Inhibitory drug interactions generally fall into two categories – "direct" and time-dependent inhibition. "Direct" inhibition occurs when a drug inhibits P450

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isoenzyme without requiring biotransformation. Traditionally, direct inhibition has been divided in three categories: competitive, noncompetitive, and uncompetitive inhibition (Madan et al. 2002). Competitive inhibition occurs when substrate and inhibitor compete for binding at the same active site at the enzyme. Based on the Michaelis-Menten kinetics, V_{max} is unchanged whereas K_m increases. In case of noncompetitive inhibition, the inhibitor and the substrate bind to different sites at the enzyme. V_{max} decreases whereas the K_m value is unaffected. Binding of the inhibitor only to the enzyme-substrate complex is described as uncompetitive inhibition. Both V_{max} and K_m decrease. Finally, mixed (competitive-noncompetitive) inhibition occurs, either the inhibitor binds to the active or to another site on the enzyme, or the inhibitor binds to the active site but does not block the binding of the substrate. Enzyme kinetics and the mode of inhibition are well described by transformation of the Michaelis-Menten equation. The binding affinity of the inhibitor to the enzyme is defined as the inhibition constant Ki, whereas the affinity, with which the substrate binds, is referred to the Michaelis-Menten coefficient Km. Michaelis-Menten kinetics base on three assumptions:

1. The dissociation of the enzyme-inhibitor or the enzyme-substrate complex is the rate-limiting step.
2. The enzyme concentration is negligible compared to the concentration of the substrate or inhibitor.
3. The free concentration of inhibitor and substrate is known or approximated by the total concentration of substrate and inhibitor.

Identifying a drug as an inhibitor of a given P450 isoenzyme does not necessarily imply that the drug will cause clinically relevant drug interactions. The clinical inhibition potential must be considered in the following context:

1. The pharmacokinetics of the inhibitory drug, particularly maximum exposure and half-life.
2. The potential of coadministering the inhibitory drug together with other drugs that are substrates of the same isoenzyme (inhibition of the comedication by the new chemical entity ("NCE") must be considered separately from inhibition of the NCE metabolism by the comedication).
3. The extent to which clearance of the comedication is dependent on the related isoenzyme.
4. The potential of saturating the capacity of the related isoenzyme.
5. The clinical consequences of alteration of the pharmacokinetics of the affected drug (depending on the drug's therapeutic index).

The K_i determination is inevitable to understand the mechanism of inhibition and for risk assessment.

The second type of drug inhibition results from "irreversible" (or "quasi-irreversible") inhibition of cytochrome P450 and often involves metabolism-dependent inhibition or suicide inactivation of cytochrome P450 (Ortiz de Montellano 1995). Irreversible or mechanism-based inactivation occurs when a compound is metabolized by a CYP to a reactive intermediate which modifies and inactivates the enzyme. At least three different mechanisms are discussed which lead to enzyme inactivation (Polasek and Miners 2007):

1. Reaction with amino acids in the active site of the CYP enzyme.
2. Reaction with the heme nitrogen.
3. Coordination to the heme iron to form a metabolite-intermediate complex (MIC).

The first two mechanisms are classified as true irreversible inhibition, because covalent modification causes inactivation whereas coordination to the heme iron is considered as quasi-irreversible since MIC formation does not actually destroy the enzyme. However, catalytically active CYP can be regenerated in vitro, e.g., by ultrafiltration, dialysis, or oxidation with ferrycanide, but not in vivo. Mechanism-based inhibition is characterized by time- and dose-dependency with a 1:1 stoichiometry, involvement of a catalytic step (NADPH-dependent), saturation of inactivation kinetics, and blockage by other substrates or competitive inhibitors. Chemical substituents frequently associated with cytochrome P450 time-dependent inhibition are aliphatic, alicyclic, or cyclopropylamine; methylenedioxyphenyl; furan; thiophene; alkene; alkyne; 2-alkylimidazole; 3-alkylindole; dihaloalkane; hydrazine; aminophenol; and phenol (Grimm et al. 2009). Due to the chemical modification of the active site, the enzyme activity is permanently lost and can only be re-established by de novo synthesis of the enzyme. Hence, the duration of clinical interactions lasts longer than the actual half-life of the MBI. Mechanism-based inactivation ("MBI") accounts for some of the most potent clinically observed drug–drug interactions (e.g., with mibefradil and tienilic acid, Zhou et al. 2007).

Quantitative prediction of drug-drug interactions bridges the gap between in vitro studies and clinics and provides valuable information to avoid toxic interactions in clinical practice.

In the presence of competitive inhibition, the AUC ratio, reflecting the fold reduction in clearance after oral coadministration of substrate ("victim") and inhibitor ("perpetrator"), is determined by the concentration of the inhibitor also called perpetrator at the enzyme site (I) and its associated equilibrium dissociation constant (K_i) as illustrated in Fig. 40.1, with the following equation: $AUC_i/AUC = 1 + I/K_i$. I/K_i reflects the strength of inhibition of a compound for a given in vivo concentration, qualifying the inhibition risk: the higher the ratio, the higher the risk. A value above 0.1 is considered positive according to the FDA guidance, reflecting a potential inhibition risk.

Regarding MBI, the relationship between AUC ratio, reflecting the fold reduction in clearance is described by the following equation: $AUC_I/AUC = 1/(k_{deg}/(k_{deg} + (I*k_{inact}))/(I + K_I))$ (Mayhew et al. 2000), with K_I, the inhibitor concentration at which half maximal inactivation rate is achieved; k_{inact}, the first-order inactivation rate constant; k_{deg}, the natural degradation rate constant for the enzyme in the liver; and I, the inhibitor concentration at the enzymatic site. However, this equation is sometimes used with caution because of the level of uncertainty linked to the natural enzyme turnover k_{deg} and the inhibitor concentrations (Grimm et al. 2009).

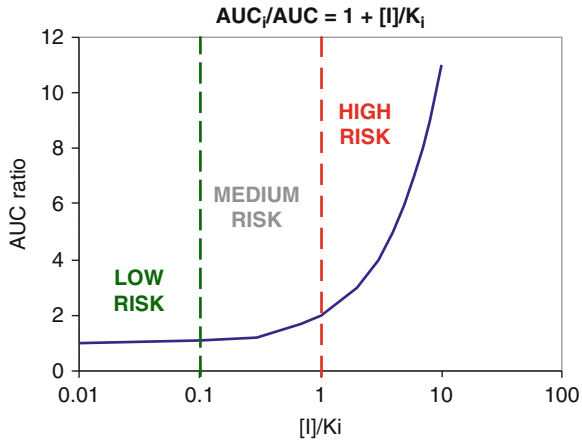

Fig. 40.1 Plot of the relative increase in exposure AUCi/AUC versus I/K_i related to potential risk for drug interaction of direct inhibitors

For CYP3A4, as an example, for which the highest number of mechanism-based inhibitors has been described, this parameter is still debated in the literature. However, once k_{deg} is selected, risk assessment can be proposed positioning the compound of interest (as an inhibitor) on an abacus, taking into account the in vitro parameters k_{inact} and K_I and the inhibitor concentration [I] (Fig. 40.2).

For more information on prediction of clinical drug-drug interactions, please refer to the literature (e.g., Mayhew et al. 2000; Ito et al. 2004; Obach et al. 2006, 2007; Venkatakrishnan and Obach 2007; Fowler and Zhang 2008).

40.1.1 Assays Available

Besides liver slices, isolated/cultured hepatocytes; purified, reconstituted P450 isoenzymes; human liver microsomes; and microsomes from cell lines transfected with cDNA encoding a given human P450 isoenzyme (recombinant P450 isoenzymes) are used for drug interaction studies, most commonly human liver microsomes and recombinant P450 isoenzymes. During the early drug discovery phase, IC_{50} determinations are typically performed in an HTS format using fluorescence marker substrates and recombinant P450 isoenzymes. The method is a fast and cost-effective way to perform thousands of IC_{50}s per year.

Since human liver microsomes contain a complex mixture of CYPs that more closely approximates an intact liver, CYP inhibition data from human liver microsomes are considered as more reliable. For large amounts of compounds, either 1 or 2 concentrations of a potential inhibitor are tested using the classical marker substrates (Table 40.2). With the development of more sensitive LC-MS/MS instruments and the ability to analyze more analytes in parallel, pooled samples for analysis or cassette substrates in incubation to assess full IC_{50}s have further enhanced the throughput of LC-MS/MS-based CYP inhibition assays in human liver microsomes (Dierks et al. 2001; Peng et al. 2003). In the area of ultrafast LC-MS/MS techniques, e.g., LDTD instruments (Phytronix) or RapidFire™ (Perloff et al. 2009; Miller et al. 2009; Luippold et al. 2011), full IC_{50} determination and screening for mechanism-based inhibition in human liver microsomes have become common already in the early

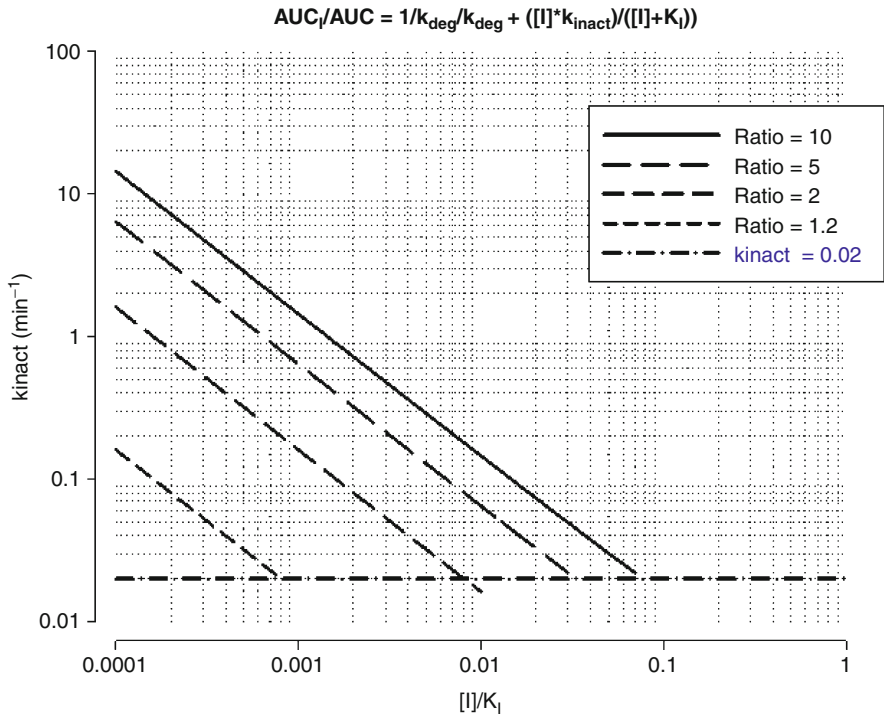

Fig. 40.2 Abacus for MBI risk assessment, assuming $k_{deg} = 0.00016\ min^{-1}$. Ratio represents the AUC ratio with and without coadministration of inhibitor. The threshold $k_{inact} = 0.02\ min^{-1}$ corresponds to the accepted lowest detectable limit using the classical in vitro test

Table 40.1 Marker substrates and typical incubation conditions using recombinant P450 isoenzymes (BD Supersomes®, BD Biosciences, Franklin Lakes, NJ)

CYP	Substrate	Substrate Conc. (μM)	Enzyme (nM)	$NADP^+$ (μM)	G6P (mM)	$MgCl_2$ (mM)	G6P-DH (U/mL)	Incubation Time (min)
1A2	3-Cyano-7-ethoxycoumarin (CEC)	5	5	8.1	0.4	0.4	0.2	15
2A6	Coumarin	3	5	8.1	0.4	0.4	0.2	10
2B6	7-Ethoxy-4-trifluoromethylcoumarin (EFC)	2.5	5	8.1	0.4	0.4	0.2	30
2C8	Dibenzylfluorescein (DBF)	1	9	8.1	0.4	0.4	0.2	30
2C9	7-methoxy-4-trifluoromethylcoumarin (MFC)	75	5	8.1	0.4	0.4	0.2	45
2C19	3-cyano-7-ethoxycoumarin (CEC)	25	5	8.1	0.4	0.4	0.2	30
2D6	3-[2-(N, N-diethyl-N-methylamino] ethyl]-7-methoxy-4-methylcoumarin (AMMC))	1.5	7.5	8.1	0.4	0.4	0.2	30
2E1	7-Methoxy-4-trifluoromethylcoumarin (MFC)	70	5	8.1	0.4	0.4	0.2	45
3A4	7-Benzyloxy-trifluoromethylcoumarin (BFC)	50	5	8.1	0.4	0.4	0.2	30
	7-Benzyloxyquiniline (BQ)	40	7.5	8.1	0.4	0.4	0.2	30
	Dibenzylflourescein (DBF)	1	1	8.1	0.4	0.4	0.2	10

Final volume: 200 μL

Table 40.2 Recommended in vitro marker substrates and inhibitors for cytochrome P450 isoforms (FDA 2006)

	Substrate				Inhibitor			
CYP	Preferred	K_m (μM)	Acceptable	K_m (μM)	Preferred	K_i (μM)	Acceptable	K_i (μM)
1A2	Phenacetin	1.7–152	Ethoxyresorufin	0.18-0.21	Furaylline[a]	0.6-0.73	α-Naphthoflavone	0.01
2A6	Coumarin	0.3–2.3			Tranylcypromine	0.02-0.2	Pilocarpine	4
	Nicotine	13–162			Methoxsalen[a]	0.01-0.2	Tryptamine	1.7
2B6	Efavirenz	17–23	Propofol				3-Isopropenyl-3-methyl diamantine	2.2
	Bupropion	67–168	S-Mephenytoin	1,910			2-Isopropenyl-3-methyl diamantine	5.3
							Sertaline	3.2
							Phencyclidine	10
							Thiotepa	48
							Clopidogrel	0.5
							Ticlopidine	0.2
2C8	Taxol	5.4–19	Amodiaquine	2.4	Montelukast	1.1	Trimethoprim	32
			Rosiglitazone	4.3–7.7	Quercetin		Gemifibrozil	69–75
							Rosiglitazone	5.6
							Pioglitazone	1.7
2C9	Tolbutamide	67–838	Flurbiprofen	6-42	Sulfaphenazole	0.3	Fluconazole	7
	S-Warfarin	1.5–4.5	Phenytoin	11.5-117			Fluvoxamine	6.9–19
	Diclofenac	3.4–52					Fluoxetine	18–41
2C19	Mephenytoin	13–35	Omeprazole	17–26			Ticlopidine	1.2
			Fluoxetinr	3.7–104			Nootkatone	0.5
2D6	Bufuralol	9–15	Debrisquine	5.6	Quinidine	0.027–0.4		
	Dextromethorphan	0.44–8.5						
2E1	Chlorzoxazone	39–157	p-Nitrophenol	3.3			Diethyldithiocarbamate	0.9.08.1934
			Lauric acid	130			Clomethiazole	12
			Anilin	6.3–24			Diallyldisulfide	150
3A4[b]	Midazolam	1–14	Erythromycin	33–88	Ketoconazole	0.0037–0.18	Azamulin[a]	
	Testosterone	52–94	Dextromethorphan	133–710	Itraconazole	0.27,2.3	Troleandomycin	17
			Triazolam	234			Verpamil	10,24
			Terfenadine	15				
			Nifedipine	5.1–47				

[a]Mechanism based inhibitor
[b]Strongly recommended to use at least two structurally unrelated substrates

discovery process. In the drug development phase, detailed CYP interaction studies to evaluate the mode of inhibition, K_i and K_I/k_{inact} determination are usually required for intermediate and potential direct and mechanism-based CYP inhibitors.

Interaction studies in suspended/cultured hepatocytes (Oleson et al. 2004; Gómez-Lechón et al. 2004) and liver slices are less common since a couple of competing reactions occur, e.g., uptake pathways or phase II metabolism of the NCE and/or marker substrate, which make it difficult to interpret the data mechanistically. However, interaction studies in human hepatocytes are recommended when drug candidates are extensively metabolized by non-CYP enzymes to prevent over- or underprediction of the interaction potential from human liver microsome studies (Parkinson et al. 2010).

40.2 "Direct" Cytochrome P450 Inhibition

40.2.1 CYP Inhibition Studies Using Recombinant P450 Isoenzymes

Combinatorial chemistry and high-throughput screening for pharmacological activity have identified a relatively large number of compounds, which have potential drug properties. Since inhibitory drug interaction has been associated with life-threatening adverse effects, an early identification for potential drug interaction of NCE is desirable. The availability of high-throughput assays for cytochrome P450 inhibition facilitates the identification of those drug candidates, which have lower potential for drug-drug interactions.

Table 40.1 summarizes typical assay conditions for CYP inhibition studies of the most relevant P450 enzymes using recombinant P450 isoenzymes (Supersomes®) which are applicable to 96 and 384 well formats. Assay conditions for additional P450 isoenzymes can be found under www.bdbiosciences.com.

40.2.1.1 IC_{50} Determination

Usually, the NCE is pipetted together with the enzyme-substrate complex, and the reaction is started with the addition of the cofactor solution. Incubation times vary between 15 and 45 min at 37°C. Afterward, the reaction is stopped by the addition of a TRIS/acetonitile solution and applied to fluorescence read-out. IC_{50} determinations are calculated using standard software tools, e.g., ExcelFit® or SigmaPlot®.

EVALUATION

The assays are usually performed in parallel to solvent control and a well-known inhibitor of the P450 isoenzyme investigated (positive control).

CRITICAL ASSESSMENT OF THE METHOD

For overexpressing P450 isoenzymes, several heterologous expression systems have been established, that includes bacteria (*Escherichia coli*), yeast (Saccharomyces cerevisiae, Schizosaccharomyces pombe), insect cells (baculovirus), and mammalian cells (V79, CHO, HepG2, NIH 3 T3, human lymphoblast cells) (Crespi and Miller 1999). High active P450 isoenzymes, overexpressed in human lymphoblast cells (BD Supersomes®, BD Biosciences, Franklin Lakes, NJ) and in insect cells (Baculosomes®, Panvera, Madison WI), have been asserted in the market and are commercially available. However, the Supersomes® and Baculosomes® have not been thoroughly characterized with respect to their kinetic properties and substrates/inhibitors specificities. Usually, the expression of the cytochrome b5 and/or NADPH-cytochrome P450 reductase varies from batch to batch, which can affect the turnover number (V_{max}) for a given enzyme, although the affinity (K_m value) of P450 enzymes toward marker substrates is generally comparable between recombinant enzymes and human liver microsomes (McGinnity et al. 1999). Moreover, the catalytically inactive apoprotein contributes significantly to the total protein concentration. On the other hand, the simplicity of the test system (separate study of the P450 isoenzyme, fluorescence labeled marker substrates, which allows a rapid, compound-independent read-out without any extraction procedures) allows a quick estimation of the interaction potential of NCEs in an HTS format with an excellent sensitivity, reproducibility, and throughput (thousands of compounds/year and person). In addition, polymorphic P450 isoenzymes with different genotypes are available, which allow detailed interaction studies. Sometimes, fluorescent excitation and emission overlaps between NCEs/metabolites, NADPH, and marker substrate, which results in

assay failure but might be overcome by alternative read-out methods (e.g., LC-MS/MS technologies).

A major disadvantage is that inhibitory metabolites generated from other CYPs are overseen in the assays (false negatives). False positives are due to enzymes involved in the metabolic turnover other than the particular one studied.

MODIFICATION OF THE METHOD

Additional fluorescence labeled marker substrates with different extension/emission wavelengths are on the market, e.g., from Invitrogen™ (www.invitrogen.com), which allow some variation if the NCE/metabolite interferes with the fluorescence read-out.

"P450-Glo™ Assays" from Promega Biosciences Inc. (www.promega.com) – a luminescent cytochrome P450 assay – was introduced as alternative CYP inhibition assay. The marker substrates are luciferin derivatives (luciferin 6′-chloroethyl-ether, luciferin 6′-methylether, 6′-deoxyluciferin and luciferin 6′-benzylether, ethylene glycol ester of deoxyluciferin, ethylene glycol ester of luciferin 6′-methylether, Cali et al. 2006), which are converted from recombinant P450 isoenzymes (Supersomes®, Baculosomes®, or yeast expression systems) to luciferin, which in turn reacts with luciferase to an amount of light that is directly proportional to P450 activity. The assay promises an exquisite sensitivity with low background signals and a broad dynamic range. Since no information of NCEs toward their luciferase inhibition potential is known, an inhibition study has to be performed in parallel. The same is true for quench effect of the NCE that might alter the read-out. The marker substrates are not specific for any single P450 isoenzyme, except for 6′-deoxyluciferin (human CYP2C9). Hence, application to HLM or cell-based assays is critical today. Marker substrates for CYP2A6, CYP2B6, and CYP2E1 are currently missing. The assay is applicable to 96-, 384-, and 1536-well format. Furthermore, the luciferase read-out reaction needs additional 20 min incubation time.

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40.2.2 CYP Inhibition Studies Using Human Liver Microsomes

Human liver microsomes contain all P450 isoenzymes expressed in human livers, although their content and genotype, especially for polymorphic P450 isoenzymes such as CYP2D6, CYP2C9, CYP2C19, and 2B6, can vary from sample to sample. To overcome the problem of variability, several individual human liver samples are pooled to get an "average" of all P450 enzymes expressed in human livers. Individual and pooled human liver microsomes are commercially available. Since all P450 isoenzymes are present in microsomes, enzyme-selective substrates must be used. Table 40.2 summarized recommended substrates.

40.2.2.1 IC_{50} Determination

Each drug, one or two concentrations (e.g., 1 and 10 μM) in the early discovery stage, or more concentrations (up to ten concentrations which cover two orders of magnitude) for detailed IC_{50} determination, is incubated with human liver microsomes in the presence of the marker substrate (FDA 2006). Reactions are initiated with addition of $NADP^+$/regeneration system or NADPH at 37°C. According to the Michaelis-Menten assumptions, the marker substrates should be

used at concentrations below or around the corresponding K_m values, which have to be determined once for each specific substrate prior to the incubation. The microsomal protein concentration should be as low as possible to circumvent unspecific binding. Cofactors, such as $NADP^+$, G6P, G6P-DH, and $MgCl_2$, are usually used at concentrations of 0.5 mM, 5 mM, 0.5 U/mL, and 6 mM, respectively, in 50 mM phosphate buffer pH 7.4. Organic solvent should be minimized as much as possible to avoid inhibitory effects of the solvents (Busby et al. 1999; Yuan et al. 2002). Addition of ice-cold acetonitrile solution containing an appropriate amount of internal standard followed by a sharp centrifugation step stops the reactions. The supernatants are either pooled or applied directly to LC-MS/MS analytics for quantification of the marker metabolite generated from the respective marker substrate. IC_{50} determinations are calculated using standard software tools, e.g., Excelfit® or SigmaPlot®.

Table 40.3 summarizes typical incubation conditions and kinetic constants of marker substrate reactions of human P450 enzymes in a pool of human liver microsomes.

40.2.2.2 K_i Determination

Based on the result from the IC_{50} determination, determination of additional kinetic parameters such as K_i for the assessment of clinical drug-drug interactions and the inhibition mode are useful. K_i experiments are typically performed with variable substrate and inhibitor concentrations, spanning at least 0.5–5 x of the expected K_i (according to $K_i = IC_{50}/2$) and K_m concentrations using the same incubation conditions as outlined above. Transformation of the Michaelis-Menten equation is used both for the calculation the K_i value as well as for graphical depiction of the type of inhibition (e.g., direct plot ([rate]/[substrate]), Dixon plot [1/rate]/[inhibitor], Linewaver-Burk plot [1/rate]/[1/substrate], or Eadie-Hofstee plot [rate]/[rate/substrate]). K_i is easily taken from Dixon plots and the inhibition mode from the Eadie-Hofstee plot (Segel 1993).

EVALUATION

The assays are usually performed in parallel to solvent control and a well-known inhibitor of the P450 isoenzyme investigated.

CRITICAL ASSESSMENT OF THE METHOD

The Michaelis-Menten assumption can be violated in the case of P450 enzymes, depending on the CYP isoenzyme on the in vitro system used. CYP3A4 and CYP2C9 are known to show atypical enzyme kinetics such as enzyme activation or partial inhibition which illustrates possible pitfalls especially when testing only two concentrations during the discovery phase. The "free" concentration of substrate or inhibitor may differ significantly from the total concentration, since microsomes usually contain a large amount of lipids and proteins that can decrease the free concentration of the drug and the marker substrate in the medium. The potency of some inhibitors is such that the free concentration of the inhibitor is in the same range as the

Table 40.3 Typical incubation conditions and kinetic constants of marker substrate reactions of human P450 enzymes in a pool of human liver microsomes (Madan et al. 2002; Bourrier et al. 1996; Transon et al. 1996; and Hesse et al. 2000)

CYP	Marker reaction	Protein(mg/ml)	Incubation time (min)	K_m (μ)	V_{max} (pmol/min/mg)
1A2	Phenacetin O-deethylation	0.1	10	30.1 ± 9.3	7,700 ± 4,500
	Ethoxyresorufin O-deethylation	0.1	10	0.26 ± 0.01	120 ± 2
2A6	Coumarin 7-hydroxylation	0.05	5	0.57 ± 0.02	1,300 ± 12
2B6	Bupropion hydroxylation	0.1	10	89 ± 14	3,600 ± 1,500
	S-Mephbenytion N-demethylation	1	30	1,700 ± 40	1,900 ± 30
2C8	Paclitaxel 6α-hydroxylation	0.1	10	14 ± 1	530 ± 30
2C9	Diclofenac 4′-hydroxylation	0.1	5	3.7 ± 0.2	3,600 ± 59
2C19	S-Mephenytion 4′-hydroxylation	1	30	35 ± 2	380 ± 4
2D6	Dextromethorphen O-demethylation	0.1	10	5.5 ± 0.5	360 ± 13
2E1	Chlorzoxazone 6-hydroxylation	0.1	10	27 ± 2	2,500 ± 100
3A1	Testosterone 6β-hydroxylation	0.1	10	110 ± 10	9,800 ± 490
	Midazolam 1′hydroxylation	0.1	10	3.3 ± 1.3	577 ± 375
4A9/11	Lauric acid 12-hydroxylation	0.1	5	7.6 ± 1.2	2,200 ± 100

enzyme concentration. This problem can be overcome by lowering the enzyme concentration (often limited by analytical sensitivity of the assay) or by estimating an "apparent" K_i, by correcting for the fraction of the inhibitor that is bound to the enzyme, which is calculated as the product of the fractional inhibition in the presence of a given inhibitor concentration and enzyme content (Gibbs et al. 1999). Note that IC_{50} values are extrinsic constants whereas K_i values are intrinsic constants. Consequently, IC_{50} values (but not K_i values) are dependent on the type of substrate and incubation conditions and are difficult to reproduce between different laboratories. On the other hand, IC_{50} determination is less time consuming, and an external quality control can be achieved by using standard inhibitors in parallel. Nevertheless, the FDA has accepted the method of predicting the potential for drug interaction by a drug based on K_i values (together with the free plasma concentration of the drug).

MODIFICATION OF THE METHOD

The most critical step in the interaction studies is a sensitive and reproducible method for quantification of the marker substrates and the corresponding metabolite. Analytical methods are usually applied to time- and resources-consuming HPLC (UV/fluorescence/radioactivity detection) or LC-MS/MS detection.

An alternative detection method was introduced by Yang (Yang et al. 1991), Bloomer (Bloomer et al. 1992, 1995), Rodrigues (Rodrigues et al. 1994, 1997; Rodrigues 1996), and Riley (Riley and Howbrook 1997). They used ^{14}C-nitrosodimethylamine, [O-methyl-^{14}C]dextromethorphan, [O-ethyl-^{14}C]phenacetin, and [N-methyl-^{14}C]erythromycin as marker substrates for CYP2E1, CYP2D6, CYP1A2, and CYP3A4, respectively, with a [^{14}C]formaldehyde or [^{14}C]acetaldehyde read-out after a simple and rapid extraction method.

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40.3 Time-Dependent CYP Inhibition

40.3.1 IC_{50} Shift Assay

A simple method to evaluate potential time-dependent inhibitors is to preincubate an NCE at various concentrations for 30 min in presence and absence of NADPH in *rh*CYP (Burt et al. 2010) or in human liver microsomes (Grimm et al. 2009, incubation conditions; see Chap. 2 "Safety Pharmacology: Introduction" or Obach et al. 2007), with or without a dilution step in between following addition of the marker substrate to determine the residual P450 enzyme activity usually by LC-MS/MS analysis. A significant IC_{50} shift indicates mechanism-based inactivation of the related P450 isoenzyme, for which K_I and k_{inact} determination are usually done as follow-up investigations. For screening purposes to identify potential MBIs, preincubation is usually done with one or two concentrations (often 1 and 10 µM) with subsequent LC-MS/MS analysis to increase throughput.

40.3.2 Time-Dependent Inhibition Screening Using Recombinant Human P450 Isoenzymes

A high-throughput assay for the evaluation on MBI potential using recombinant P450 isoenzyme was introduced by Abecassis (2003), deploying a change of the apparent IC_{50} value with time. According to Maurer (2000), who derived a mathematical relationship between the inhibitory potency at any time $IC_{50}^{(t)}$ as outlined in Fig. 40.3, a decrease of the IC_{50} value with time indicates a mechanism-based inhibition whereas competitive inhibition is time independent and shows no changes of the IC_{50}. The slope value has a direct proportionality to K_I/k_{inact}, and slope divided by IC_{50} is directly proportional to k_{inact}.

Serial dilution of an NCE (5–10 concentrations) is pipetted together with the enzyme-substrate complex and cofactor solution (saturated conditions, e.g., 12 nM for rhCYP3A4, 7-benzoquinoleine 10 µM (~K_m), 4 mM $MgCl_2$, 3.3 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, 0.1 mg/mL BSA in 0.3 M potassium phosphate buffer pH 7.4 in a total volume of 100 µL on a 96-deep-well plate. A well-known mechanism-based inhibitor, e.g., mifepristone, and – if feasible – a competitive inhibitor are investigated as positive and negative controls in parallel. Reaction starts with addition of 1 mM NADPH. Fluorescence (excitation/emission wavelength: 410 and 538 nm) is measured in a real-time course every 30 s for 15 min on a Fluoroscan Ascent Labsystem (ThermoFisher Instruments). IC_{50} values at each time point are automatically fitted by standard software tools, and the ln(IC_{50}) is plotted versus the incubation time.

40.3.3 Determination of the Apparent Partition Ratio

Alternatively, the MBI potential can be determined based on the apparent partition ratio according to Lim et al. (2005). The enzyme activity is measured after 1-h incubation time with an NCE in presence and absence of NADPH in comparison to a known positive control. A decrease of the enzyme activity after preincubation of the NCE in presence of NADPH indicates MBI. The primary incubation usually contains 2 mg/mL HLM, various concentrations of the test compound, 10 mM $MgCl_2$, 2 mM EDTA, 100 mM potassium phosphate

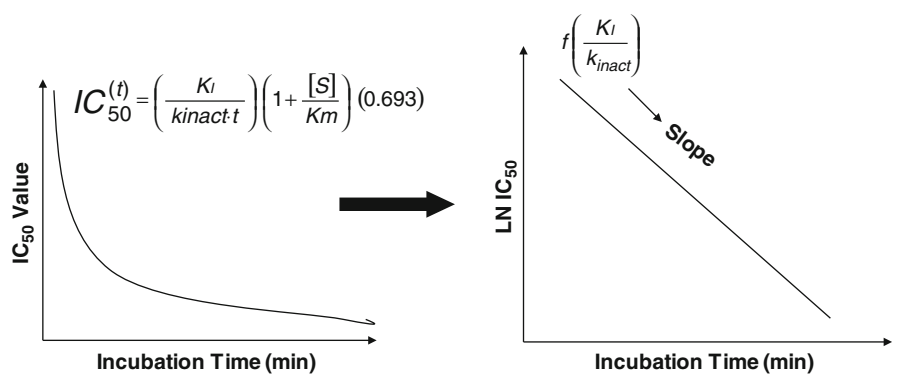

Fig. 40.3 Mathematical relationship between the inhibitory potency with time and kinetic parameter. IC_{50} values decrease with incubation time due to strong/irreversible interaction with reactive intermediate and the P450 enzyme

buffer pH 7.4, 1 mM $NADPH^+$, 10 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate dehydrogenase in a total volume of 200 µL. The reactions are initiated by adding NADPH-regenerating system and incubated for 1 h at 37°C to ensure complete inactivation. To analyze the residual catalytic activity, an aliquot of the incubation solution (usually 10–20 µL) is transferred to a mixture containing the identical concentration of cofactors as described above and the marker substrate at saturated concentration (5 × Km) instead of the test compound (1:20 dilution). The secondary incubation is incubated for additional 20 min and stopped afterward with acetonitrile containing internal standard. Microsomal proteins are pelled by centrifugation, and an aliquot of the supernatant is analyzed by LC-MS/MS analytics. The APRs are calculated plotting percent activity remaining as a function of the molar ratio of the test compound to P450 isoenzyme (Fig. 40.4). Values from the respective control incubation were set to 100%. Investigation on the reversibility of the inactivation can be achieved either by oxidation with ferricyanide or by dialysis to distinguish between true MBIs and quasi-irreversible inhibitors (Lim et al. 2005).

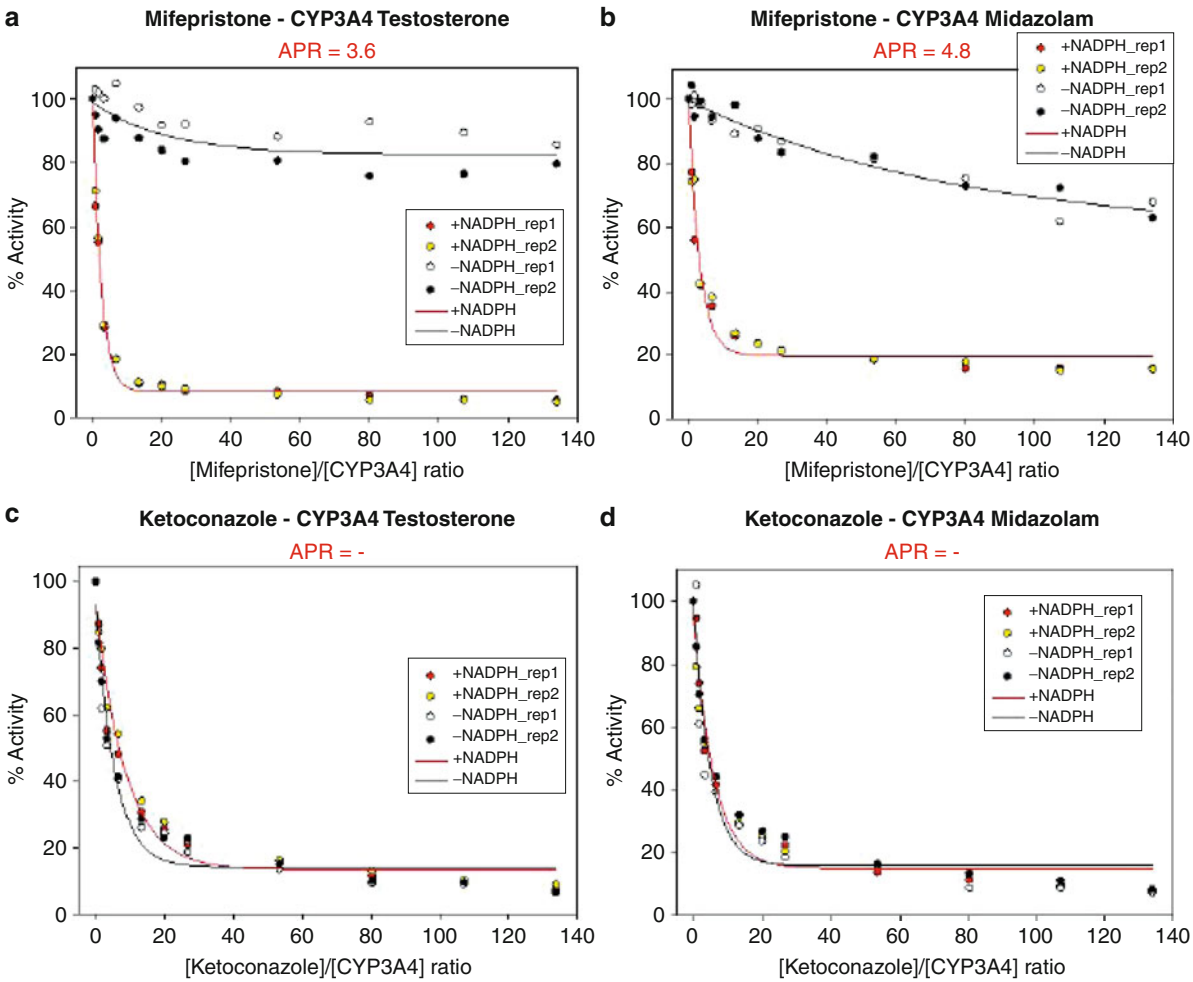

Fig. 40.4 Curves corresponding to the percentage of the remaining enzyme activity in presence and absence of NADPH-regenerating system were plotted as function of the molar ratio of test compound to P450 isoenzyme (data related to CYP3A4, using testosterone and midazolam as CYP3A4 marker substrate). Mifepristone serves as positive control (graph A and B) and ketoconazole as negative control (graph C and D)

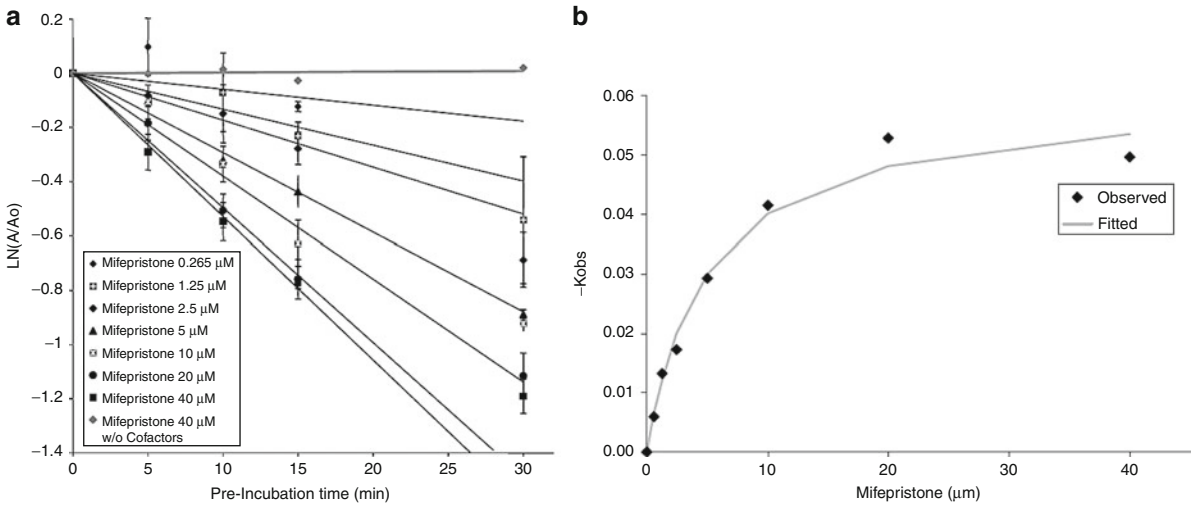

Fig. 40.5 (**a**) Time-dependent inhibition of midazolam hydroxylase activity by Mifepristone in HLM. (**b**) Observed inactivation rates at different mifepristone concentrations

40.3.4 K_I/K_{inact} Determination

K_I and k_{inact} determination are principally done according to Lim et al. using additional preincubation times (e.g., 5, 10, 15, 30 min depending on the inhibitor). Kinetic parameters (k_{inact}, K_I) are obtained by plotting the natural logarithm of the residual enzyme activity (Ln A/A0) against the preincubation time (Fig. 40.5a and b). The inactivation rate K_{obs} is determined as the negative slopes of the lines reflecting the inactivation process. Apparent K_I and k_{inact} values are determined using Kitz-Wilson plot and by nonlinear regression analysis of

$$-K_{obs} = k_{inact} * [I]/K_I + \{[I]$$

where K_{obs} (min^{-1}) is the inactivation rats for a given test compound concentration,
I in µM is the concentration of the test compound,
k_{inact} (min^{-1}) is the maximal rate of enzyme inactivation, and
K_I (µM) is the concentration required to achieve the half-maximal rate of the enzyme inactivation.

EVALUATION

The assays are usually performed in parallel to solvent control, a well-known mechanism-based inhibitor as positive and a competitive inhibitor as negative control of the respective P450 isoenzyme. To increase throughput, samples are either pooled or analyzed separately with ultrafast LC-MS/MS techniques. Assay variability and the analytical accuracy must be small enough to identify IC_{50} shifts produced by low-potency TDI substances.

CRITICAL ASSESSMENT OF THE METHODS

In terms of MBI, discrepancies between human recombinant CYPs and HLM have been observed internally and are reported by various authors (e.g., Palamanda et al. 2005; Polasek and Miners 2007). A different CYP: oxidoreductase molar ratio of approximately 1:1 in *E. coli* expression system versus 1:10 or 1:20 in HLM might be one reason for these differences as reactive intermediates are likely to be generated more efficiently in recombinant CYPs because of higher rates of electron transfer by the advantageous CYP: oxidoreductase molar ratio (Polasek and Miners 2007). Additionally, competition between multiple CYPs for the oxidoreductase in human liver microsomes could lead to comparably less catalytic turnover to reactive intermediates. This may clarify why the differences in MBI occurs prevalently with drugs that form alkylamine MICs (Benoussan et al. 1995; Polasek and Miners 2007). N-terminal modifications are frequently incorporated into wild-type cDNAs to increase expression levels of human CYP in *E. coli* (Boye et al. 2004). Alteration in this region may affect membrane anchoring but can also influence the aggregation

characteristics of CYP with the oxidoreductase and other CYP enzymes (Backes 2003; Polasek and Miners 2007).

Dilution factors in the IC_{50} shift assay needs to be selected carefully: IC_{50} values for direct-acting inhibitors vary with the dilution factor unless they are based on the final (postdilution) inhibitor concentration, whereas the IC_{50} values for mechanism-based inactivators vary with the dilution factor unless they are based on the initial (prediluted) concentration (Parkinson et al. 2011). Preincubation times and the numbers of preincubation times in case if K_I/k_{inact} determination have to be chosen properly – potent TDI usually requires short incubation times, whereas for less effective TDIs, longer preincubation times are necessary to get sufficient data on enzyme inactivation. Metabolites, which are more potent inhibitors than the parent substances, appear like TDIs in the IC_{50} shift assay. For the prediction of clinical drug interaction studies, kinetic data are usually corrected for nonspecific binding.

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In-Silico ADME Modeling

41

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41.1 Introduction

The pressure on research efficiency and cost in the pharmaceutical industry has resulted in a paradigm shift to bring active molecules earlier to the market (Wess 2002; Lawrence 2002). Increasing expenses by attrition in late stage development are partially attributed to an inadequate understanding of pharmacokinetic and toxicological behavior of drugs (Prentis et al. 1988;? Kennedy 1997; Drews 2000). The conversion of biologically active molecules into effective and safe pharmaceuticals adds substantial value to the drug discovery process. Consequently, the improvement of a compound profile toward a clinical candidate is one of the essential skills in integrated drug discovery teams. Those candidate requirements include multiple parameters including potency and efficacy, selectivity against related proteins or "antitargets," favorable physicochemical and pharmacokinetic properties leading to the required bioavailability after oral administration, and an acceptable half-life of elimination of the final candidate. A simultaneous optimization of multiple parameters in carefully planned iterations is therefore required to arrive at molecules with suitable properties and profiles.

The requirement for shortening pharmaceutical discovery caused the integration of pharmacokinetic and developmental efforts into earlier research to focus on molecules with increased optimization potential. It is mandatory to initiate time-consuming optimization programs only for those series which show a potential to be convertible into drug molecules.

Within the discovery process, in silico approaches to identify or improve chemical structures are utilized in the lead generation and optimization phase. First, lead

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series are identified with activity and selectivity in relevant assays (Wess et al. 2001; Bleicher et al. 2003). In this phase, mainly global in silico models are applied. The following lead optimization phase is the most challenging part of drug research (Wess et al. 2001; Wess 2002). Its ultimate object is to turn an efficacious molecule into an effective and safe drug. This is accomplished by structural variations to improve the molecule such that its profile fulfills defined criteria for its therapeutic application. During this phase, the ADME (absorption, distribution, metabolism, excretion), as well as the toxicity properties of the drug candidate, is being optimized. These activities are mainly supported by local in silico models. Although the clinical development is also supported by in silico tools to describe the pharmacokinetic behavior of the drug candidate, a description of these tools is beyond the scope of this chapter.

The introduction of several new technologies like protein crystallography, ADME assays, medicinal chemistry automation, and others has added not only new opportunities but also more complexity to the lead optimization phase. Consequently, it is vital for success to conceive lead optimization as simultaneous multidimensional optimization of compound properties rather than addressing one parameter at a time. Traditionally, binding affinity is first optimized, while ADME parameters are addressed later in this process (Oprea 2002; Zamora et al. 2003a). This approach, however, showed only limited success as optimizing for affinity only can result in chemical classes, where further improvement becomes impossible. The efficiency of the drug discovery process is expected to improve if both aspects are considered simultaneously. Besides high biological activity in vitro, it is of great importance to simultaneously ensure sufficiently high drug concentrations at the target site in vivo by seeking for favorable ADME properties. To this end, various in vitro assays have been introduced to monitor compounds as early as possible for their ADME characteristics. However, all these techniques require the synthesis of the compound to be tested. To save time and chemical resources, it is desirable to introduce in silico tools into the drug development process, allowing the reliable assessment of compound properties prior to synthesis and subsequently the drawing up of a priority list of the most promising compounds to be synthesized. Thus, facing the requirements of modern drug development, rational approaches to alter molecules being originally directed by quantitative structure-activity relationship (QSAR) and structure-based design become more and more a tight interplay between multiple disciplines: medicinal chemistry, structural biology, pharmacology, and pharmacokinetics.

This chapter discusses selected computational methods and strategies toward the generation and application of in silico models in drug discovery. In silico models can be classified in general and local, chemotype-specific models. This grouping broadly reflects the range and diversity of chemotypes incorporated in the training set for establishing the model. The underlying assumption is that general models might find wider applicability to novel structures for prediction, while local models are focused toward a particular class. Thus, general models might be of interest in earlier phases of a discovery project (library design, hit exploration) without much experimental ADME data for a particular chemotype. Local models on the other hand are based on previous knowledge for one series and are more useful in the lead optimization phase. Frequently, experimental data for one particular ADME property is then available to build a model. Then, this information can be turned into a more focused model with, of course, less wide applicability, but the resulting model certainly will help more to direct further synthesis efforts.

It is beyond the scope of this chapter to provide an exhaustive list of software tools, descriptors, models, and approaches to address multiple ADME issues. We rather discuss a selected list of approaches which have shown to be valuable in several drug discovery projects. The entire field, the methods, and descriptors are changing rapidly, and the interested reader thus is referred to recent review articles for this purpose (Clark and Pickett 2000; Clark 2001; Matter et al. 2001; van de Waterbeemd and Gifford 2003; Dearden 2007; Hou and Wang 2008; Vaz et al. 2010; Tarcsay and Keserü 2011; Gleeson et al. 2011a, b).

Any approach to correlate chemical structures to experimental data requires first an appropriate description of structures by molecular descriptors and second a robust statistical technique unveiling those structural features correlated with the ADME property. These quantitative structure-activity relationships (QSARs) result in models quantifying the influence of various descriptors on estimating the activity. However, it is of utmost importance to realize that statistical approaches can only highlight correlations between descriptors

and activity, but cannot prove any causality. Hence, in silico models require careful validation to avoid overfitting and erroneous conclusions.

The presented computational approaches can be grouped based on their description of molecular structures: (a) simple physicochemical or topological descriptors like PSA (polar surface area); (b) alignment-free descriptors capturing global information from 3D molecular representations like VolSurf and QikProp descriptors; (c) combinations of physicochemical, structural, and topological descriptors from 2D molecular representation like MOE or DRAGON descriptors; and (d) 3D descriptors, which depend on the alignment of molecules to obtain spatially resolved information on regions affecting the interesting property, like CoMFA and GRID descriptors. This range of different descriptors is analyzed using a variety of statistical methods to establish predictive models, for example, using linear regression models like PLS, nonlinear regression models, decision trees, and regression trees. In contrast to these QSAR-based approaches, a method named MetaSite (Cruciani et al. 2005; Vaz et al. 2010) is described for in silico prediction of the site of metabolism (SOM) in a ligand by distinct cytochrome P450 enzymes. This approach does not depend on a training set of molecules. In contrast, this method derives its prediction from a combination of the lability of ligand hydrogen atoms plus orientation effects of this ligand in the CYP450-binding site, which is estimated by available 3D structures of a CYP450 enzyme from X-ray crystallography or homology modeling.

In the following sections, this overview provides examples for both global and local ADME models to highlight different approaches. Datasets for intestinal human absorption, human serum albumin binding, plasma protein binding, and CYP2C9 inhibition will be discussed in detail, while predictive models for other important ADME properties have also been developed. The prediction of the site of metabolism will be discussed with some examples. In addition, the development and application of a local QSAR model addressing intestinal absorption from an in vivo perspective for a congeneric series will also be outlined.

It has to be pointed out that prediction failures of general ADME models are often related to two major sources, namely, the quality of experimental data used to derive the model and the interpretation of the final model. These problems are discussed in depth by Stouch et al. (2003). Some models fail as they were built from data collected from different sources and laboratories. Although this might work for some robust assays, it could produce incomparable data for others. Such problems have been reported, for example, for Caco-2 assays run in different laboratories. Even if experimental data are collected in a single laboratory or under strictly harmonized conditions, as nowadays routinely performed within the pharmaceutical industry, another potential source of errors can occur if such a model is transferred to another place where a slightly different assay protocol is utilized to monitor the predictivity of the model. Furthermore, it is ambitious to derive a truly general model for a wide range of chemotypes. Often the limited coverage of training set chemical space plus the presence of novel compound classes to be predicted in research projects which are very dissimilar to any training molecule might also significantly limit the scope of a particular model. Finally, a classification model should not be used to rank order compounds but only to filter undesirable compounds. Any other application would lead to an overinterpretation of the model's precision. Furthermore, it has to be emphasized here that adding more compounds with additional experimental data to an existing general model does not guarantee that its predictivity will increase. This is because the addition of new compounds might introduce new modes of interaction and/or new reaction mechanisms.

Although the ADME space is of lower dimensionality that means there are less descriptors building the structure-property model, ADME properties in practice are more difficult to predict than biological receptor affinities, as corresponding experimental screens are often multimechanism rather than single-mechanism systems (Lipinski 2000). In contrast, biological assays for the majority of pharmacological targets are typical single-mechanism systems for which computational models to correlate structural descriptors are easier to develop and resulting predictions tend to be more robust. Computational models based on experimental data combining multiple underlying biological mechanisms tend to get worse if more data for more diverse molecules are integrated into the training set. This is mainly due to the fact that the increase of assay data relates to an increase of underlying mechanisms, on which those data have been obtained and the noise level rises for each individual mechanistic component (Lipinski 2000). For smaller,

structurally, and thus probably mechanistically homogeneous datasets, acceptable correlations are obtained, while the ability of descriptors to capture a more diverse experimental dataset is limited. Although the same descriptors might still have statistical significance and thus explain trends for inhomogeneous data, their predictivity is often too low to be useful for lead optimization purposes. This only allows implementing validated filters based on property distributions. Hence, it is mandatory to use high-quality single-mechanism ADME experimental data for building single-mechanism predictive models (Lipinski 2000). Alternatively, the assumption might be valid that congeneric series behave similar in multiple mechanism assays so that a local model for this series is primarily capturing the main trend of this series.

To further illustrate this point, one should be aware that poor intestinal absorption of a lead structure, for example, can be attributed to either insufficient physicochemical properties or poor membrane permeation but also to the net result of efflux mediated by transporter proteins including the multidrug resistance protein MDR1 (or P-glycoprotein, P-gp) or multidrug resistance-related proteins (MRPs) in the intestinal membrane. Cell lines with only one single efflux transporter are currently engineered for in vitro permeability assays to get suitable data for reliable QSAR models. In addition, successful efforts to gain deeper insight into P-gp and other ATP-binding cassette (ABC) transporters on a structural basis have been reported (Litman et al. 2001; Rosenberg et al. 2003; Demel et al. 2008; Broccatelli et al. 2011). These examples further illustrate the complexity when dealing with different assays and molecules.

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41.2 Computational Approaches

41.2.1 Polar Surface Area (PSA)

PURPOSE AND RATIONALE

As for most drugs, the preferred route of administration is *per os*; many efforts have been made to identify physicochemical properties that favor intestinal absorption as single component to oral bioavailability. While for many ADME properties only qualitative in silico assessment can be realistically expected, more quantitative rankings are possible with regard to passive drug permeability across the intestinal epithelium and the endothelium of the blood-brain barrier (Pickett et al. 2000; Artursson and Bergström 2003). Among the strategies to predict intestinal absorption in silico, the use of molecular surface areas was among the first ones and is still employed due to speed and simplicity. Molecular surface areas have been used 40 years in the modeling of solvation and partitioning processes (Hermann 1972; Pearlman 1980). More recently, ADME properties were predicted using a term derived from the molecular surface known as the polar surface area (PSA). Some reviews highlight the importance of this simple descriptor with physicochemical relevance (Artursson and Bergström 2003) to understand passive transport problems across membranes.

PROCEDURE

The PSA of a molecule is defined as van der Waals surface from all oxygen and nitrogen atoms including attached hydrogen atoms; it relates to the capacity to form hydrogen bonds as essential determinant for passive intestinal absorption.

There are different methods to calculate PSA values. The dynamic polar surface area PSA_d is obtained from a Boltzmann-weighted average from low-energy conformers (Palm et al. 1996; 1997; van de Waterbeemd et al. 1996; Krarup et al. 1998). To this end, all low-energy conformations from a conformational analysis with a certain energy cutoff are taken into account. The probability of a molecule to adopt a certain conformation is calculated from a normalized Boltzmann distribution (Palm et al. 1996; Lipkowitz et al. 1989). The energy cutoff value of 2.5 kcal/mol ensures that all conformations that contribute more than 1% to the conformational space are considered.

The polar surface area of a single conformer PSA_s is calculated from the global minimum conformation of a molecule (van de Waterbeemd et al. 1996; Clark 1999a). Alternatively, the 3D structure of the molecule could be generated by 2D/3D converters like CONCORD (Pearlman 1987; Balducci et al. 1699), or CORINA (Sadowski et al. 1992, 1994a), followed by energy minimization. Finally, the PSA of the minimized conformation is calculated, for example, by MOLVOL (Dodd and Theodorou 1991) or other approaches.

While these descriptors above rely on the 3D molecular structure, the topological polar surface area (TPSA) is calculated by simply summing up tabulated surface contributions of polar fragments rapidly obtained from SMILES representations (Daylight Chemical Information Systems Inc) of 2D chemical structures (Ertl et al. 2000). For that purpose, a set of fragments comprising 43 polar atom types was defined. The contributions of these atom types were determined by least-squares fitting to the single conformer 3D PSA (PSA_s) of a large subset of drug-like structures from the World Drug Index (World Drug Index Database). From this database, all molecules with apparent valence errors, molecular weights outside the interval of 100–800, and molecules not having at least one oxygen, nitrogen, sulfur, or phosphorus atom were removed. The remaining set used for the least-squares fitting consisted of 34,810 reasonably drug-like molecules. A comparison of PSA_s and PSA_d of β-adrenoreceptor blocking drugs revealed that correlations between surface properties of the global minimum conformations and permeability were only slightly poorer than correlations obtained using the dynamic properties (Palm et al. 1996). Furthermore, the efficient 2D-TPSA showed a very good correlation with PSA_s ($r = 0.99$) and PSA_d ($r = 0.98$). Finally, the TPSA method has proven its applicability to predict not only intestinal absorption but also blood-brain barrier penetration and Caco-2 cell permeability (Ertl et al. 2000), providing results of the same quality as with PSA_s and PSA_d data.

EVALUATION

Two of the major physicochemical determinants of passive absorption are lipophilicity and hydrogen bonding potential. PSA is a good predictor for hydrogen bonding potential (Palm et al. 1997) while it is not directly related to lipophilicity, as shown by Clark (1999a) considering a series of aliphatic alcohols. In this series, PSA is constant, while the lipophilicity increases with increasing carbon chain length. Thus, one could expect that a combination of lipophilicity data and PSA data should result in an even better predictor to guide drug design toward orally available compounds. In fact, modern approaches to derive in silico ADME models often include informative combinations of multiple descriptors, while individual descriptors capturing hydrogen bonding and lipophilicity are often among the very informative ones to derive an individual structure-activity relationship.

CRITICAL ASSESSMENT OF THE METHOD

Using small datasets, it was initially shown that the dynamic PSA is strongly correlated with the membrane permeability of a series of β-adrenoreceptor blocking agents (Palm et al. 1996). Subsequently, it was found that PSA_d is able to predict the absorption after oral administration to humans of 20 structurally diverse drug molecules (Palm et al. 1997). The resulting sigmoidal correlation between fractional dose absorption and PSA_s for 20 drug molecules is given in Fig. 41.1. From these results, it was concluded that drugs with complete oral absorption should have a PSA_d value of <60 Å^2, while >140 Å^2 is seen as threshold for compounds with low absorption. For molecules with a higher PSA value, a poor absorption is very likely if no active transport mechanisms are involved. However, low PSA values are necessary, but not a sufficient criterion for good absorption. For example, highly hydrophobic compounds may have

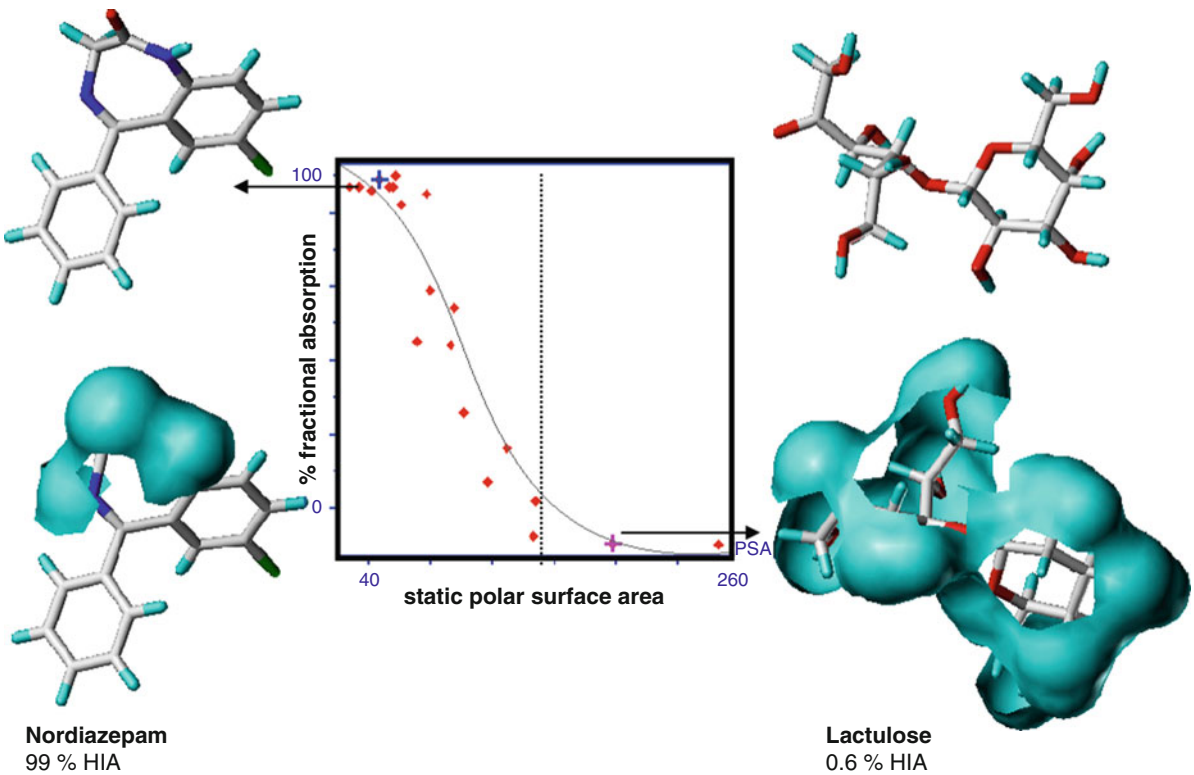

Fig. 41.1 Sigmoidal correlation between human fractional dose absorption and PSA_d for 20 drugs (Data from Palm et al. 1997). The influence of the polar surface area on fractional absorption is illustrated by comparing PSA regions for the orally available nordiazepam (*left*) to the nonavailable lactulose (*right*)

a low PSA value, but due to their low solubility, their intestinal absorption is low. Therefore, it is important not to use PSA naively as a guide to absorption but to couple it to other information and existing knowledge.

These findings were confirmed by Kelder et al. (1999) calculating the PSA values for approximately 2,000 orally available drug-like molecules that have reached at least clinical phase II. They concluded that orally active drugs that are transported passively by the transcellular route should not display a PSA > 120 Å^2. In addition, oral drugs tailored to brain penetration were shown to have PSA values of <70 Å^2. A very similar finding was reported by Clark (1999b), who derived a simple quantitative structure-activity relationship (QSAR) for brain penetration from a combination of logP and PSA. The resulting interpretation that smaller PSA values are more favorable for brain penetration is reasonable as it is known that the endothelial cell monolayer of the BBB forms a much tighter barrier than the intestinal epithelial cell barrier (Artursson and Karlsson 1991; Pardridge 1996).

MODIFICATIONS OF THE METHOD

It has been estimated that the scatter in the correlation between PSA and transcellular membrane permeability is likely to increase with more chemotypes from different therapeutic classes to be incorporated. Hence, to improve predictivity, PSA was combined with other predictive molecular descriptors such as logP (Clark 1999b; Egan et al. 2000a) and number of rotatable bonds (Veber et al. 2002). However, the effect in particular of rotatable bonds was discussed controversially in the medicinal chemistry literature (Lu et al. 2004).

In addition to the PSA, a nonpolar surface area (NPSA) can be computed by taking the total surface area minus the PSA into account. A linear combination of PSA and NPSA was reported to give satisfactory models for analogue series like dipeptides and endothelin receptor antagonists, where PSA alone was not a satisfactory predictor of passive membrane permeability (Stenberg et al. 1999a, b). Collectively, these results suggested that a more general model of passive membrane permeability should incorporate different molecular descriptors capturing information about polarity and lipophilicity.

This led to the concept of fragmentation of the total molecular surface area in combination with multivariate analysis (Stenberg et al. 2001) toward predictive models of drug permeability for more complex datasets. Permeability models were established based on so-called "partitioned total surface area (PTSA)" descriptors. Each of the PTSA descriptors corresponds to the surface of a certain atom type, differentiated by hybridization, which results in individual descriptors, for example, sp^3, sp^2, and sp carbon atoms. The resulting permeability model based on 19 descriptors finally consisted of oxygen, nitrogen, and polar hydrogen surfaces, while the main contribution for prediction of Caco-2 permeability was attributed to PSA. In addition, some more lipophilic contributions were seen as relevant to obtain a significant PLS model.

This work was expanded toward a classification of drug molecules into the established biopharmaceutical classification system (BCS) on the basis of PTSA descriptors alone, which included separate models for solubility and permeability (Bergström et al. 2003). The PLS models reported for solubility and permeability resulted in a correct biopharmaceutical classification for as many as 85% of the studied compounds, while an external FDA standard drug test set was correctly classified with 77% accuracy.

While models to understand a single chemotype were reported to include only a few informative descriptors from this surface area family for larger and more diverse datasets, multivariate analysis of multiple descriptors might be more useful.

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41.2.2 Alignment-Free 3D Descriptors (VolSurf)

PURPOSE AND RATIONALE

As described above, molecular surface areas were successful to predict important ADME properties like passive membrane permeability and intestinal absorption. This concept was taken further toward the development of a set of descriptors capturing a wide range of physicochemical relevant properties extracted from molecular interaction fields of three-dimensional (3D) structures. To this end, Cruciani et al. developed a tool called VolSurf (Cruciani et al. 2000a, b, 2003), integrating enhanced molecular descriptors with multivariate statistical tools for the analysis of physicochemical and pharmacokinetic data. VolSurf transforms the information present in 3D molecular interaction fields into a limited set of descriptors. Those have been shown to carry relevant information related to ADME properties, like polarity, hydrogen bonding, lipophilicity, size, polarizability, and others. As these descriptors are easy to calculate, to understand, and to interpret from a chemistry point of view, they provide further design guidelines for chemical optimization after a linear model has been established.

PROCEDURE

The interaction of drug molecules with biological membranes is a three-dimensional recognition that is

mediated by surface properties. Information about surface properties of drug molecules and other solutes can be extracted from 3D molecular interaction fields. In VolSurf, the information from calculated molecular interaction fields is extracted and compressed in a few informative descriptors, as outlined in Fig. 41.2. Those descriptors capture various aspects of physicochemical molecular properties and thus can be successfully correlated to pharmacokinetic properties. To this end, the 3D grid maps of interaction energies between drug molecules with particular probe atoms are calculated using the GRID force field (Goodford 1985). This advanced force field is able to calculate energetically favorable interaction sites for a variety of chemically relevant probe atoms and functional groups around a molecule in a given 3D conformation. As the information contained in 3D molecular fields is related to the interacting molecular partners, the amount of information captured in those interaction fields is generally superior to global descriptors or those computed from only the 2D chemical structure.

In most cases, the GRID water, the hydrophobic (DRY), and the carbonyl oxygen (O) probes have been utilized for computing molecular interaction fields which are then analyzed using VolSurf to extract meaningful descriptors. GRID uses a potential based on the total energy of interactions (Lennard-Jones, H-binding, and electrostatic terms) between a target molecule and a probe and can be used to characterize putative polar and hydrophobic interaction sites around target molecules. The water probe simulates solvation/desolvation processes, while the DRY and the O probes encounter drug-membrane interactions. This treatment of hydrophobic interactions is done at each grid point by a combination of the following terms: $E_{Entropy} + E_{Lennard\text{-}Jones} - E_{H\text{-}bonding}$. $E_{Entropy}$ is

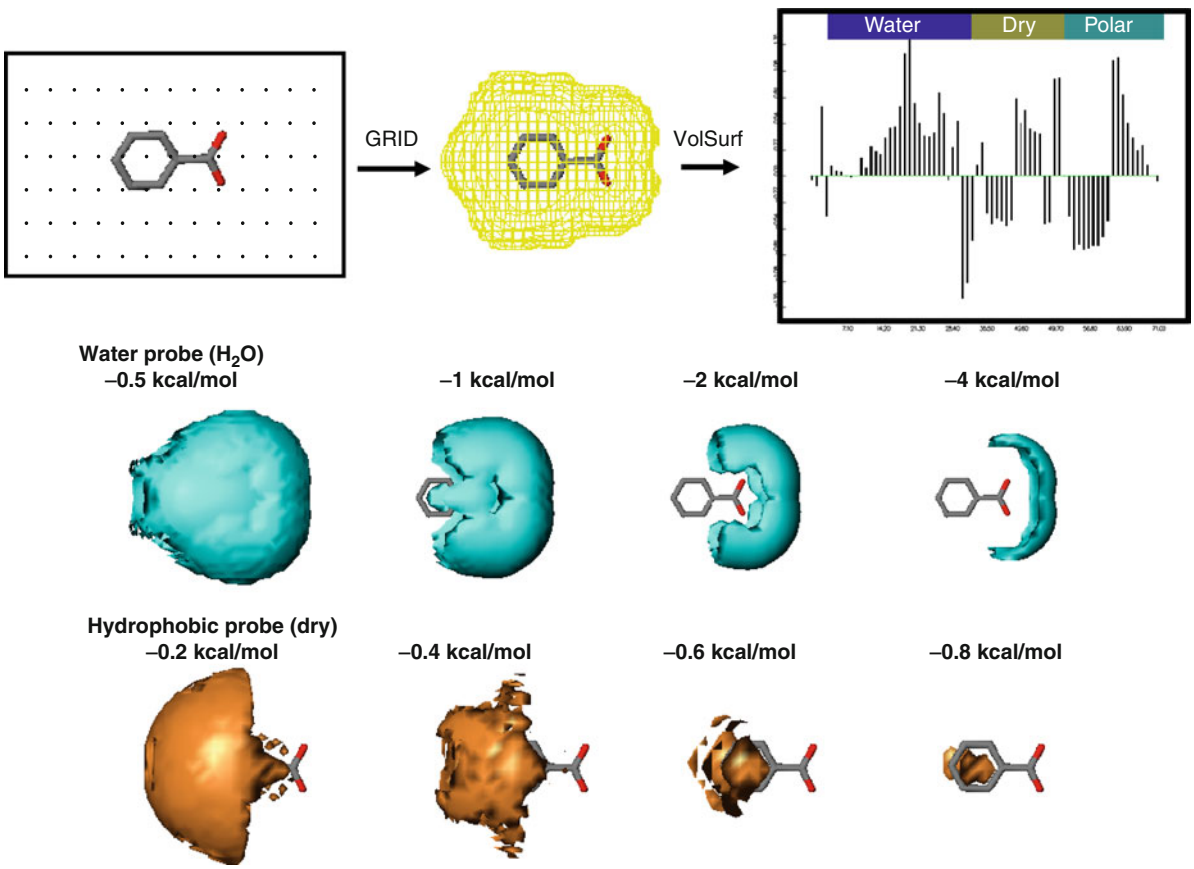

Fig. 41.2 Computation of VolSurf descriptors (Cruciani et al. 2000a) derived from GRID molecular interaction fields. For any molecule, interactions with GRID water and dry probes at different energy levels are used for contouring. Those levels serve to compute vectors of 72 volume, size, and surface-related descriptors

the ideal entropic component of the hydrophobic effect in aqueous environment, $E_{Lennard\text{-}Jones}$ accounts for induction and dispersion interaction occurring between any pair of molecules, and $E_{H\text{-}bonding}$ subtracts the effect of hydrogen-bonding contributions between water and polar parts of the solute surface.

Each drug molecule is characterized by its potential hydrogen bonding, polar and hydrophobic interactions, starting from its three-dimensional structure, but without the necessity to bring different molecules into an alignment by atom-based or field-based superposition methods.

Subsequently, VolSurf extracts information present in these 3D molecular fields and transforms it into a limited number of descriptors, as shown in Fig. 41.2. These descriptors capture relevant molecular properties which are related to ADME and pharmacokinetic properties, like polarity, hydrogen bonding, lipophilicity, size, shape, polarizability, and others. In particular, the size and shape of hydrophilic and hydrophobic molecular regions at different interaction energy levels and the balance between these regions are extracted by VolSurf. Other descriptors for quantitative ADME models are the amphiphilic moments and critical packing parameters. A detailed account of these descriptors and the approach to extract shape and surface information are given by Cruciani et al. (2000a).

The originality of VolSurf resides in the fact that surfaces, volumes, and other descriptors can be obtained from 3D molecular fields without complex algorithms of trigonometric projections, recursive generations, and tessellations. In total, the analysis of molecular recognition is achieved by image analysis approaches, while the image compression step involves chemical knowledge by selecting an appropriate parameterization according to the 3D map of interest. The resulting set of descriptors thus has a clear chemical meaning.

EVALUATION

The descriptors generated by VolSurf have been successfully correlated using multivariate statistical techniques like partial least-squares projection to latent structures (PLS) in literature and in internal studies with bioavailability, blood-brain partitioning, membrane transport, and other relevant ADME and pharmacokinetic properties (Alifrangis et al. 2000; Crivori et al. 2000; Cruciani et al. 2000c, 2003; Guba and Cruciani 2000). One earlier example is the study by Guba and Cruciani to unravel essential molecular features correlated to human intestinal absorption of 20 drug molecules (Guba and Cruciani 2000). The correlation of VolSurf descriptors for 20 diverse drugs to this pharmacokinetic parameter is shown in Fig. 41.3. The interpretation of PLS models for factors influencing permeability and absorption is in agreement with earlier, qualitative findings based on global molecular properties represented by descriptors like logP and others. However, due to the nature of the descriptors, this approach allows to better understand physicochemical requirements for a pharmacokinetic effect and to use it for design, for example, the balance between lipophilic and hydrophilic parts in combination with size, volume, and other effects. A special emphasis in the analysis of VolSurf models can be placed on the interpretation after multivariate statistics because any rational design to improve molecular properties depends on the understanding of how molecular features influence physicochemical and ADME properties.

The successful use of VolSurf was also reported for predicting absorption properties (Cruciani et al. 2000c) from drug permeability data of 55 molecule compounds (Irvine et al. 1999) in Caco-2 cells (human intestinal epithelial cell line derived from a colorectal carcinoma) and MDCK (Madin-Darby canine kidney) cell monolayers. In this interesting case, it was shown that models including counterions for charged molecules clearly show significantly better statistical quality and overall performance. The final model was also able to correctly predict to a great extent the relative ranking of molecules from another Caco-2 permeability study by Yazdanian et al. (1998).

This model recently has been extended (Cruciani et al. 2003) to a dataset of 450 carefully selected Caco-2 data from the literature, excluding compounds showing high efflux rates which might indicate active transport by P-glycoprotein (P-gp) through the intestinal membrane. This attempt was done to compile a dataset of molecules having a similar mechanism of transport. However, as a quantitative comparison of Caco-2 data from different laboratories is nearly impossible due to experimental uncertainties and variations of conditions, the molecules were classified in low and high permeable compounds. The use of partial least-squares discriminant analysis (DA-PLS) resulted in a statistically significant model after crossvalidation which allows discriminating in the 2D PLS score plot between compounds with low and high permeability

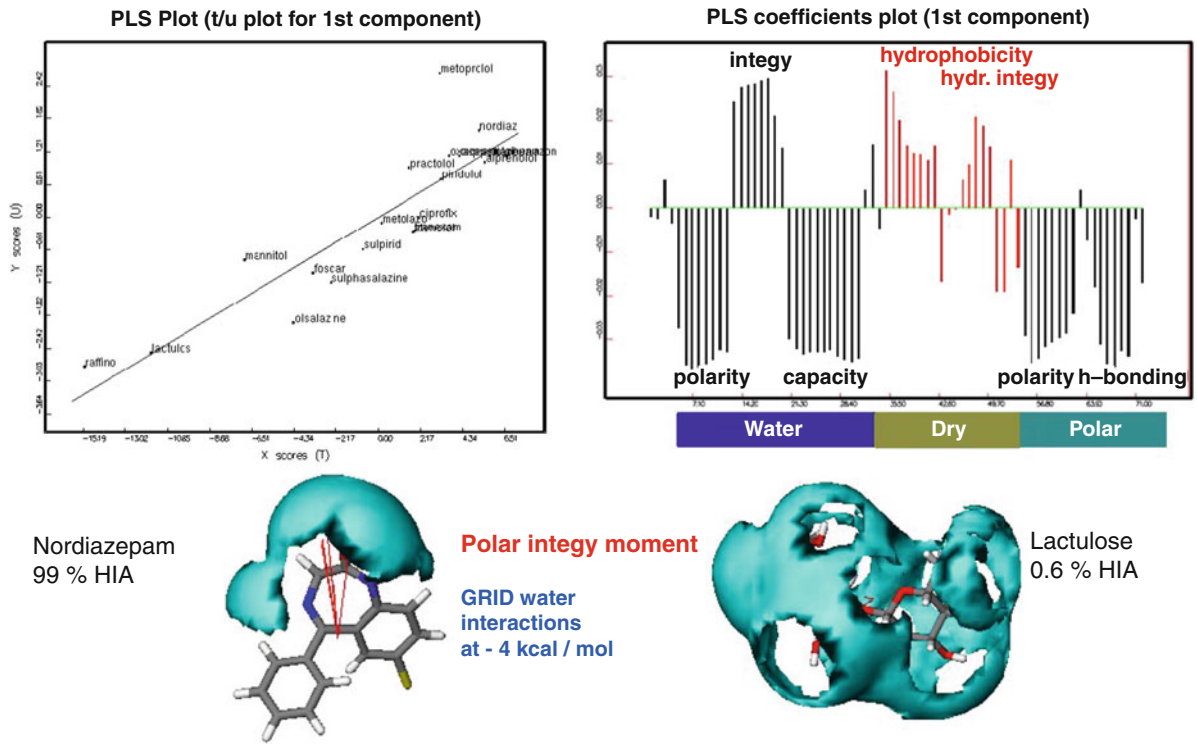

Fig. 41.3 Correlation of VolSurf descriptors with human intestinal absorption using multivariate statistics (PLS) based on 20 drug molecules as reported by Guba and Cruciani (2000). The PLS plot (u1 versus t1) and the corresponding PLS coefficient plot are shown. Different interaction patterns with the GRID water probe are displayed for the orally available nordiazepam (*left*) versus the large area for the nonavailable lactulose (*right*)

while compounds at the interface of both classes could not be predicted with high certainty. Classification models like this are valuable for earlier phases of the lead optimization process, as external compounds can be projected in the chemical space represented by such a model in order to classify Caco-2 permeability potential for novel, untested molecules. Of course, the prediction accuracy of classification models is lower, and results are only meant to be used in a qualitative sense for design and decision making.

The suitability of VolSurf to describe the interaction of compounds with membranes was reported by Alifrangis et al. (2000). They established a structure-property model for membrane partitioning for 20 peptides, with experimental data tested in two chromatographic systems with phospholipids as stationary phase, immobilized artificial membranes (IAMs), and immobilized membrane chromatography (ILC). The relationship between these measures and the sets of calculated descriptors derived from molecular surface area, MolSurf, and VolSurf were analyzed using PLS, showing that the VolSurf-derived model was significantly better than both other models. Especially the VolSurf critical packing descriptor, developed to describe the interaction of amphiphilic molecules with membranes, was found to be important to explain the peptide membrane partitioning ability.

In addition to the above mentioned properties of VolSurf, no alignment of the compounds is necessary prior to the calculation of the molecular interaction fields and the following generation of the VolSurf descriptors. Furthermore, this approach is often hardly influenced by conformational sampling and averaging (Mannhold et al. 1999; Crivori et al. 2000; Cruciani et al. 2000a; Guba and Cruciani 2000). This is probably due to the peculiarity of the GRID force field which allows for the conformational flexibility of external groups, hydrogen atoms, and electron lone pairs. In general, the automated protocol consisting in a simple 2D-to-3D structure conversion followed by energy minimization produces good results, without the need of molecular dynamic sampling or Boltzmann

averaging. This makes VolSurf descriptors computationally efficient and well suited for fast quantitative structure-property relationship studies, especially when dealing with a large number of compounds.

VolSurf descriptors were also successfully used to build a classification model for predicting blood-brain barrier (BBB) permeability for drug-like molecules (Cruciani et al. 2000c; Crivori et al. 2000). Based on 72 descriptors, a discriminant PLS analysis was performed to build a model for qualitative experimental BBB permeation data for 110 molecules. Another 120 molecules served for model validation by external prediction. For racemic compounds, all stereoisomers were used to generate descriptors based on the reasonable assumption of negligible stereoselectivity in passive membrane permeation. Based on both datasets and combined discriminant PLS, a new model with 229 molecules was derived. It correctly predicts 90% of the BBB data, and the interpretation of the PLS coefficients provides insights to drug design, pharmacological profiling, and screening. Descriptors encoding polarity such as the hydrophilic water-accessible surface regions, capacity factors (volume/total surface at distinct energy levels, that is, number of polar interactions per surface unit), and hydrogen bonding are inversely correlated with BBB permeability. Hence, BBB permeability decreases with increasing polar surface in agreement with the PSA model discussed above. Some more quantitative information can be extracted, such as the influence of charge distribution and electron lone pairs. The influence of capacity factors means that although diffuse polar regions are tolerable, dense and localized polar regions are detrimental for BBB permeation. All descriptors encoding hydrophobic interactions show less influence but are directly correlated with BBB permeation. While size and shape of the molecules have no influence, the critical packing and hydrophilic-lipophilic balance are important showing that a balance of nearly all descriptors is relevant for BB permeation. Utilizing all molecules from another dataset (Luco 1999) for external prediction, the VolSurf model was able to correctly predict the BBB permeability range for more than 75% of the compounds (Cruciani et al. 2000c), which is encouraging, as BBB permeation is dependent not only on passive diffusion but also on active transport and metabolism.

Other important applications include the generation of a model to predict thermodynamic water solubility (Cruciani et al. 2003). This model is based on consistent solubility data from literature plus additional measurements for 970 compounds. Its quality allows to differentiate between very poorly, poorly, medium, highly, and very highly soluble molecules while exact rankings within individual classes are not possible. However, given the different factors influencing experimental thermodynamic solubility data, it is not likely that significantly improved models for this key property in pharmaceutical sciences can be derived.

VolSurf was also used in a study to obtain information about metabolic stability in human liver preparations (Cruciani et al. 2003). A training set of 500 compounds with data from metabolic stability testing of the compounds in human CYP3A4 cDNA-expressed microsomal preparations was used to build a model using principal component analysis (PCA). Although this statistical approach did not incorporate any experimental information about compound stability, a grouping of metabolically instable molecules in one focused region in the final model was observed upon inspection of the PCA score plots. This model has also been improved (Crivori et al. 2004) by adding other GRID-derived descriptors in addition to VolSurf. These descriptors named GRid-INdependent Descriptors (GRINDs, Pastor et al. 2000) are implemented in the program Almond (see below). On an extended training set of 1,800 compounds tested in one laboratory at a pharmaceutical company, a classification model using discriminant PLS was established and validated using different test sets. This extended model correctly predicted 75% internal compounds and 85% public drugs and revealed an overall precision of 86% correct selection of metabolically stable compounds.

CRITICAL ASSESSMENT OF THE METHOD

Many published and internal VolSurf applications underscore the assumption that these 3D molecular descriptors capture relevant information about physicochemical and pharmacokinetic properties of drug molecules. Other studies have shown that the method is not significantly depending on the chosen conformation, as long as they are consistently generated. Often molecular 2D/3D converters like CONCORD (Pearlman 1987) and CORINA (Sadowski et al. 1992, 1994b) followed by energy minimization can be applied. Hence, these descriptors are extending concepts like PSA and logP by capturing similar

information to those in a series of carefully chosen descriptors. In combination with multivariate statistics, the resulting models then allow a more quantitative understanding of factors influencing the physicochemical or ADME property of interest, which in combination with its intrinsic chemical interpretability provide important guidelines for compound optimization.

The suitability of these descriptors for database mining was investigated in a comparative analysis (Cruciani et al. 2002). While 2D descriptors like Unity fingerprints and MACCS keys were shown to outperform VolSurf descriptors and logP in studies related to pharmacodynamics (clustering behavior on chemically homogeneous series with different pharmacological effect), VolSurf shows the most realistic clustering for pharmacokinetic aspects exemplified by solubility and blood-brain partitioning behavior.

MODIFICATIONS OF THE METHOD

While VolSurf has first been developed to model pharmacokinetic properties, it has recently been shown to capture relevant information for protein-ligand interactions (Zamora et al. 2003b). Steric, hydrophobic, and hydrogen-bonding interactions from these descriptors were also useful for describing ligand binding affinities to various proteins. Significant PLS models for diverse set of protein-ligand complexes and a congeneric series of ligands to a single binding site were reported, which would allow for a simultaneous optimization of ligand binding affinity and pharmacokinetic properties using a framework of consistent descriptors.

Additional shape descriptors in an extended VolSurf approach have been applied to model biological activity of a series of quinolones using a quantitative structure-activity relationship (QSAR) approach (Cianchetta et al. 2004). The molecular shape function used to augment the VolSurf approach is constructed from probe-drug interaction energies from the GRID force field. It can be seen as a combination of steric vector descriptors with one extreme in the center of mass of a molecule and with the other located in the molecular surface. These extended VolSurf descriptors now might allow for a simultaneous optimization of affinity and pharmacokinetic properties using multivariate statistical analysis using two or more dependent variables (i.e., biological properties) to model. Any resulting model then might allow identifying favorable descriptor combinations for both properties under investigation and thus enabling a more focused lead optimization by concentration on those regions in chemistry space, which result in acceptable properties simultaneously.

Another modification of the VolSurf approach to analyze molecular interaction fields was described by Pastor et al. (2000). The same information previously been used to compute VolSurf descriptors now served to compute GRIND descriptors. In contrast to VolSurf, these descriptors capture information about pharmacophore distances within individual molecules. These descriptors represent favorable interaction energy regions where groups of a potential protein would interact favorably with the ligand. Hence, this set of descriptors is ideally suited to model ADME properties related to molecular recognition events between a protein and a series of ligands like, for example, inhibition of or substrate binding to cytochrome P450 isoforms (Afzelius et al. 2002a, 2004a), as well as binding to transporters like P-gp (Cianchetta et al. 2005; Crivori et al. 2006), PEPT1 (Larsen et al. 2008), and among others.

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41.2.3 3D-QSAR (Comparative Molecular Field Analysis, CoMFA)

PURPOSE AND RATIONALE

The relationship between chemical properties like solvent partitioning and biological activity was first discovered over a century ago (Meyer 1899), while the field of quantitative structure activity relationship (QSAR) was opened by Hansch and Fujita (1964) with statistical correlations of descriptors like logP, molar refractivity, and shape to activity of congeneric series. Numerous applications of QSAR over the following four decades highlighted the fundamental relation between chemical structure and biological activity, which allows estimating the activity of untested molecules based on a validated "QSAR equation." However, the identification of important global descriptors often does not lead to a design of new compounds, as these descriptors do not capture specific, spatially resolved information about molecular recognition events. The approaches discussed in previous sections are describing properties like passive permeability which are related to fundamental physicochemical behavior. But the understanding of structural requirements for protein-ligand recognition in ADME like binding and inhibition of drug molecules to the family of cytochrome P450 enzymes or specific transporters like P-gp or Pep-T1 requires a more specific description of molecules and their putative modes of interaction with their macromolecular partners. The idea that three-dimensional molecular interaction fields (Goodford 1985) can be combined with multivariate statistics like PLS (Wold et al. 1984; Dunn et al. 1984) led to a popular approach for 3D-QSAR named comparative molecular field analysis (CoMFA) (Cramer et al. 1988a; Clark et al. 1990). Statistical relationships are derived between molecular property fields of aligned compounds and biological activities. Electrostatic and steric interaction energies are computed between each ligand and a probe atom located on

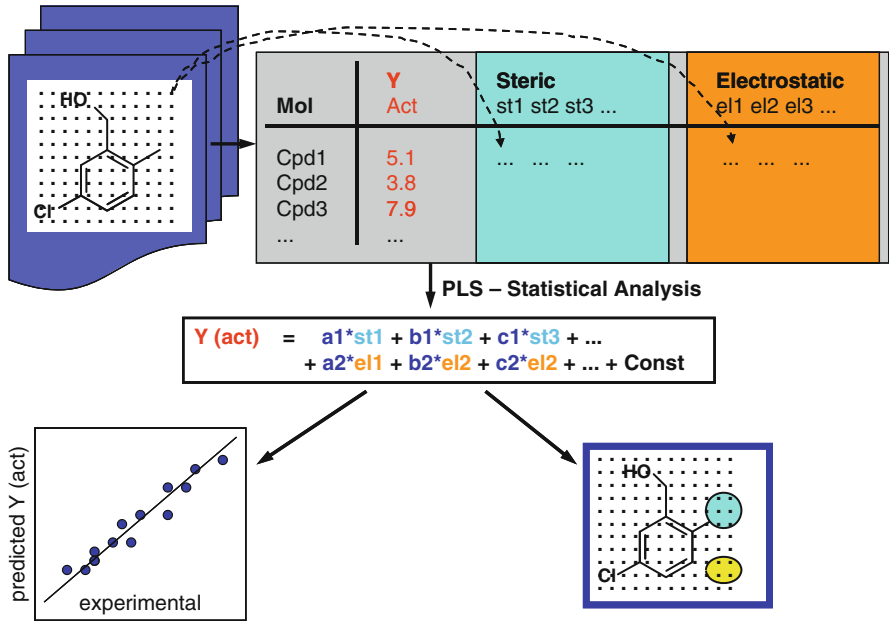

Fig. 41.4 The CoMFA process consists of individual steps (Cramer et al. 1988b). First, interaction energies are computed between all molecules in alignment and a probe atom on a regular grid. This information is stored in a particular format in order to allow for multivariate statistical analysis. The PLS method then produces a statistical model which can be interpreted and used for prediction

predefined grid points for CoMFA, as summarized in Fig. 41.4. These fields have been successfully replaced or augmented by alternative approaches to compute molecular interaction fields, for example, various probes from the GRID force field (Goodford 1985; Baroni et al. 1993), lipophilic fields (Kellogg et al. 1991), or the comparative molecular similarity index analysis (CoMSIA, Klebe et al. 1994a).

PROCEDURE

The first step of a 3D-QSAR study is to superimpose all molecules, which is critical for the success of this investigation. There are multiple approaches toward a reasonable alignment including the use of X-ray crystallography for template molecules (Waller et al. 1993), docking into experimentally determined binding sites or homology models, an automated pharmacophore-based alignment (Martin et al. 1993; Greene et al. 1994; Sprague 1995; Jones et al. 1995), field fit similarity methods (Clark et al. 1990; Klebe and Abraham 1993), flexible molecular alignments (Klebe et al. 1994b; Lemmen et al. 1998), and manual alignment strategies based on conformational searching and chemical intuition. While sometimes a simple alignment onto a common core produces a chemically meaningful model, this only allows interpreting the influence of different substitution properties to biological activity. The real value of 3D-QSAR, however, is that not only models for congeneric series can be established but also that different series could be merged into a single model, given that a reliable alignment of core structures could be obtained. This step is mainly driven by the identification of pharmacophore points, namely, those molecular features that are likely to interact with the protein binding site. The 3D-QSAR statistical models might then allow discriminating between different alignment hypotheses simply on the basis of the comparison of the predictivity of both alternative models. Although the dependency of grid-based 3D-QSAR methods of the alignment is often seen as disadvantage, a careful and stepwise alignment procedure might produce deeper insights than might be extracted from quantitative information about pharmacophore hypotheses (Kellogg and Semus 2003).

After superposition, steric and electrostatic interaction energies between a probe atom and every molecule are calculated at surrounding points of a predefined grid box. The nature of the probe is depending on the property to be mapped. Typically, this is based on a volume-dependent lattice with 1 or 2 Å grid spacing, a positively charged carbon atom, and a distance-dependent dielectric constant. The magnitude of the regions is typically defined to extend the superimposed conformers by 4.0 Å along the principal axes. The maximum field values have to be truncated for mathematical reasons at cutoff values, often 30 kcal/mol for steric and +/− 30 kcal/mol for electrostatic energies. For points "inside" a molecule

(steric energy above the cutoff), no electrostatic energy is computed; those values were set to the mean of the related column. Consequently, these maps can be perceived as 3D profiles, which might correspond to binding site recognition requirements in terms of steric, electrostatic, and hydrophobic complementarity.

In CoMSIA, molecular interaction fields are replaced by fields based on similarity indices between probe atoms and each molecule. The same alignment can be used to compute steric, electrostatic, and hydrophobic similarity index fields for CoMSIA. Hydrophobicity in this context is based on Crippen's partial atomic hydrophobicities (Ghose and Crippen 1986). The advantage of CoMSIA fields is that no singularities occur at atomic positions due to a Gaussian-type distance dependence of the physicochemical properties; thus, no arbitrary cutoffs are required. Similarity indices (Kearsley and Smith 1990) are typically computed using a probe with charge +1, a radius of +1, a hydrophobicity of +1, and an attenuation factor α of 0.3 for the Gaussian-type distance dependence.

Equal weights for different CoMFA or CoMSIA fields were assigned using the CoMFA_STD scaling or block scaling option, while columns, that is, grid points with no or little variations, are often rejected prior to statistical analysis. Then PLS is used as multivariate statistical approach to derive a linear relationship for highly underdetermined matrices, while crossvalidation (Wold 1978) is used to check for consistency and predictivity. A faster approach to crossvalidation has been implemented in SAMPLS (Sheridan et al. 1994).

The resulting std*coeff (standard deviation * PLS coefficient) contour maps from 3D-QSAR models enhance the understanding of electrostatic, hydrophobic, and steric requirements for ligand binding, guiding the design of novel molecules to those regions where variations altering steric or electrostatic fields reveal a significant correlation to biological properties.

EVALUATION

A survey on 3D-QSAR literature (Oprea 2004) reported more than 1,100 entries in the Chemical Abstracts database on CoMFA, 3D-QSAR, and related keywords. As the number of potential targets in drug discovery is steadily increasing, it is likely that 3D-QSAR models will continue to be developed in the future. Successful applications were not only reported to understand target-related affinity, but also for some ADME relevant targets like transporters and cytochrome P450 isoforms. For example, a 3D-QSAR model for peptide substrates of the mammalian H^+/peptide cotransporter PEPT1 (Gebauer et al. 2003) showed a high degree of internal consistency and was able to correctly predict a test set of 19 peptide derivatives. In another example, structural features for the P-glycoprotein (P-gp)-mediated transport of a series of glucocorticoids were recently identified by means of CoMFA and CoMSIA models (Yates et al. 2003). Further applications in the area of ADME modeling include the generation of 3D-QSAR models for cytochrome P450 2C9 inhibitors (Rao et al. 2000; Yasuo et al. 2009), 2D6 inhibitors (Vaz et al. 2005), and 2D6 substrates (Haji-Momenian et al. 2003) to predict potential drug-drug interactions.

CRITICAL ASSESSMENT OF THE METHOD

As for all statistical approaches, there is a need for robust validation to ensure that the resulting models are not misleading due to chance correlation and that these models are predictive for novel molecules. Hence, their successful application depends on the chosen validation strategy plus its rigorous application to independent external test sets. Furthermore, the choice of descriptors leading to directly interpretable 3D-QSAR models is crucial for interpretation and discussions with medicinal chemists.

The use of 3D-QSAR is based on a series of particular approximations (Oprea 2004), as given in the following. The ligand and not a metabolite nor a covalently linked inhibitor must be responsible for the biological effect. Ligands should also be modeled in their presumable "bioactive" conformation. No significant conformational changes should occur upon binding to the target, and the binding site should be the same for all ligands. Kinetic effects are neglected which requires the system to be in equilibrium. The entropic contributions to the free energy of binding should be comparable across the entire ligand series, while solvent effects, temperature, diffusion, transport, pH, and salt concentrations known to contribute to the binding energy are neglected.

The alignment rule significantly impacts results and predictivity of the final 3D-QSAR models. Hence, the use of consistent alignment rules for novel molecules that are less similar to the training site molecules is one of the main prerequisites of this approach. It has clearly been recognized that the definition of a justified

alignment rule matching a user-defined pharmacophore is important to avoid wrong conclusions and errors in CoMFA studies (Kubinyi 1998). Whenever possible, experimentally determined alignment should be given preference in 3D-QSAR. Despite those limitations, the arsenal of 3D-QSAR methods is an important tool in the daily practice of drug discovery programs, especially in the absence of experimental 3D structure of macromolecular receptors or ADME antitarget in complex with representative ligands.

MODIFICATIONS OF THE METHOD

Other approaches are also employed to compute molecular interaction fields and derived descriptors on a regular grid, like the GRID force field (Goodford 1985; Baroni et al. 1993), lipophilic fields (Kellogg et al. 1991), or comparative molecular similarity index analysis (CoMSIA, Klebe et al. 1994a). Further additions to the family of 3D-QSAR methods include 4D-QSAR (Dunn and Hopfinger 1998), CoMPASS (Jain et al. 1994), receptor surface models (Hahn and Rogers 1998), the pseudoreceptor approach (Gurrath et al. 1998), and comparative molecular surface analysis (CoMSA, Polanski et al. 2002). As there is still no general solution of the alignment problem, further research led to the development of alignment-free 3D descriptors like, for example, GRIND descriptors (see above; Pastor et al. 2000) or Topomer CoMFA (Cramer 2003). The latter approach, for example, combines the CoMFA methodology with a highly standardized method to generate fragment conformers, so-called topomers. A series of input structures is disconnected into two or more fragments at a central acyclic single bond while removing any core fragment structurally common to the entire series. Individual steric and electrostatic field descriptors are then generated for each fragment and combined in a standard PLS analysis. Successful applications of this automated 3D-QSAR method and its application to virtual screening for alternative substituents have been reported (Cramer 2003; Cramer et al. 2008). One interesting recent application is the combination of Topomer CoMFA with a quantitative series enrichment analysis (Wendt and Cramer 2008) to automatically build models from large chemogenomic spaces extracted from public databases like ChEMBL, PubChem, and ChemBank (Wendt et al. 2011a). This approach was, for example, applied to successfully build and utilize a Topomer CoMFA model for a series of toluidinesulfonamides, as HIF-1 inhibitors with CYP2C9 inhibition as undesirable side effect using CYP2C9 structure-activity data from the PubChem database (Wendt et al. 2011b).

Furthermore, the use of predictive and validated CATALYST pharmacophore models (Greene et al. 1994; Sprague 1995) has also been found wide application to understand structure-activity relationships. This approach has also been successfully applied to ADME relevant targets like human cytochrome P450 datasets (Ekins et al. 2001) to understand structural requirements of their active sites on the basis of inhibitor and substrate series in the absence of crystal structures for these human enzymes.

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41.2.4 Predicting the Site of Drug Metabolism by Cytochromes (MetaSite)

PURPOSE AND RATIONALE

The oxidative metabolism of drug molecules is significantly mediated by cytochrome P450 (CYP) enzymes, which constitute a large superfamily of heme-containing monooxygenases occurring in most living organisms. CYP-mediated biotransformations are involved in various physiological and pathophysiological processes (Brown et al. 2008). While this includes the process of detoxification of potentially harmful xenobiotic compounds, it could also result in the generation of toxic reactive intermediates (Tarcsay and Keserü 2011). Seven of the known 57 human CYP isoforms are responsible for the metabolism of ~90% of the current pharmaceutical substances (de Groot 2006). Several aspects of these enzymes such as rate and site of metabolism have to be taken into account to support lead optimization efforts. Hence, it would be valuable to have computational approaches addressing these individual aspects. In particular, the fast and reliable computational estimation of the site of metabolism (SOM) could support optimization by blocking a labile substructure or altering essential features of the CYP substrate molecular recognition. Furthermore, any predicted metabolite might serve as starting point to assess its potential toxicological side effects.

The underlying mechanisms responsible for drug metabolism have been extensively reviewed (Guengerich 1991; Tarcsay and Keserü 2011), including our knowledge on the CYP catalytic cycle, as supported by X-ray structures of crucial intermediates (Schlichting et al. 2000; Denisov et al. 2005). Kinetic isotope effect studies and computational results have shown that the insertion of the heme-iron-bound oxygen into the bound substrate is the rate-limiting step of this cycle (Guengerich et al. 2004; Bach 2010). This catalytic process for CYP-mediated biotransformations is both influenced by steric effects (e.g., recognition of the substrate in its CYP binding site) and electronic effects (e.g., abstraction of hydrogen radicals).

Various sites of metabolism prediction tools capture different states of the catalytic cycle and can be classified as (Tarcsay and Keserü 2011) (1) orientation effect-based methods which predict the first substrate recognition step using information either from the protein structure or the spatial alignment of known ligands, (2) mechanism-based methods, which focus on the electronic effects of the rate-limiting step by calculating hydrogen-bond abstraction energy in model systems, (3) combined models which take orientation effect as well as mechanism-based electronic effects into account, and (4) empirical models which consider the outcome of the catalytic cycle as represented in the database of known biotransformations. The availability of multiple X-ray structures of important human cytochromes today including CYP3A4 (Yano et al. 2004; Williams et al. 2004; Ekroos and Sjogren 2006; Sevrioukova and Poulos 2010) led to an increasing number of approaches focusing on the CYP substrate recognition step based on atomic coordinates of relevant CYP binding sites.

As comprehensive overviews of in silico approaches representing all four classes have recently been given (Vaz et al. 2010; Tarcsay and Keserü 2011), we focus in this section on one promising approach combining X-ray structures with substrate reactivity, which was successfully applied in internal projects, namely, the program MetaSite (Zamora et al. 2003a; Cruciani et al. 2005). MetaSite utilizes a molecular interaction field similarity metric as well as reactivity combined with specific correction terms for the type of biotransformation reaction plus the CYP isoforms. This approach relies first on the spatial alignment of the interaction pattern of the CYP active site and its substrate to explore the ligands' substructure exposed to the CYP heme group. Chemically reactive moieties in the neighborhood of the heme iron then will be assigned as SOM. There are different algorithms with different requirements on the accuracy of the protein active site geometric structure, allowing for evaluating the binding pose of a substrate within the CYP binding site. MetaSite relies on a description of the CYP binding site by molecular interaction fields (MIFs) using the program GRID (Goodford 1985), which requires high-resolution structural information of the protein. Favorable regions for interaction are those obtained from analysis of these interaction fields. This approach represents not only a simplification but also an important modification compared to docking, as multiple CYP X-ray structures reveal significant degrees of binding site flexibility, which is partially considered using this simpler and fuzzier representation. Hence, MetaSite provides an interesting alternative to regular QSAR methods, as it does not depend on an a priori

training dataset, but it is based on a combination of first-principle arguments to predict potential sites of metabolism.

PROCEDURE

In detail, MetaSite evaluates the complementarity of molecular interaction fields of cytochrome P450 substrate binding site and its ligand while exploring different binding poses of the ligand with respect to the orientation of the heme iron (Zamora et al. 2003b; Cruciani et al. 2005). GRID-derived molecular interaction fields are computed using a hydrophobic (DRY), an amide donor nitrogen (N1), and a carbonyl acceptor oxygen (O) probe atom (Goodford 1985). This computational step allows for limited protein site chain flexibility by using the GRID-directive MOVE, which mimics the limited mobility of amino acid residues to slightly accommodate for ligand binding. The GRID interaction points are then filtered by interaction energy. In a next step, the distance between those points to the oxygen atom attached to the heme iron in *compound I* of the catalytic cycle (Schlichting et al. 2000; Tarcsay and Keserü 2011) is used to generate a new set of descriptors that represent the interaction energy in a distance-binning fingerprint. The maximum of the GRID interaction energy for each distance bin is used as descriptor after conversion into a fingerprint. Multiple crystal structures of homology models for those cytochromes without an X-ray structure can be easily incorporated, and they definitively influence the outcome of the MetaSite predictions.

The descriptor generation for the ligand molecule is performed in a similar manner, replacing the distance to the heme-bound oxygen species with distances to each individual ligand hydrogen atom, as they will be replaced by the active oxygen in the CYP hydroxylation process. Hence, for each hydrogen atom as reference, a different distance-binning scheme is generated for the same ligand and converted into a separate fingerprint.

The binning-derived fingerprint descriptors for the protein and the ligand with different hydrogen references are then evaluated by computing their fingerprint similarity. The intramolecular environment of a ligand candidate atom in a molecule to its CYP binding environment around the heme-bound oxygen is thus matched. The fundamental idea of this approach then is that the reference hydrogen atom, for which the fingerprint shows the closest match to the protein binding site descriptor, is the predicted SOM. Atoms that closely match the heme environment are presumably more likely to be oxidized.

Ranking of different substrate atoms assigned as SOM is then performed on the basis of their in silico reactivity, as computed from a mixture of molecular orbital calculations and fragment recognition. The reactivity R_i of a particular atom represents the activation energy required to produce the reactive intermediate (e.g., for hydrogen radical abstraction). This reactivity depends on the ligand 3D structure and on the mechanism of the reaction.

In addition to the accessibility and reactivity components to the MetaSite assessment of individual atoms, versions 3.0 and higher have incorporated a new term accounting for the ease of hydrogen abstraction. Furthermore, every metabolic reaction can have a component factor for each individual CYP, indicating the propensity of a particular isoforms toward a particular reaction, like the increased likelihood for CYP3A4 to participate in N-demethylation reactions compared to CYP2C9 (Vaz et al. 2010).

EVALUATION

For metabolically labile compounds with an experimentally confirmed or an in silico hypothesis about the SOM, chemically blocking the site of metabolism is a very common approach during lead optimization to modulate substrate turnover and thus improving metabolic stability. The interpretation of MetaSite results may allow formulating a hypothesis on the site of metabolism and on the molecular features, which are involved in substrate interactions to its binding site.

When possible metabolites are experimentally found by mass spectrometric methods, the MetaSite contribution plot is an important tool providing guidance for further optimization. The MetaSite prediction of metabolites for midazolam is shown as an example in Fig. 41.5. The score plot (upper panel) provides information on the likelihood of individual atoms in midazolam to be metabolized by CYP3A4 from the MetaSite analysis using the internal CYP3A4 structure in MetaSite version 3.0. The 1'-product, which is most likely, is highlighted by a blue circle in the chemical structure on the left. The second likely product is the 4'-product; both are indeed experimentally observed (Gorski et al. 1994; Khan et al. 2002). Some other labile atoms are also detected by MetaSite, as indicated by light red labels, while they are less likely to result in

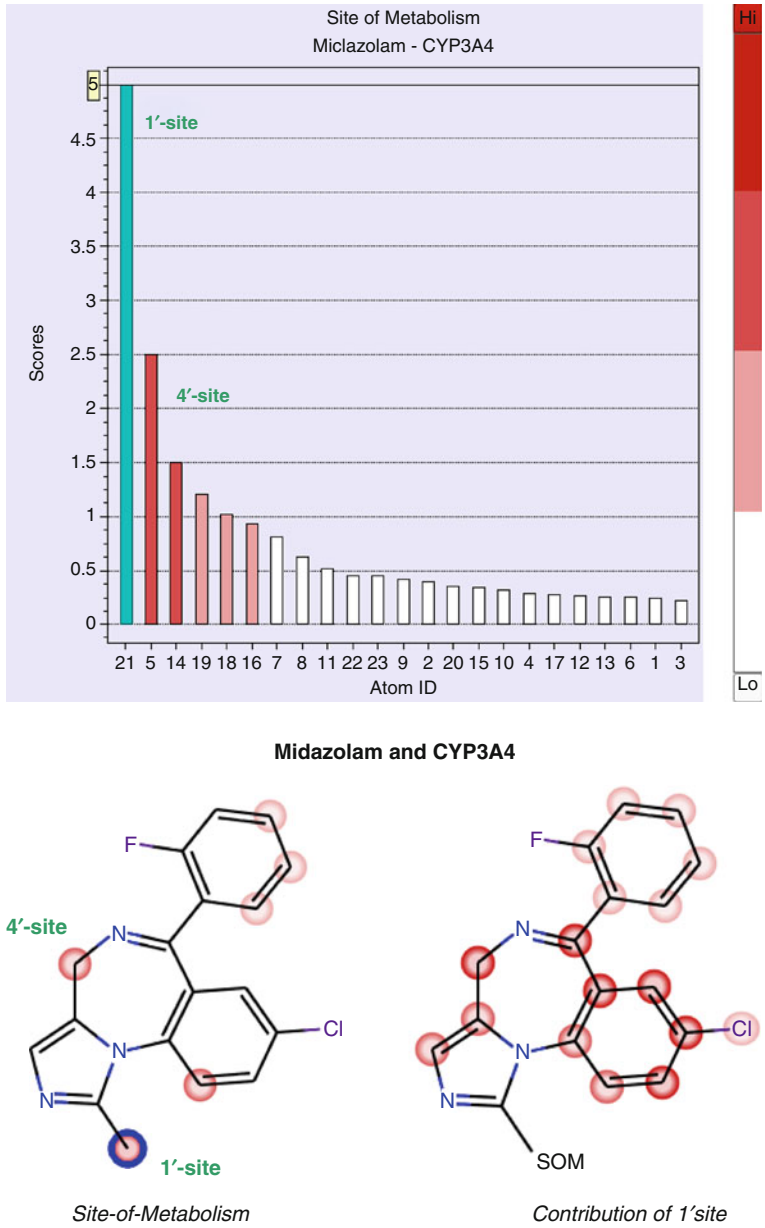

Fig. 41.5 MetaSite prediction of CYP3A4 metabolites for midazolam. The score plot (*upper*) indicates the likelihood of atoms to be metabolized by CYP3A4. The 1'-product is highlighted by a *blue circle* in the site-of-metabolism plot (*left*); different colors indicate likelihoods of atoms for metabolization. The contribution plot (*right*) indicates which groups contribute significantly to the recognition to the CYP binding site for a particular SOM

a corresponding biotransformation product. The typical use of MetaSite also includes applying different CYP3A4 X-ray structures for reference compounds with experimentally known metabolism from the same series to evaluate, which X-ray structure matches best the experimental metabolite pattern when using it for MetaSite. This structure then is routinely used for further predictions in a particular series.

In addition to this site-of-metabolism plot, the contribution plot for midazolam is shown in Fig. 41.5 on the right. This plot displays the contributions of interactions of ligand groups with the CYP enzyme in the particular pose, which results in the 1'-product. This might be useful, exploring those interaction patterns of different SOMs. Interestingly, comparing the 4'- and 1'-product, the contribution of the CYP3A4 interaction with the fluorobenzene ring is weaker for the formation of the 1'-product (Vaz et al. 2010).

In addition, MetaSite CYP3A4 predictions are shown in Fig. 41.6 for the indomethacin derivative

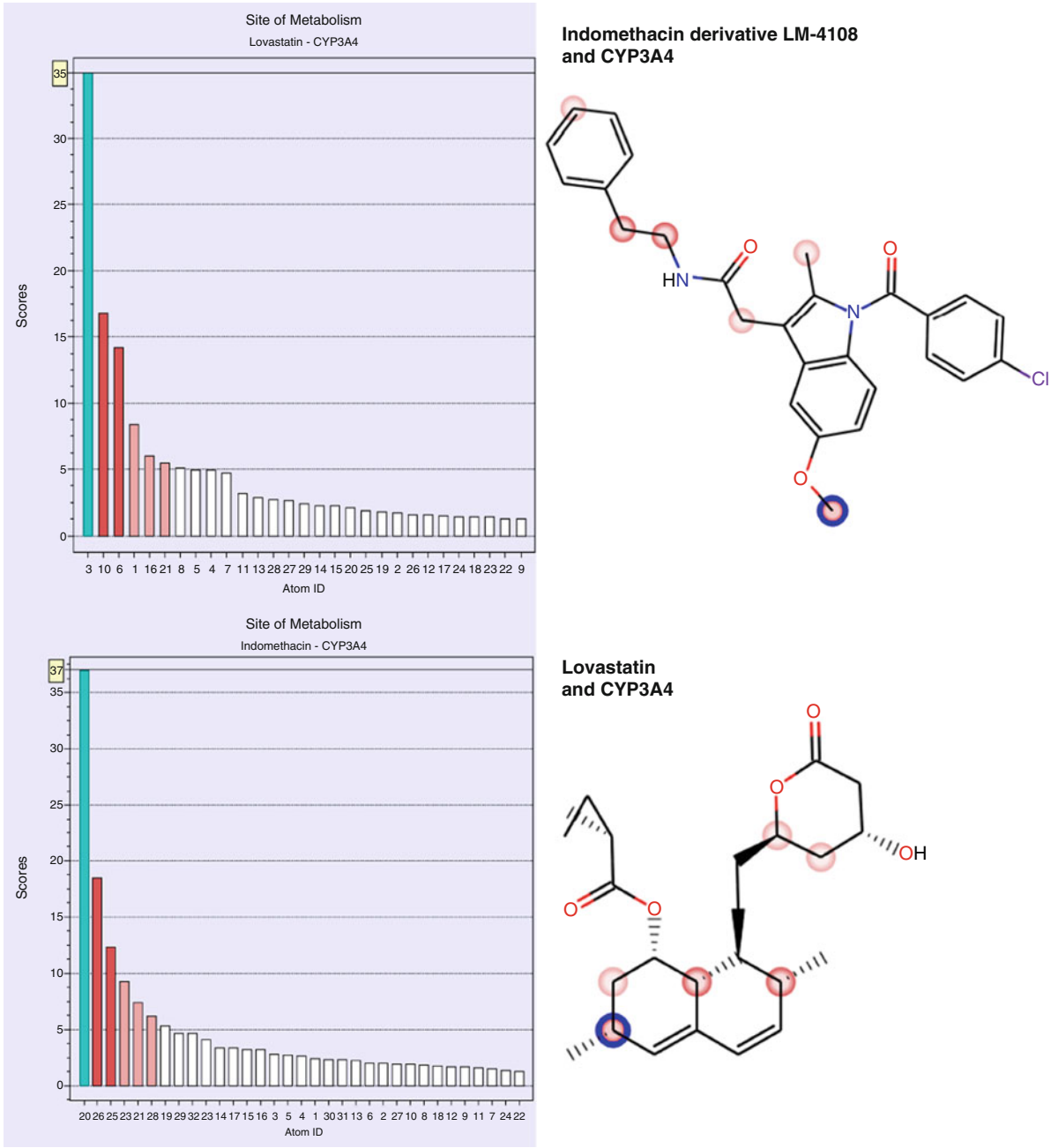

Fig. 41.6 MetaSite prediction of CYP3A4 metabolites for the indomethacin derivative LM-4108 (*upper panel*) and lovastatin (*lower panel*)

LM-4108 (upper panel, Remmel et al. 2004; Boyer et al. 2009) and the cholesterin-lowering drug lovastatin (lower panel, Caron et al. 2007). For LM-4108, the addition of the reaction mechanism-based probability in MetaSite suggests the benzylic hydrogen position as labile and as probable SOM. In addition, the methoxy group at the indole was detected as most probable SOM (blue circle in Fig. 41.6, upper panel). The third most important position is the CH_2 group, next to the amide nitrogen. Indeed, all three positions correspond to experimentally detected metabolites for this molecule (Remmel et al. 2004). Based on this

knowledge of potentially labile positions, several metabolically more stable analogues were synthesized, primarily by more polar modifications at the benzylic and amide CH_2 positions (Boyer et al. 2009).

For lovastatin, three main metabolites are experimentally reported; two of them (hydrogen elimination and hydroxylation) are in agreement with the most likely SOM from the MetaSite analysis, as indicated by a blue circle in Fig. 41.6 (lower panel). The other potential SOMs are much less likely. However, the third experimental metabolite, a hydroxylation in the aliphatic ester side chain, is given only a low priority.

CRITICAL ASSESSMENT OF THE METHOD

Tremendous progress has been made in the area of in silico prediction toward the identification of the site of metabolism in oxidative CYP-mediated metabolism in the past few years. The demonstrated agreement for these few MetaSite results in Figs. 41.5 and 41.6 is representative, as not always all experimentally detected SOMs are predicted. A broad comparison of prediction quality for MetaSite, although an older version, to other approaches is provided in a comprehensive overview by Tarcsay and Keserü (2011), further underscoring the value of using MetaSite for various CYP isoforms.

A recent comprehensive investigation on 227 known CYP3A4 substrates with a total of 325 metabolic pathways was carried out using MetaSite in comparison to docking studies (Zhou et al. 2006). Using either the CYP3A4 crystal structure or a homology model, both with the reactivity factor included, resulted in a prediction success of 78% correct prediction. The validation of the SOM prediction was based on counting the number of experimentally reported metabolic pathways that were found among the first, second, and third SOM sites as ranked by this approach. The employed docking method had relatively lower prediction accuracy (57%), although the value of such a docking-based approach is that it might provide useful insights into protein-substrate interactions, which could be additionally explored in future compound design (Zhou et al. 2006).

Site-of-metabolism prediction might significantly contribute to the drug discovery process, especially during earlier phases of research projects. At such an early project phase, when many potential hit series are evaluated as promising starting point for lead optimization, SOM predictions might contribute to this evaluation, as there are too many compounds to be characterized by experimental metabolite identification (ID) studies employing mass spectrometric methods despite their rapid turnaround times today. This in silico profiling might also be used for evaluating and ranking compound libraries. During the following optimization phase, SOM predictions are useful, complementing these rapid metabolite ID studies, in particular in those cases where experimentally an SOM can only be attributed to a large substructure at this early phase of discovery. A combination of metabolite ID and SOM prediction then clearly helps to formulate a hypothesis and thus stimulates chemical synthesis for specifically addressing this site.

MODIFICATIONS OF THE METHOD

In particular, the application of MetaSite for in silico enumeration of metabolites to aid metabolite identification appears to be very promising and further extends the use of this approach. A robust soft spot identification procedure combining MetaSite prediction rankings and rapid mass spectrometric confirmation has been described (Trunzer et al. 2009). While MetaSite's first rank predictions were experimentally confirmed in only 55 of the cases, for additional 29% of the investigated compounds, the second or third ranked SOM was detected. The proposed automatic and high-throughput reprioritization of a likely SOM thus significantly increases the likelihood to focus the next compound design to the correct SOM to more than 80%. This information allows to rapidly establish structure-metabolism relationships and to explore them by further chemical synthesis.

In another study, a recent modification of MetaSite was combined with mass spectroscopic data to mimic the rationalization of fragment ions routinely performed by a metabolism scientist during structure elucidation (Bonn et al. 2010). For 96% of the metabolites, the chemical structures were correctly assigned. Furthermore, for ~80% of the cases, this correctly assigned structure was selected at first rank in comparison to manual inspection of the data supported by additional mass spectroscopic data. Hence, this MetaSite modification might be a very promising addition, allowing to automatically assign the chemical structure to a majority of experimentally detected metabolites (Bonn et al. 2010).

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41.3 Examples for Global and Local In Silico ADME Models

This section will provide an overview on global and local ADME models from our group to illustrate our approach for building predictive models on structurally diverse training sets. We will discuss datasets for human intestinal absorption, human serum albumin binding, and CYP2C9 inhibition more in detail as examples for the construction of general models. Significant models for other relevant ADME properties have also been obtained. The main application of these models is seen in earlier phases of the drug discovery process related to library design and lead identification when no or little information on analogues is available. In addition, we will also show the generation of local, chemotype-specific models using permeability prediction for thrombin inhibitors and prediction of systemic exposure following oral administration of MMP-8 inhibitors. These models exemplify the typical situation in lead optimization where a collection of analogues was experimentally profiled. Those models are typically used in combination with

appropriate QSAR models tailored for affinity and selectivity for individual projects in the frame of multidimensional lead optimization.

41.3.1 Prediction of Human Intestinal Absorption

PURPOSE AND RATIONALE

The knowledge of the extent of human intestinal absorption is an important factor in the design of novel drug candidates. Several in silico models to predict human oral absorption have been reported in the literature (Agatonovich-Kustrin et al. 2001; Deretey et al. 2002; Egan et al. 2000b; Fu et al. 2001; Klopman et al. 2002; Norinder and Österberg 2001; Oprea and Gottfries 1999; Raevsky et al. 2000; Sugawara et al. 1998; Zhao et al. 2001). Simple models are based on only few physicochemical descriptors like logP, logD, or polar surface area (PSA). Consistently, all these models are only applicable if the compounds are passively absorbed. In case of absorption via active transporters or if efflux is involved, prediction of absorption is still not successful. This is a typical example where the attempt to predict data related to multiple underlying mechanisms do not produce significant models.

Prediction of systemic exposure or bioavailability from molecular structure is much more difficult since these parameters depend on solubility, absorption, and first-pass clearance. To this end, Yoshida and Topliss (2000) generated a QSAR model by applying "fuzzy adaptive least squares," using logD at pH 7.4 and 6.5 as physicochemical properties and the presence of key functional groups as structural descriptor. They achieved a classification of drugs into one of four bioavailability categories with an overall accuracy of 60% (Yoshida and Topliss 2000). Another recent approach uses neural networks and a collection of chemical descriptors to arrive at a model with good predictability (Turner et al. 2004).

While the majority of published models are based on a limited number of drug molecules, especially the study of Zhao et al. (2001) provides the most extensive compilation from available literature data and a statistical model derived from those using Abraham descriptors. We used this carefully selected dataset to build a quantitative model for human intestinal absorption employing VolSurf descriptors (see Sect. 41.2.2 and Cruciani et al. 2000a).

PROCEDURE

A dataset of 169 drugs and drug-like molecules was extracted from the compilation of literature data from Zhao et al. (2001). These molecules are considered to have reliable data about human intestinal absorption. Biological data were used as percent human intestinal absorption (%HIA) for statistical analysis. All molecules were treated as neutral and converted into their 3D structures using CORINA (Sadowski et al. 1992). The molecules were not aligned to each other. After 3D conversion, no further energy minimization was employed. Molecular interaction fields were computed using the GRID force field in VolSurf employing the water, dry, and carbonyl oxygen probes. Subsequently, a series of 72 VolSurf descriptors was computed from analysis of these three molecular interaction fields. These descriptors encode the following information (Cruciani et al. 2000b):

- Total volume (at an energy level of 0.25 kcal/mol)
- Total surface (at an energy level of 0.25 kcal/mol)
- Rugosity as ratio between total volume/total surface
- Globularity
- Volumes of interactions with GRID water probe at eight energy levels (−0.2 to −6.0 kcal/mol)
- Integy moments as distance from the center of mass to the center of interaction with the water probe eight energy levels
- Capacity factors as ratio of water volumes of interactions to total surface at eight energy levels
- Local interaction energy minima and distances
- Volumes of interactions with GRID dry probe at eight energy levels (−0.2 to −1.6 kcal/mol)
- Integy moments as distance from the center of mass to the center of interactions with the dry probe at eight energy levels
- Hydrophilic versus lipophilic balance (from −3, −4 kcal/mol water probe to −0.6, −0.8 kcal/mol dry probe)
- Amphiphilic moment as vector from hydrophobic to hydrophilic domain center
- Critical packing parameter defined as the volume of the hydrophobic part divided by the product of the surface of the hydrophilic part times the length of the hydrophobic part
- Volumes of interactions with GRID carbonyl oxygen probe at eight energy levels (−0.2 to −6.0 kcal/mol) to model hydrogen bonding capacity

All descriptors were normalized using autoscaling and subjected to multivariate statistical analysis

using PLS. From initial models encompassing all 72 descriptors, a set of 40 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000c).

EVALUATION

This procedure led to a predictive PLS model for 40 VolSurf descriptors and four relevant PLS components with a crossvalidated r^2(cv) value after leave-one-out crossvalidation of 0.662 and a conventional r^2 value of 0.709[1]. Further statistical validations using leave-two-out and leave-multiple-groups-out crossvalidation procedures further underscore the significance of the final model. The overall statistical quality of this model is similar to the model reported by Zhao et al. (2001; model 5; r^2: 0.74; r^2(cv): 0.72). This model also favorably compares to a previously reported VolSurf model for human intestinal absorption based on 20 drug molecules only. This earlier model with one PLS component showed an r^2(cv) of 0.726 and a conventional r^2 value of 0.782 for 72 descriptors without variable selection (Guba and Cruciani 2000; cf. Fig. 41.3), while the structural range of molecules in the extended dataset from Zhao et al. (2001) certainly encompasses more chemotypes and relevant functional groups.

The correlation of VolSurf descriptors to the human intestinal absorption for 169 drugs is shown in Fig. 41.7 from the final four component PLS model. On the left panel, the plot of experimental versus predicted %HIA is displayed. Although the data points do not fall onto a straight line, the model clearly is able to discriminate between compounds with high, medium, or low intestinal absorption. The analysis of PLS coefficients from this model allows for a chemical interpretation of the importance of individual descriptors contributing to the model, as shown in Fig. 41.7 on the right panel. Factors like rugosity (ROH2), integy moment derived from water interactions (Iw*OH2), hydrophobic contributions at lower energy levels (D2DRY), and hydrogen bonding (HB*O) are positively correlated to human intestinal absorption. Thus, if a chemical modification of the underlying structure increases any of these descriptors, it is likely that the intestinal absorption will be positively affected. In contrast, the increase of polarity from both water and carbonyl O probes (W*OH2, W*O), the associated capacity factor (Cw*OH2), and the hydrophobic integy moment are detrimental for intestinal absorption. These conclusions for factors influencing permeability and thus intestinal absorption are in agreement with earlier findings pointing to the positive impact of hydrophobicity, integy moment, shape, and hydrogen bonding, while polarity derived using the GRID water and carbonyl O probes as well as the capacity factors from polar interactions on the entire surface is detrimental. Previous work also suggests H-bond donors, acceptors, and polar surface contributions as important to model human intestinal absorption (Clark 1999a; Wessel et al. 1998; Palm et al. 1997). However, due to the nature of the VolSurf descriptors in combination with multivariate statistical analysis, this approach led to an enhanced understanding and quantitative modeling of physicochemical requirements influencing this pharmacokinetic observable. In particular, the balance between lipophilic and hydrophilic parts in combination with size, volume, and other effects guides the design of new compounds.

CRITICAL ASSESSMENT OF THE METHOD

VolSurf descriptors are able to predict absorption for a diverse set of drugs. The presented model is derived using a consistent frame of relevant chemically interpretable descriptors, which find applications in different local and general models. However, absorption is not only controlled by passive membrane permeability. There are other factors influencing in vivo human absorption, namely, the in vivo dissolution rate in small intestinal fluid and the dose used for the human study. Furthermore, active transport or efflux mechanisms are difficult to rule out and can only be identified by additional in vitro experiments. These important pieces of information should be known before any QSAR analysis is attempted on human absorption. This lack of consistent information throughout the literature is difficult to overcome, in particular for human studies. Hence, this study for the dataset from Zhao et al. (2001) provides a reasonable attempt to address these problems to carefully selecting members of the final dataset.

[1] r^2(cv), which is also known as q^2, indicates the crossvalidated r^2. Each compound is removed once from the final model, then a submodel is generated, and the removed compound is predicted using this submodel. The subsequent analysis of all prediction errors produces r^2(cv) (Wold 1978; Cramer et al. 1988b).

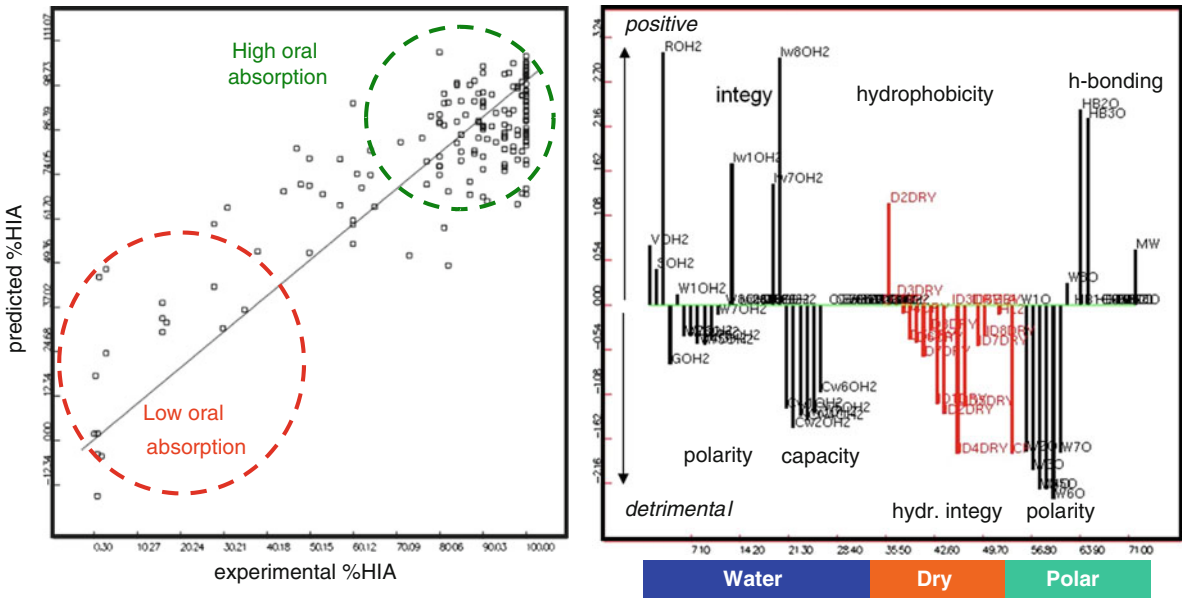

Fig. 41.7 Correlation of VolSurf descriptors with human intestinal absorption for 169 drug molecules. *Left*: predicted versus experimental %HIA (human intestinal absorption) from final 4-component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of human intestinal absorption

Another problem for this and related datasets is the skewed distribution of the biological observable. There are too many compounds with high absorption, as this information gets primarily published after a successful drug discovery program. Compounds with no or low absorption by design or as failures are much harder to find in the public domain. Furthermore, this model is built within a limited chemical space which certainly does not reflect the chemical diversity in typical corporate libraries within the pharmaceutical industry. Although individual members of the training set are quite diverse to each other, the global coverage of chemical space is limited.

This collection of limiting factors clearly influences the quality of the model and its predictive power. In view of these sources of inconsistencies and errors in determining the intestinal absorption in humans, it is impressive that the quality of the model is as good as it is. In particular, this model was shown to be of particular use as general model in earlier phase for classification of molecules. Any significant improvement is likely not to come from statistical or computational methods but from more consistent data on a broader collection of drug-like molecules, preferably by an in vitro system eliminating some sources of experimental errors.

MODIFICATIONS OF THE METHOD

From a recent compilation of relevant human intestinal absorption data by Hou et al. (2007), a significantly larger dataset from public sources became available to us for model building. From a total of 648 chemical compounds from this work, 579 were believed by the authors to be transported by passive diffusion (Hou et al. 2007). From those molecules, we selected a training set of 453 molecules and a test set of 98 molecules by statistical design after elimination of some structures. However, this new dataset does still not overcome the skewed distribution of intestinal absorption data, as observed for the smaller dataset described above (Zhao et al 2001). After compound standardization and 3D conversion, a total of 184 descriptors were computed using the software package MOE (CCG). We then employed the program Cubist (RuleQuest) to derive regression tree models (Quinlan 1992; Butina and Gola 2003) based on the training dataset and all 184 descriptors. Cubist first builds a collection of rules to partition the training dataset into nodes in analogy to a decision tree structure. Each rule, however, has an associated multiple linear regression (MLR) model describing the SAR for all molecules as members of this rule. Thus, Cubist effectively classifies a set of

compounds according to structural parameters and evaluates a separate SAR model for each of these subsets rather than attempting to build a single model for the entire dataset (Quinlan 1992; Butina and Gola 2003). After the first predictive regression tree model from this approach, we then used a genetic algorithm to select useful descriptors in order to improve the statistical quality of the training set in terms of regression coefficients and to show the best performance on the test set. The finally selected regression tree model is based on four rules and 20 descriptors selected by our GA procedure after 1,000 iterations. While for the training set a r^2 value of 0.810 and a crossvalidated r^2(cv) value of 0.689 (tenfold crossvalidation) were obtained, the predictive r^2 of 0.846 for the test set of 98 molecules suggests a significant and useful model, which we then have implemented for design.

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41.3.2 Predicting Human Serum Albumin and Plasma Protein Binding

PURPOSE AND RATIONALE

Problems related to poor systemic exposure of drug molecules can also be related to their volume of distribution, which is indirectly related to plasma protein binding. Human plasma contains more than 60 different proteins of which the major components are serum albumin (HSA, 60%) and glycosylated proteins like α-acid glycoprotein (AGP). Human serum albumin (66 kDa) is the most abundant protein in blood plasma with concentrations of 0.53–0.75 mM. Eighteen different variants arising from single amino acid mutations have been identified, accounting for different protein binding. Allelic variation makes data consistency difficult and hence modeling of the resultant data less reliable (Kariv et al. 2002). Considering the high concentration of albumin and the wide range of effective concentrations of therapeutic drugs from nanomolar to millimolar, the free concentration for a therapeutic effect can be significantly reduced for drugs with high binding to plasma proteins, independent of the fact that the affinity of drugs to their target protein is often higher than for plasma proteins. A total of eight hydrophobic binding sites for fatty acids have been identified, while for drug-like compounds, two high affinity binding sites were postulated in subdomains IIA and IIIA. The analysis of HSA X-ray structures (Bhattacharya et al. 2000; Petitpas et al. 2001) also showed that increased ligand concentrations led to binding to all known binding sites with different affinities and different pharmacological relevance.

There have been only a limited number of attempts to understand the molecular factors influencing drug binding to HSA (Colmenarejo et al. 2001; Colmenarejo 2003; Kratochwil et al. 2002; Saiakhov et al. 2000; Hajduk et al. 2003). Although it has been assumed that plasma protein binding is only related to ligand lipophilicity, a study by Kratochwil et al. (2002) demonstrated that lipophilicity is rather poorly correlated to HSA binding for a diverse set of molecules, in contrast to congeneric series, where lipophilicity is often found to be the dominant factor. This suggests that specific molecular recognition elements plus other physicochemical properties are involved in binding. This led these authors to derive a model based on pharmacophore similarity for 138 molecules and a comprehensive list of pharmacologically relevant first HSA association constants from the literature.

Colmenarejo et al. (2001, 2003) reported another significant and predictive computational model to predict binding to HSA based on a series of 95 drug-like molecules, again aiming to cover a large region of the chemical space. A set of consistent experimental data from a single laboratory were generated using high-performance affinity chromatography with HSA-immobilized stationary phases. We decided to use this homogeneous experimental data for HSA binding to build a quantitative model. Since our rational is to build many different models of ADME relevance with a consistent frame of informative descriptors, we employed the VolSurf descriptors (Cruciani et al. 2000a) already used for correlation of 3D structures to human intestinal absorption.

PROCEDURE

The set of 95 drugs and drug-like molecules from the study of Colmenarejo et al. (2001) was used. From experimental HPLC retention times, the $\log K_{(HSA)}$ values were used for statistical analysis. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry, and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000b) was computed and analyzed as described above. All descriptors were normalized using autoscaling and subjected to PLS analysis. In the process of model building, two structurally unique outliers were rejected (Ebselen, Captopril). From initial models with only 93 compounds, a set of 47 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000c).

EVALUATION

This procedure led to a predictive 6-component PLS model for 47 VolSurf descriptors with a crossvalidated $r^2(cv)$ value after leave-one-out crossvalidation of 0.671 and a conventional r^2 value of 0.755. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation procedures underscores the significance of the final model. The graph of experimental versus calculated $-\log K_{(HSA)}$ values is shown

in Fig. 41.8 on the left. This model quality corresponds to the model reported by Colmenarejo et al. (2001).

The predictivity of this model to external compounds was further evaluated by splitting the dataset into a training set of 83 compounds and a test set of 10 molecules. One approach was to use the test set from Colmenarejo et al. (2001; model B), while another test set was generated using statistical design after a principal component analysis (PCA) on VolSurf descriptors (model A). The design was done by selecting the most descriptive compounds from the PCA scores (Hudson et al. 1996a). Scaling and variable selections were done independently for these smaller training sets, resulting in significant PLS models. For model A, a 6-component PLS model with a crossvalidated r^2(cv) value of 0.668 and a conventional r^2 value of 0.763 indicates a statistically significant model. The standard error of prediction of 0.233 for 10 external compounds counts for a model with high predictivity (41.9 left). For model B, a crossvalidated r^2(cv) value of 0.646 and a conventional r^2 value of 0.745 with an SDEP of 0.274 were observed (Fig. 41.9 right), leading to the same conclusion. For both models, the graph of experimental versus calculated $-\log K_{(HSA)}$ values is shown in Fig. 41.9. Both test sets are indicated by filled squares. In both cases, the agreement between experiment and prediction was very good.

The analysis of PLS coefficients from the final model allows to assess the importance of individual descriptors contributing to the model. The corresponding PLS coefficient plot for the final model is shown in Fig. 41.8 on the right. Factors like volume (VOH2), shape and rugosity (ROH2), integy moment from water interactions (Iw*OH2), hydrophobic contributions at lower energy levels (D3DRY), hydrophobicity (D3DRY), and molecular weight (MW) are positively correlated with HSA binding of drug molecules. In contrast, polarity (W*OH2), integy moment from the dry probe (Iw*DRY), and hydrogen bonding terms (HB5O) are negatively correlated to albumin binding. Any increase in those molecular properties is likely to decrease binding of drug-like molecules to HSA.

The interpretation for human serum albumin is in accord with literature findings and X-ray information on warfarin binding to the first drug-binding site in HSA (Petitpas et al. 2001). The influence of hydrophobicity in combination with geometry factors like shape, volume, and size is often discussed (Colmenarejo et al. 2001; Colmenarejo 2003). It also is in accord with the findings of Kratochwil et al. (2002) demonstrating that lipophilicity influences albumin binding, but certainly not to a very significant extent. Obviously, factors like pharmacophore similarity which might translate into different shape-related VolSurf descriptors are more

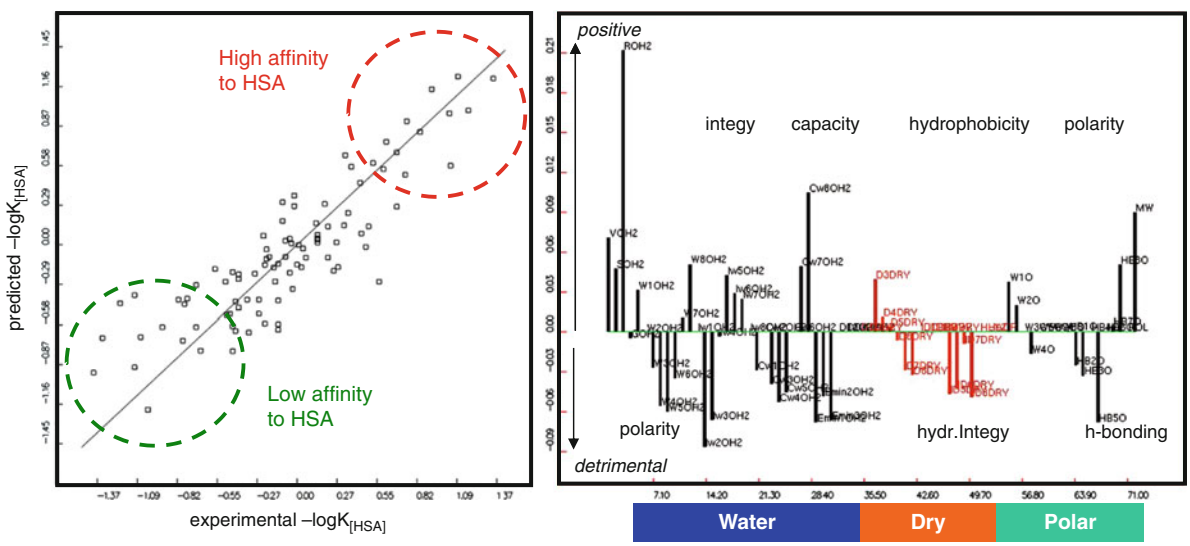

Fig. 41.8 Correlation of VolSurf descriptors with human serum albumin binding affinity for 93 drug-like molecules. *Left*: predicted versus experimental $-\log K_{(HSA)}$ from the final 6-component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of human serum albumin binding

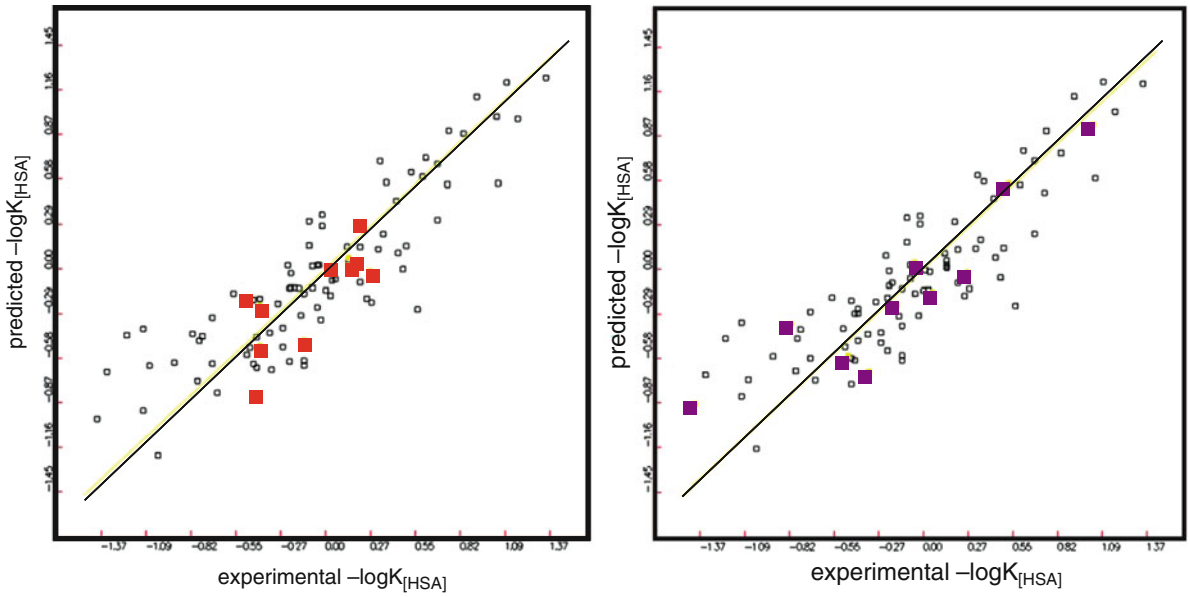

Fig. 41.9 Validation of VolSurf model for human serum albumin binding. Two submodels have predictive ability to external test sets. Ten compounds were removed by either experimental design on PCA scores (*left*) or following the literature (*right*)

important. Any increase in polarity reduces HSA binding which can be understood by the mentioned physicochemical requirements of the albumin drug-binding sites. The striking effect of shape descriptors on the other hand corresponds to the topological requirements of the binding sites.

CRITICAL ASSESSMENT OF THE METHOD

The presented general in silico model for HSA binding is built on a dataset from a solid in vitro assay conducted in a single laboratory. Hence, the quality of data certainly is not a limitation for this model. However, this assay is intrinsically not able to differentiate between drugs binding to different albumin binding sites. This uncertainty might affect the overall quality of the model and adds another mechanistic dimension to the data. Hajduk et al. (2003) reported a detailed statistical analysis of 889 diverse molecules binding to the binding site in albumin subdomain IIIA only. This analysis was based on data from heteronuclear NMR correlation and fluorescence spectroscopy which enabled the measurement of dissociation constants to only one binding site. However, approaches like these are not routinely used in experimental profiling for this property.

Before such a model can be applied to another binding site or a different pharmaceutical company, careful validation and harmonization studies have to be conducted in order to ensure that the experimental data used for building the model correspond to a certain extent to those data used to evaluate it. Typical assays in this area even do not differentiate between drugs binding to albumin or other serum proteins like α-acid glycoprotein. Furthermore, there is a dynamic equilibrium between albumin-bound and free drug molecules. While binding is focused on one aspect in the transport of drug molecules, the kinetic aspect of k_{on} and k_{off} rates is equally important to understand the complex equilibrium between bound drug, free drug, and drug bound often in a tighter fashion to its target protein. Those aspects have not been incorporated into such a model.

The present model is significant, as it allows guiding library design and hit exploration on the basis of solid in vitro data without knowledge of close analogues. As drugs with high affinity for albumin exhibit markedly reduced efficacy in vivo, this model offers a method to reduce albumin binding of potential candidates while maintaining high affinity at the therapeutic target. However, the real significant of such a model can only be assessed in the context of the experimental setup for drug discovery within a particular organization.

MODIFICATIONS OF THE METHOD

Other models to predict HSA binding have been reported, including a predictive group contribution

model involving 74 chemically intuitive fragments linked to serum albumin binding from the detailed analysis of 889 compounds at Abbott (Hajduk et al 2003). However, human plasma protein binding (PPB), as combined experimental parameter, is experimentally determined more often for novel structures instead of HSA binding alone, causing multiple computational models to be developed for this complex property as well. In a large and comprehensive QSAR model building investigation, Votano et al. (2006) compiled training and test sets of 808 and 200 compounds with PPB data from various public sources. Four modeling approaches (multiple linear regression, artificial neural networks, k-nearest neighbors, and support vector machines) involving topological descriptors were successfully applied for model building in this case on this larger dataset. Gleeson (2007) established PLS models for human and rat PPB in-house data from GlaxoSmithKline, employing training sets of 686 and 1081 compounds, respectively. A collection of 30 different descriptors including TPSA, molecular weight, and various pKa- and logD-derived properties were employed for model building. The final models displayed promising predictive ability on large test and validation sets. This analysis, however, also revealed significant species differences with respect to PPB.

Recently, a large compilation of publicly available human clinical pharmacokinetic data was reported by Obach et al. (2008), where the authors collected and compiled i.v. data for 670 drugs. These data encompass clearance, volume of distribution at steady state, mean residence time, terminal phase half-life, and plasma protein binding data given as fraction unbound (fu) to PPB. PPB data are available for 551 individual compounds in this database.

From this collection, we then selected a training set of 496 molecules and a test set of 55 molecules by statistical design. The PPB fraction unbound (fu) data were normalized according to Gleeson (2007) to result in a pseudoequilibrium constant logK = log ((1−fu)/fu). After a compound regularization and standardization process and 3D conversion, a total of 184 descriptors were computed using the software package MOE (CCG). We then applied the program Cubist (RuleQuest) for regression tree models (Quinlan 1992; Butina and Gola 2003) based on the training dataset. Cubist builds a collection of rules to partition the training dataset. Each rule has an associated multiple linear regression (MLR) model (see above). Based on a first model, we applied a genetic algorithm to select informative descriptors to improve the statistical quality of the model. The selection is driven by the training set correlation coefficient plus the performance on the test set. The final regression tree model for the PPB dataset is based on six rules and 47 descriptors. The correlation of MOE descriptors with plasma protein binding is shown in Fig. 41.10 on the left for the training set and on the right for the test set. While for the training set an r^2 value of 0.757 and a crossvalidated r^2(cv) value of 0.578 (tenfold crossvalidation) are obtained, the predictive r^2 of 0.792 for the test set indicates a significant and useful model, which we have implemented for design. This model was applied to a novel set of 40 internal molecules with high-quality PPB data, which results in a predictive r^2 value of 0.547, thus underscoring the value of such a model for further design. Interestingly,

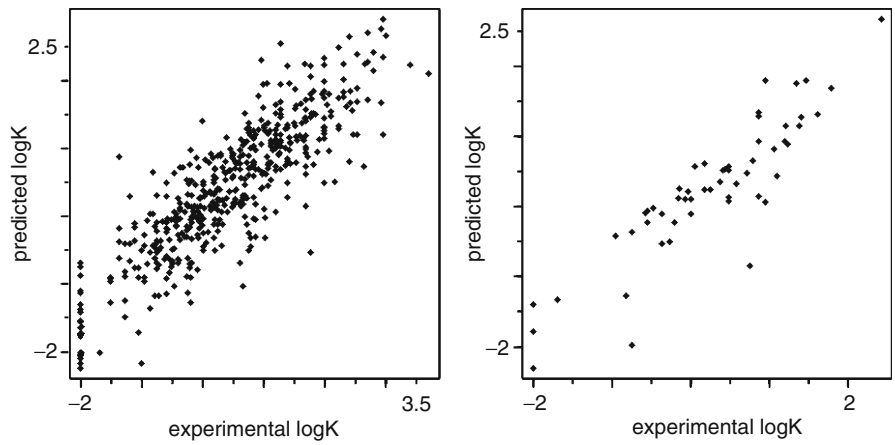

Fig. 41.10 Correlation of MOE descriptors with human plasma protein binding data (logK derived from fraction unbound). *Left*: predicted versus experimental logK values for the training set of 496 molecules. *Right*: predicted versus experimental logK values for the test set of 55 molecules

the application of simple descriptors like logD or logP does not provide the same prediction quality for this dataset.

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41.3.3 Prediction of Cytochrome P450 2C9 (CYP2C9) Inhibition

PURPOSE AND RATIONALE

Mammalian cytochrome P450s (CYPs) represent a class of membrane-associated heme-containing proteins that metabolize a range of xenobiotics (see above). The major human isoforms contribute to the oxidative metabolism of approximately 90% of pharmaceuticals, while CYP2C9 is responsible for about 18% of all catalyzed reactions (Smith et al. 1997; Rettie and Jones 2005; de Groot 2006). While typical CYP2C9 substrates belong to the class of nonsteroidal anti-inflammatory drugs (e.g., diclofenac, ibuprofen, naproxen, flurbiprofen, piroxicam), polar acidic drugs, progesterone, hypoglycemics, and coumarin-based anticoagulants are also metabolized. Any interference of potential drug candidates with these metabolism pathways might cause undesirable drug-drug interactions upon comedication (Rettie and Jones 2005), which can be a major hurdle to the clinical development of new drug molecules. Therefore, a reliable prediction of CYP2C9 inhibition potential for novel chemical structures would be useful in early discovery phases.

Earlier investigations studied smaller chemical series to establish the structure-activity relationship for CYP2C9 substrates and inhibitors (Jones et al. 1993, 1996a; Mancy et al. 1995). 3D-QSAR approaches were also employed for model building on smaller datasets (Jones et al. 1996b; Rao et al. 2000; Ekins et al. 2000; Afzelius et al. 2002b, 2004b). Subsequently, the 3D structure of CYP2C9 has been first deduced from X-ray studies of the homolog rabbit CYP2C5 (Williams et al. 2000). Some years later, Williams and coworkers

(2003) indeed determined the structure of human CYP2C9 with S-warfarin, while Wester et al. (2004) crystallized a different human CYP2C9 construct with flurbiprofen at a resolution of 2.0 Å. Here, a particular conformation of the helix B to helix C region allows Arg108 to form hydrogen bonds with Asp293 and Asn289 and to interact with the flurbiprofen carboxylate, thus positioning the substrate for regioselective oxidation in the site (Wester et al. 2004). Site-directed mutagenesis also supports the importance of Asn289 plus Ser286 in the CYP2C9 binding site for interacting with diclofenac and ibuprofen (Klose et al. 1998), which also explains its preference for smaller, acidic, and lipophilic ligands.

In order to detect inhibitors for major human CYP450 enzymes early in discovery, we set out to develop fast QSAR models for this (Byvatov et al. 2007) and other CYPs as "antitarget," namely, CYP3A4 and CYP2D6. To be of practical use for internal discovery, any model should be based on experimental data obtained under *identical* conditions for a wide range of diverse compounds. The purpose of our model differs from classical QSAR such that many diverse structures and chemical series provide the basis for its development, and it will be applied to predict diverse candidate molecules. For larger datasets, the use of descriptors requiring information about a putative alignment of 3D structures is often not possible due to structural diversity of the ligands and the lack of a consistent alignment hypothesis. Consequently, we have used two sets of descriptors known to capture information relevant for protein-ligand interactions in our own model building approach (Byvatov et al. 2007).

First, we computed three-point pharmacophore (3PP) fingerprints from 2D structures using MOE (CCG), where each bit encodes the presence of a pharmacophore triangle with specific distances. For this descriptor, the support vector machine statistical approach (SVM) (Cortes and Vapnik 1995) was found to be useful for model building, as it can handle large numbers of descriptors (Byvatov and Schneider 2003), while the application of PLS (Wold et al. 1984; Dunn et al. 1984) did not result in useful models. In order to build PLS models, we additionally employed a collection of descriptors (CMQA) consisting of 163 substructure keys following the MACCS key definitions, 150 CATS topological pharmacophore descriptors (Schneider et al. 1999), pKa values for basic and acidic groups from ACD/Labs, and surface-based descriptors computed using QikProp (Duffy and Jorgensen 2000, Schrödinger). This collection was useful in other projects for deriving significant models.

PROCEDURE

A dataset of 1,338 compounds representing multiple chemical series from various discovery programs was extracted from our internal database, for which IC_{50} values for CYP2C9 inhibition were previously determined using a harmonized protocol employing human recombinant CYP2C9 and 7-MFC (7-methoxy-4-trifluorocoumarin) as substrate (Byvatov et al. 2007). IC_{50} data were converted into pIC50 values. The quality of some data points was limited by low solubility, as estimated by prediction or experimental solubility testing. Any interpretation in those cases was done with care. All molecules were treated as neutral; counterions and salts were removed. Canonical 3D structures were generated using CORINA (Sadowski et al. 1992). The dataset was divided into a training set of 1,100 and a test set of 238 molecules using statistical design. Classification for actives and inactives was done using an IC_{50} threshold of 10 μM.

EVALUATION

Linear SVM regression with 3PP fingerprints produced a model with a crossvalidated r^2(cv) of 0.34 (leave-25%-out crossvalidation) and an r^2 value of 0.81, which correctly predicts the test set of 238 compounds (predictive r^2 value of 0.63). For CMQA descriptors, a PLS model with six components results in a crossvalidated r^2(cv) of 0.34 (leave-one-out method), a conventional r^2 of 0.48 and a predictive r^2 value of 0.55 for 238 test molecules. The training and test set predictions are shown in Fig. 41.11 for the SVM model (upper) and the PLS model (lower) with test set predictions shown on the right.

Most outliers are localized in three regions: very active compounds ($IC_{50} > 0.4$ μM, upper right circle), medium active compounds (central circle), and inactive compounds (lower left circle). Inactive outliers are often due to insufficient solubility at assay concentration. A similar observation was made for some medium active compounds with IC_{50} values greater than 2 μM. Again low solubility is the limiting factor to obtain a more accurate value. In contrast, some compounds with high affinity were predicted to be only moderately active; on the other hand, some of

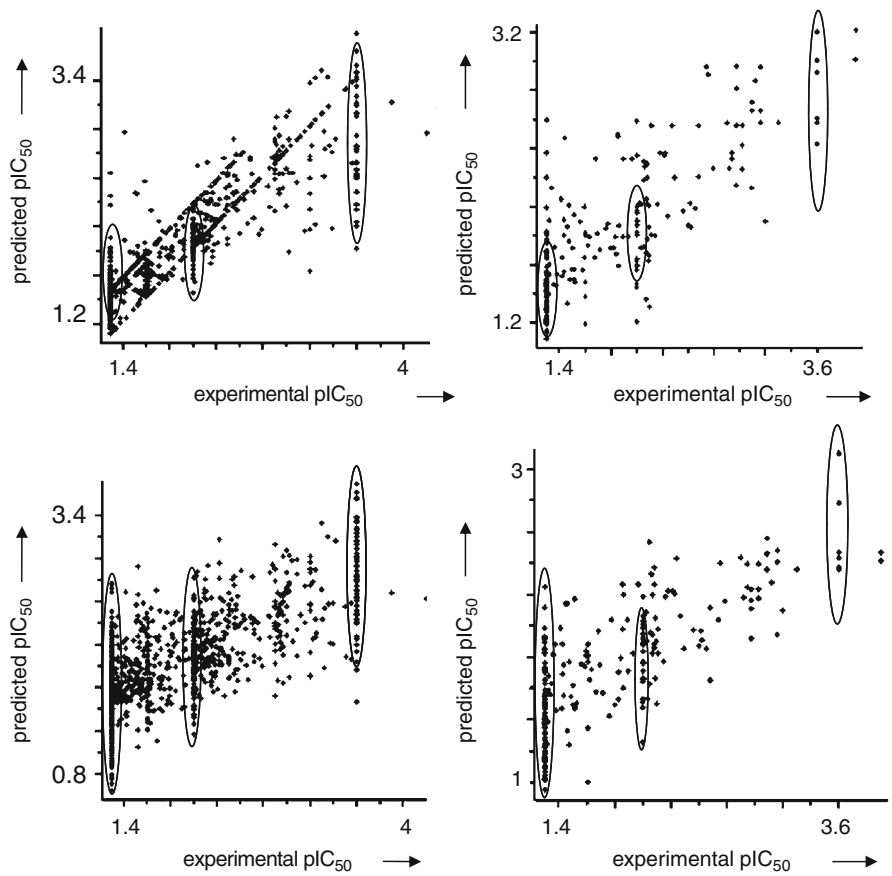

Fig. 41.11 Predicted versus experimental pIC_{50} values for selected QSAR models to identify potential CYP2C9 inhibitors. *Upper panel*: SVM-3PP based model with training set (1,100 molecules, *left*) and test set performance (238 molecules, *right*). *Lower*: PLS-CMQA-based model with training (*left*) and test set performance (*right*)

the inactive compounds were predicted to have binding activity for CYP2C9—both a consequence of the complexity of interactions with CYP2C9.

For further model validation, we employed a second in-house dataset with 344 compounds from a focused library for G protein-coupled receptor (GPCR) targets and data from the same CYP2C9 inhibition assay (Byvatov et al. 2007). In addition, a third dataset by Afzelius et al. (2002, 2004) with 34 active and 49 inactive molecules and CYP2C9 inhibition Ki values from AstraZeneca was used. In both cases, the performance was monitored using receiver operating characteristic (ROC) curves as index of the ability of a classifier to discriminate between actives and inactives. While for the 238 compounds test set 6% false positives using SVM-3PP and 3% using PLS-CMQA were observed, the number of false negatives is significantly higher with 16% and 22%, respectively (Byvatov et al. 2007). This picture is related for the GPCR dataset with 5% false positives for SVM-3PP and PLS-CMQA, but 24 and 26% for the GPCR library, respectively. Furthermore, a total of 75% from the Afzelius dataset was correctly classified, providing additional evidence for the reliability of this model.

CRITICAL ASSESSMENT OF THE METHOD

Both computational models from a consistent dataset with values from a single laboratory are able to identify true CYP2C9 inhibitors with a relatively small rate of false positives and thus provide relevant filters to detect potential CYP2C9 inhibitors early in discovery (Byvatov et al. 2007). This fits to the primary application scenario in an industrial setting, where active compounds should be flagged with high reliability and inactive CYP2C9 inhibitors will be subsequently tested. Thus, additional experimental validation is required for those molecules passing the initial filter to identify false negatives. These types of filters are usually the ones, which will be implemented in multidimensional compound design and optimization.

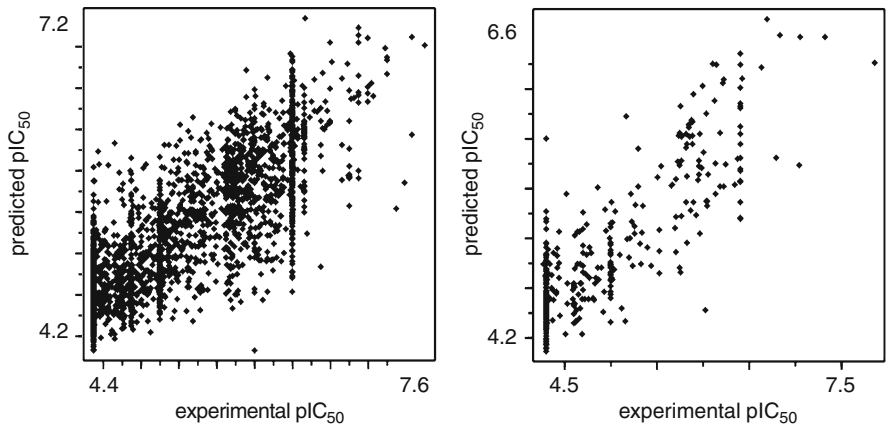

Fig. 41.12 Correlation of MOE descriptors with human CYP2C9 inhibition (pIC_{50}). *Left*: predicted versus experimental pIC_{50} values for the training set of 2484 molecules. *Right*: predicted versus experimental pIC_{50} values for the test set of 454 molecules

MODIFICATION OF THE METHOD

Following our positive experience upon prospective application of this CYP2C9 inhibition model after a certain period of time, we extracted a significantly larger dataset from our internal collection of CYP2C9 data accumulated after a period of experimental testing for numerous discovery projects. From this pool, we selected a representative training set of 2,484 molecules and a test set of 454 molecules employing statistical design. As our experience using the combination of MOE descriptors plus Cubist-based regression trees was also very positive, we decided to apply this combination of methods for this updated dataset.

After compound standardization and 3D conversion, a total of 184 descriptors were computed using the software package MOE (CCG). We then used Cubist (RuleQuest) to derive regression tree models (Quinlan 1992; Butina and Gola 2003) based on the training dataset and all descriptors. As previously mentioned above, Cubist builds a collection of rules with associated multiple linear regression (MLR) models to partition the training dataset and capture the SAR for all members of a particular rule. We used a genetic algorithm to select useful descriptors from an initial model toward an improvement in terms of regression coefficients and to obtain a model with reasonable test set performance.

The final regression tree model after this procedure is based on 18 rules and 56 descriptors. While for the training set an r^2 value of 0.674 and a crossvalidated r^2(cv) value of 0.504 (tenfold crossvalidation) were obtained, the predictive r^2 value of 0.723 for the test set of 454 molecules indicates a useful model, which we have implemented for design. The plot of experimental versus predicted pIC_{50} values for the model is shown in Fig. 41.12. We now use this model in a semiquantitative way for classification of potentially active CYP2C9 inhibitors.

The further availability of consistent inhibition data from globally harmonized CYP450 inhibition assays for CYP3A4 and CYP2D6 together with a broad coverage of chemical space also prompted us to establish predictive models using the same setup for these "antitargets." Model building was done accordingly to CYP2C9, which resulted for CYP2D6 in a model with 23 rules and 60 descriptors from 1,862 training set and 484 test set molecules. The r^2 value for the training set is 0.706, the crossvalidated r^2(cv) value is 0.504, while the predictive r^2 value is 0.792. A related result was obtained for CYP3A4 with a training set of 2,435 and a test set of 426 compounds. Here, the final model consists of 20 rules and 69 descriptors with a training set r^2 value of 0.640, a crossvalidated r^2(cv) value of 0.436, and a predictive r^2 value of 0.672. Both models are characterized by high predictive r^2 values and thus were investigated further in prospective applications. In total, we have now implemented significant and predictive QSAR models to identify potential inhibitors for the three major CYP450 isoforms: CYP2C9, CYP2D6, and CYP3A4.

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41.3.4 Predicting Paracellular Intestinal Permeability for Thrombin Inhibitors

PURPOSE AND RATIONALE

During lead optimization, the use of chemotype-specific models offers advantages over general models, including the coverage of the relevant chemistry space for a discovery project and the availability of experimental data for a series of analogues usually from a single laboratory. Even if these data are not strict single-mechanism data, there is often evidence that molecules on a particular chemotype share a particular mechanism of action.

Many pharmaceutical companies are actively working on the development of orally active inhibitors of the serine protease thrombin as one important enzyme in the blood coagulation cascade. In many cases, however, the systemic exposure to thrombin inhibitors

following oral administration is limited due to low intestinal permeability rather than low solubility or metabolic instability. Most synthetic inhibitors require a highly basic functional group like benzamidine or guanidine for favorable interactions to the S1 pocket of thrombin, thus gaining a significant portion of its binding affinity. However, these moieties often cause low intestinal permeability.

To obtain more insight into the problem of permeability for benzamidine derivatives, Sugano et al. (2000) studied the membrane permeation of 51 benzamidine-based thrombin inhibitors in a rat-everted sac permeability model. They reported significant membrane permeability in this in vitro model, which they attributed to passive paracellular transport, an absorption mechanism being different to transcellular permeability. Using Caco-2 cell experiments, they confirmed that opening the tight junctions increases permeability of some compounds which underscores that this paracellular pathway is dominating for this chemotype. Using physicochemical descriptors, the authors obtained significant models to understand structural determinants for paracellular intestinal permeability. This homogeneous experimental dataset for permeability of a congeneric series was used by us to build a quantitative local model employing VolSurf descriptors (Cruciani et al. 2000a). Such a chemotype-specific model in combination with similar models for related properties like solubility and others could be valuable during multidimensional compound optimization in the lead optimization phase. We use this model as an example to illustrate the generation and use of related local models in optimization.

PROCEDURE

The set of 51 benzamidine-based thrombin inhibitors was taken from the study of Sugano et al. (2000). Experimental rat-everted sac permeability was expressed as log(ESA) value[2]. The experimental permeability in this assay is expressed as ratio of outer (mucosal side) concentration of the drug and inner (serosal side) concentration after 1-h incubation of the everted sac of rat small intestine. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry, and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000b) was computed and analyzed as described above. All descriptors were normalized using autoscaling and subjected to PLS analysis. No further variable selection was performed (Cruciani et al. 2000c).

EVALUATION

This procedure led to a predictive 4-component PLS model for 72 VolSurf descriptors and 51 thrombin inhibitors. A crossvalidated r^2(cv) value of 0.599 after leave-one-out crossvalidation and a conventional r^2 value of 0.812 were obtained. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation procedures underscores the significance of the final model. The graph of experimental versus calculated log(ESA) permeability values is shown in Fig. 41.13 on the left. The overall model quality corresponds to the model reported by Sugano et al. (2000).

Based on the study of Sugano et al. (2000) and our predictive VolSurf model for this series, it can be concluded that factors like size and shape previously reported to affect paracellular permeability are indeed important to explain the local structure-permeability relationship of this chemotype. Usually, permeability via paracellular aqueous pore diffusion depends on the size of the solute and its diffusion coefficient in water. Another important factor is lipophilicity. Between intestinal absorption and both volume and lipophilicity, a negative correlation was reported for this series of thrombin inhibitors. In addition, hydrogen bonding properties and polarity are factors that determine the permeability of these benzamidine analogues. These findings are in good agreement to the interpretation of the VolSurf model from inspection of the PLS coefficient plot for the four-component model (Fig. 41.13 right). This plot is the result from the multivariate statistical analysis showing that factors like hydrophilicity from water interactions (W*), capacity factors (Cw*), and hydrogen bonding properties (HB*) are positively correlated with log(ESA). Increasing these descriptors will positively affect the passive paracellular permeability in this series. In contrast, factors like volume (V), surface area (S), shape (R), lipophilicity (D*), polarizability (POL), and molecular weight (MW) are negatively correlated with permeability.

[2]According to the authors, everted sac permeability was abbreviated by ESA.

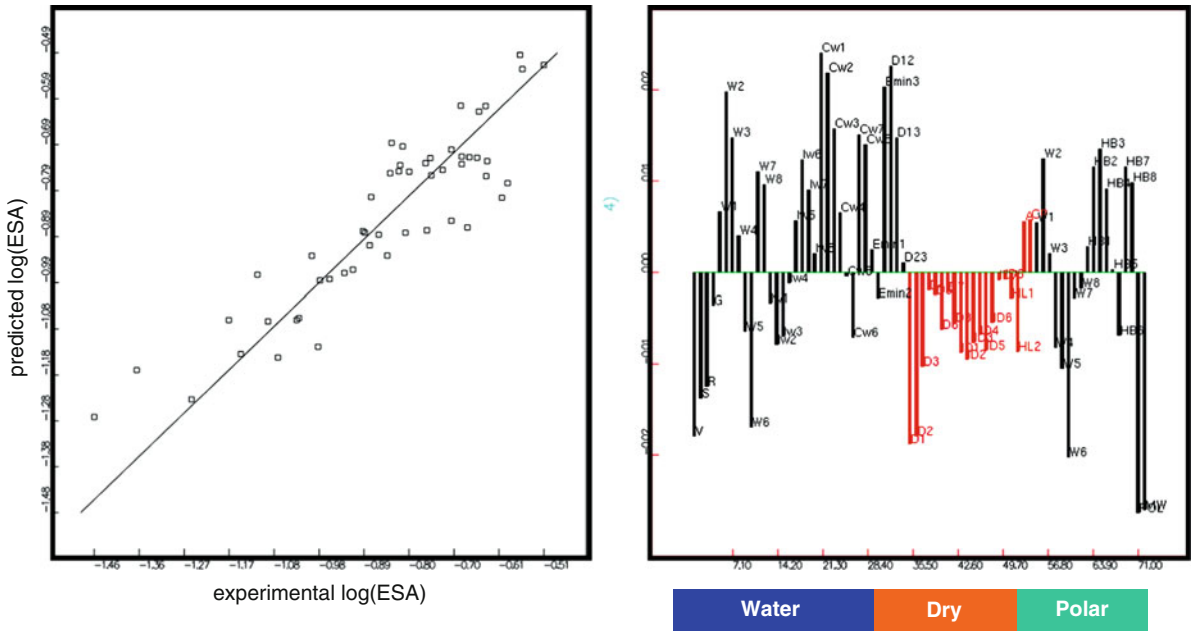

Fig. 41.13 Correlation of VolSurf descriptors with intestinal paracellular passive absorption for 51 benzamidine-based thrombin inhibitors. *Left*: predicted versus experimental permeability log(ESA) from the 4-component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of paracellular permeability

CRITICAL ASSESSMENT OF THE METHOD

This example shows that a quantitative structure-permeability relationship could be established in a local series of thrombin inhibitors predominantly following a particular route of intestinal permeability via the paracellular pathway. The presented VolSurf model does not only agree to dominant factors discussed in the literature but also builds a statistical basis to quantify individual influences by informative descriptors of physicochemical relevance. Although permeability in general is related to different underlying mechanisms, there is evidence that in this local series the permeability follows only a single route. The detailed structure-permeability relationship on the other hand will guide drug discovery project teams to introduce structural variations into the presented thrombin inhibitor scaffold and quantitatively assess the potential for paracellular permeability before synthesis. However, it is an intrinsic limitation of such a chemotype-specific model that predictions for structurally less related structures are not possible. Furthermore, it cannot be ruled out that a particular derivate does not follow any longer this primary route of permeability which is assumed to form the basis for the present investigation. Besides the molecular modeling aspects of this model, the clinical relevance of paracellular transport, as shown by this example, remains to be elucidated.

Hence, local statistical models provide a qualitative ranking of candidates and, thus, are valuable for optimization of pharmaceutically relevant compounds, especially if combined with additional models to understand affinity, selectivity, or any particular pharmacokinetic behavior.

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41.3.5 Predicting Rabbit Systemic Exposure for MMP-8 Inhibitors

PURPOSE AND RATIONALE

In search for potent and systemically available inhibitors of the matrix metalloproteinase MMP-8 (Matter et al. 1999, 2002) following oral administration, a local ADME model was derived to support lead optimization. For an internal series of inhibitors on the tetrahydroisoquinoline scaffold, hydroxamic acids for zinc ion binding in three position are essential for MMP affinity in first-generation inhibitors. However, those compounds are characterized by insufficient pharmacokinetic properties and low systemic exposure following oral administration. Driven by X-ray and 3D-QSAR studies (CoMFA), alternative Zn^{2+} binding groups like carboxylates were investigated. The expected loss in affinity for some members of this series was compensated by filling the MMP-8 S1' pocket close to the catalytic zinc ion. The design and structure-activity relationship (SAR) for this series are in agreement with protein binding site requirements and monitored multiple properties including selectivity against the undesirable MMP-1.

We decided to build a chemotype-specific VolSurf model (Chap. 2.2 "Safety Pharmacology: Introduction" and Cruciani et al. 2000a) for 49 MMP-8 inhibitors to better understand molecular and physicochemical factors controlling the systemic exposure following oral administration in a rabbit animal model. This study provides an example where in vivo data have been employed fulfilling certain requirements to build a model for some pharmacokinetic aspects. However, one has to be aware that the obtained experimental data after oral dosing to rabbits do certainly not fulfill the requirement of representing only a single mechanism. Hence, in the initial phase of model building, compounds with metabolic instability from in vitro S9 assays and very low solubility were rejected from the dataset in order to primarily focus on the absorption aspect of the systemic exposure in rabbits following oral administration. This model illustrates the use of local models from in vivo data after filtering by in vitro properties in lead optimization.

PROCEDURE

The systemic exposure of a series of 49 MMP-8 inhibitors was determined following oral administration to rabbits under consistent conditions for inhibitor dosing and formulation. These inhibitors represent different scaffolds and zinc ion-binding functionalities. From the pharmacokinetic profiles of these animal studies, the total area under the curve $AUC_{(oral)}$ was converted into $-\log(AUC_{(oral)})$ values and used to build the statistical model. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry, and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000b) was computed and analyzed as described above. All descriptors were normalized using autoscaling and subjected to PLS analysis. From initial models, a set of 46 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000c).

EVALUATION

For these 49 MMP-8 inhibitors, the systemic exposure in rabbits following oral administration was successfully correlated to VolSurf descriptors. A semiquantitative four-component PLS model using 46 descriptors was obtained with a crossvalidated r^2(cv) value of 0.424 after leave-one-out crossvalidation and a conventional r^2 value of 0.646. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation underscores the significance of the model. The validation suggests applying the final model in a semiquantitative way by classification of novel compounds into those with low, medium, or high systemic exposure. The graph of experimental versus calculated $-\log(AUC_{(oral)})$ permeability values is shown in Fig. 41.14 on the left.

Further validation of this model was done by splitting the dataset into a training set of 39 and a test set of 10 compounds using statistical design after a principal component analysis (PCA) on VolSurf descriptors following the most descriptive compound (MDC) algorithm (Hudson et al. 1996b). A 3-component PLS model with a crossvalidated r^2(cv) value of 0.457 and a conventional r^2 value of 0.673 indicates a statistically significant model. The standard error of prediction for 10 external compounds indicates medium predictivity but reasonable classification into low, medium, and high systemic exposure.

The analysis of PLS coefficients from the final model allows to assess the importance of individual descriptors contributing to the systemic exposure in rabbits following oral administration in this series. The corresponding PLS coefficient plot is shown in

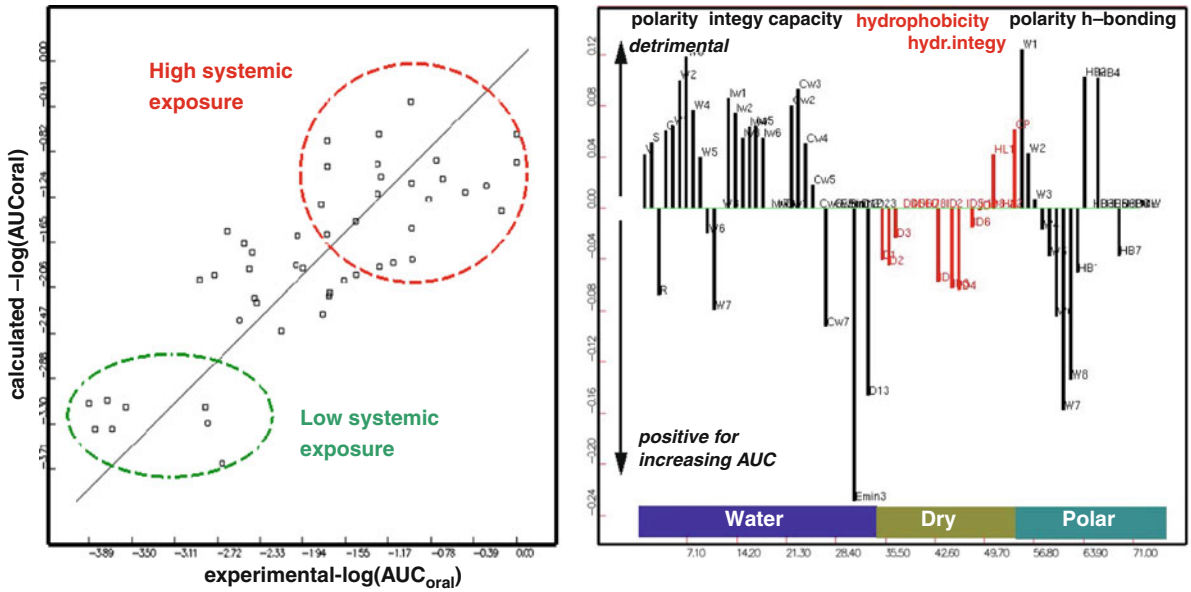

Fig. 41.14 Correlation of VolSurf descriptors with systemic exposure after oral administration in rabbits for 49 structurally diverse MMP-8 inhibitors. *Left*: predicted versus experimental systemic exposure $-\log(AUC_{(oral)})$ from the 4-component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of the systemic exposure in rabbits

Fig. 41.14 on the right. Please note that due to the chosen logarithmic scale for AUC values, favorable descriptors are now located in the lower area of this plot. Factors like shape (R), strength of interaction energy with water (Emin3), hydrophobicity (D*), integy moment from hydrophobic interactions (ID*), and interactions to the carbonyl O probe (W*) are favorable for the systemic exposure. Increasing these descriptors might lead to increased AUC after oral dosing. In contrast, polarity from the water probe interactions (W*), integy moment from the water probe (Iw*), capacity factor (Cw*), and hydrogen-bonding terms (HB*) are negatively correlated to the systemic exposure.

This interpretation, although derived for a local series of MMP inhibitors, is in qualitative agreement to models for permeability and intestinal absorption. The effects of polarity versus hydrophobicity and the importance of hydrogen-bonding interactions are consistently highlighted in comparison to general absorption model but with different importance of individual descriptors which have been tailored by PLS to the problem under study.

CRITICAL ASSESSMENT OF THE METHOD

While the chemical interpretation of this model qualitatively agrees to general models for permeability and absorption, those conclusions were derived only for a small, limited series of analogues on representative scaffolds. Furthermore, all published models in this field either describe human intestinal absorption or in vitro permeability assay data, while there are significant differences between rabbits, rats, and humans in terms of permeability and metabolism. Consequently, it cannot be ruled out that this model is very focused to the rabbit situation, because important animal studies to profile MMP-8 are conducted using rabbits. Although this model is not based on single-mechanism data, the focus on a particular series of related scaffolds might help to partially solve this problem. The quality of the model also suggests that more than one mechanism actually is responsible for the observed pharmacokinetic profile for which the AUC is only one appropriate way to summarize this behavior. Thus, a combination of such a final model from in vivo studies combined with some appropriate filters from informative in vitro assays might help to resolve this problem.

The resulting semiquantitative model was used in conjunction with structure-based docking and scoring, 3D-QSAR-based affinity and selectivity predictions, and in silico ADME models to estimate membrane permeability, solubility, and other key properties for the optimization process in this series. Hence, in

this as well as in other series, multiple models can be collectively applied for ranking and prioritizing synthesis candidates and focused virtual libraries during advanced stages of multidimensional compound optimization.

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41.4 Conclusion and Outlook

In recent years, the pharmaceutical industry has come under increasing pressure to reduce the time as well as the expenditure for the discovery and development of novel drugs. As a consequence, sophisticated in vitro assays have been introduced aiming at the simulation and prediction of the in vivo situation. However, besides the generation of huge sets of data all these assays still need the synthesis of the compounds. To overcome the issue of handling the generated information as well as to support medicinal chemists in selecting the most promising compounds for synthesis, in silico approaches revealed to be indispensable tools. in silico tools can be used to extract relevant information from large datasets, thereby assisting in the improvement of the understanding of factors controlling affinity, selectivity, as well as ADME and physicochemical compound properties. Furthermore, in silico approaches are extremely valuable tools for the design of new compounds with improved properties during a multidimensional optimization phase. Hence, these tools represent a key technology in the transformation from time-consuming trial-and-error strategies to faster prediction approaches.

Facing the growing number of in silico approaches, the list of tools discussed in this chapter can only represent a selection to illustrate application areas. The tools described in more detail have proven their suitability to support the success of drug discovery project teams. The ADME models discussed in this chapter are dominated by physicochemical molecular properties. Models for other ADME properties like transporter binding have not been discussed because they are dominated by complementarity in protein-ligand interactions. Although also important, the discussion of these models was beyond the scope of this chapter.

In different phases of a drug discovery project, different in silico models are applied, namely, general models in earlier phases and chemotype specific models later when more experimental information about analogues is available for a research team. Hence, the effective generation and storage of meaningful data in databases to address specific ADME and pharmacokinetic properties are essential for timely success of a project. That does not only imply the use of advanced in vitro assay technology toward single-mechanism ADME data but also a data infrastructure for re-using valuable data and knowledge in subsequent design phases to effectively support lead optimization.

The problems of today's in silico models for ADME properties are mainly associated with data availability, consistency, and quality to solve particular problems. Accurate and consistent date is an indispensable prerequisite for predictive models. In particular the use of multi-mechanism data often results in failures in model building and application. Using complex data might only be possible, if congeneric series are monitored as related compounds might act by similar mechanisms.

Finally, it is important to note that neither a single approach is likely to solve all problems for a chemotype nor is an approach equally well suited for every problem. The challenge lies in the selection of the most suited model for a given problem and in the effective integration of experimental and computational tools. Once the suited in silico tools are established, they can make a major contribution to the successful lead optimization process resulting in a novel drug.

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Metabolism Studies In Vitro and In Vivo

42

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42.1 Introduction

In the era of combinatorial chemistry and high-throughput screening, a huge number of hits and structural analogs potentially interesting as new chemical entities (NCEs) can be produced in a short period of time. Drug metabolism is a decisive determinant of the pharmacokinetic behavior of these compounds. Approximately three quarters of the top 200 prescribed drugs in the United States in 2002 are cleared by metabolism, one-third are cleared via the kidney, while biliary clearance of unchanged drug plays only a minor role (Williams et al. 2004). Thus, understanding and description of the metabolism of a new chemical entity is an essential part of the submission dossier (Weaver and Jochemsen 2009) as well as an important optimization parameter in drug discovery programs to reduce attrition in drug development (Kola and Landis 2004).

In vitro biotransformation tests are one piece of the puzzle to understand the pharmacokinetic characteristics of a given compound, to optimize PK parameters, and to select the most drug-like compounds that will progress into development (Eddershaw et al. 2000; Li 2004; Masimirembwa et al. 2003; Wang 2009; Baranczewski et al. 2006). Relatively recent prospect of obtaining equivalent data from in vitro and in vivo studies has provided the pharmaceutical industry with an incentive to validate in vitro models with respect to increase throughput and/or to replace animal studies where appropriate. Moreover, in vitro test systems are the only humanized models in early development (Coleman et al. 2001). An early assessment using animal in vitro and in vivo data together with

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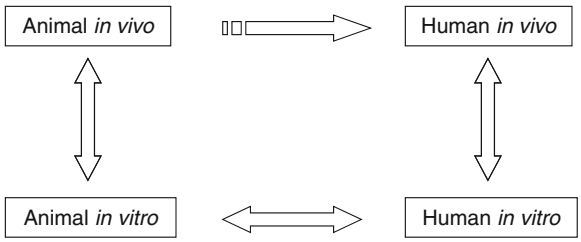

Fig. 42.1 Qualitative prediction of compound properties in humans from animal models using in vitro and in vivo data

human in vitro data allows a qualitative prediction whether humans will act in similar (path-) ways as did the animal models (Fig. 42.1).

In vivo biotransformation studies play a role later in development in both animals and humans (Gupta and Atul 2000; Inskeep and Day 1999; Pool 1999). Use of transgenic animals facilitates understanding the role of drug-metabolizing enzymes in the organism (Gonzalez and Kimura 2003). However, animal studies cannot entirely replace clinical studies in predicting all responses in human, particularly because of pronounced species differences between humans and the important laboratory animals (Martignoni et al. 2006), but, for ethical reasons, the risk to human volunteers participating in early clinical studies should be minimized (Cross and Bayliss 2000). This is supported by a variety of in vitro metabolism studies.

Metabolic stability tests can be performed in higher throughput (White 2001), especially due to the development of more sensitive LC-MS/MS instruments and the ability to analyze more analytes in parallel. They allow ranking of compounds and ensure that the molecules resulting from the optimization process retain favorable metabolic properties. In addition, they give rise to set up computational models predictive for the in vitro test which helps to speed up the selection and optimization processes although applications on the biotransformation of drugs are still limited (Lipscomp et al. 2008; Chohan et al. 2006; Li 2002; Gombar et al. 2003; Yu and Adedoyin 2003; Bugrim et al. 2004; see also Chap. 51, "In Silico Methods").

Metabolite structure elucidation studies enable the identification of toxicologically relevant biotransformation ("hot spots," Kalgutkar et al. 2005; Prakash et al. 2008) and positions of metabolic attack ("soft spots"). During discovery programs, this is mainly based on in vitro studies to support medicinal chemists in improving metabolic characteristics (Nassar and Talaat 2004a) because of high efforts due to intense sample clean-up procedures and the effort to generate the samples.

Investigation of metabolic profiles in vivo ultimatively proves the validity of pharmacological and toxicological data obtained in animal models for humans. The toxicological aspect is highlighted in a series of publications on "metabolites in safety testing" (Baillie et al. 2002, 2003; Hastings et al. 2003; Davis-Bruno and Atrakchi 2006; Guengerich 2006; Smith and Obach 2006; Humphreys and Unger 2006) followed by regulatory guidances published by FDA (US-FDA 2008) and ICH (ICH 2009). The impact on the strategies applied in industry to meet these regulations has been expressed in technical considerations (Luffer-Atlas 2008; Zhang et al. 2009; Gao et al. 2010) as well as strategy proposals to organize the sequence of metabolism studies differently along the drug development phases (Obach et al. 2012). This has impact on the conduct and timing of the radioactive human ADME study by classical means (Krone et al. 2011 and literature cited therein) or the use of accelerator mass spectrometry (AMS) in microtracer studies (Smith 2011; Lappin et al. 2008 and literature cited therein, Garner 2000; Garner et al. 2000).

In vitro metabolism studies are indispensible for predicting drug–drug interactions (US-FDA 2012; EMA 2012; Tucker et al. 2001; Bjornsson et al. 2003; Zhang et al. 2007) and variability in exposure due to pharmacogenetic differences in the population. Besides studies on enzyme inhibition (see Chap. 40, "Drug -Drug Interaction – Enzyme Inhibition") and induction (see Chap. 39, "Drug -Drug Interaction – Enzyme Induction"), information has to be generated on the enzymes involved in the biotransformation of a drug particularly for drugs which are subject to high metabolic clearance in the liver. This reaction phenotyping is essential part of submission dossiers. A stepwise approach is recommended comprising of correlation analyses in individual livers, incubations in recombinant enzymes, and incubation in human liver microsomes using inhibitors or antibodies specific for the particular isozyme (Wienkers et al. 2003; Lu et al. 2003).

Metabolism of many xenobiotics, including drugs and toxic compounds, occurs mainly in the liver (De Kanter et al. 1999). Until now, in vitro studies on the metabolism of xenobiotics were usually performed using liver preparations as a first step such as isolated perfused livers,

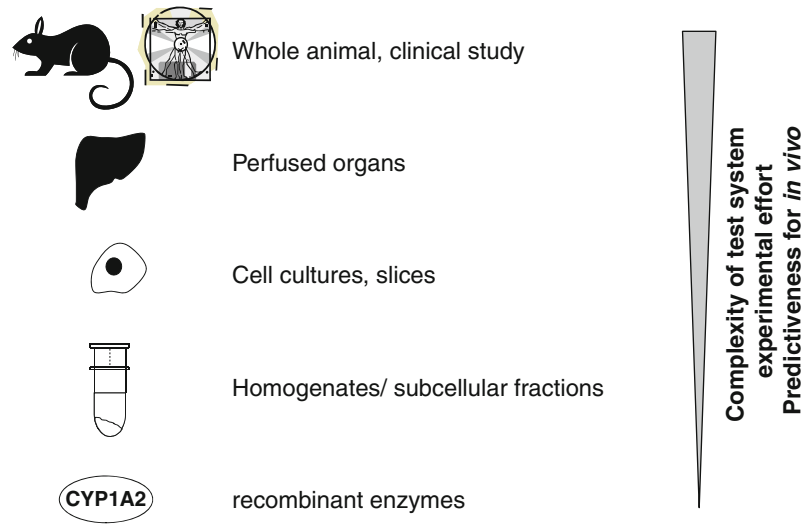

Fig. 42.2 Test systems for studying drug metabolism

Table 42.1 Comparison of in vitro test systems to study biotransformation (in part from Plant 2004)

In vitro system	Pros	Cons
Perfused organs	All metabolic active enzymes for phases I and II present including cofactors, whole metabolic profile observed, best correlation to in vivo	Expensive, ex vivo animal trial, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Slices	All metabolic active enzymes for phases I and II present including cofactors, whole metabolic profile observed, good correlation to in vivo	Expensive, ex vivo animal trial, diffusion controlled, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Cells in primary culture (e.g., hepatocytes)	All metabolic active enzymes for phases I and II present including cofactors, whole metabolic profile, population pools for cryopreserved hepatocytes possible, good correlation to in vivo	Expensive, batch variability, quality control, complex methodology, high technical effort, limited use for multiple compounds
S9 fraction	Easy to use, cheap, phases I and II present, whole metabolic profile observed	Addition of cofactors, lower enzyme activity than microsomes/supersomes
Microsomes	Easy to use, cheap, "population" pools	Addition of cofactors, only membrane-bound metabolizing enzymes such as CYPs, FMOs, and UGTs partial metabolic profile
Cytosol	Easy to use, cheap	Addition of cofactors, only soluble metabolizing enzymes such as alcohol dehydrogenases, sulfotransferases, glutathione *S*-transferase, *N*-acetyl transferases partial metabolic profile
Supersomes	Easy to use, moderately cheap, single enzyme only	Currently only CYPs, FMOs, UGTs, GSTs, and SULTs, single enzyme only, different CYP: oxidoreductase molar ratio, accuracy of kinetics questionable

liver slices, liver homogenates, isolated hepatocytes, subcellular liver fractions (S9, cytosol, microsomes), or overexpressed recombinant metabolizing enzymes, particularly cytochrome P450 isozymes. The decreasing order of tissue organization goes in parallel with the technical effort of using these models (Fig. 42.2).

Each of these in vitro systems has their specific values and limitations regarding availability of tissues,

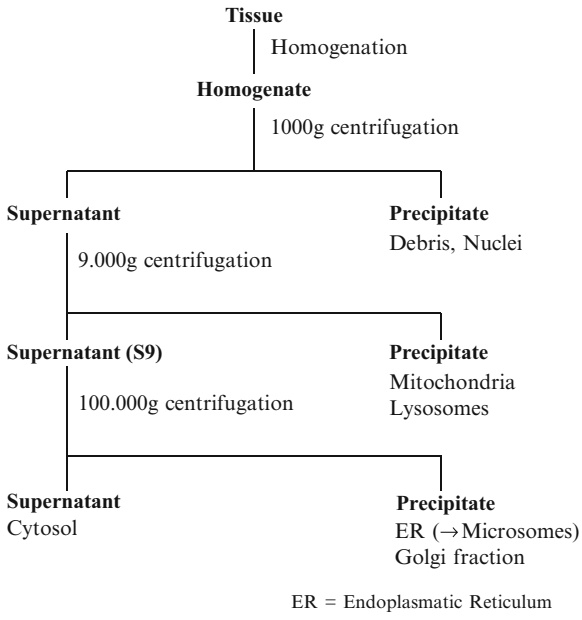

Fig. 42.3 Preparation of subcellular organ fractions by differential centrifugation (according to Ekins et al. 1999)

costs, completeness of enzymatic capability, ease of handling, and sensitivity to population differences (Plant 2004). Pros and cons have to be considered under the specific application intended, e.g., if higher throughput even in an automated environment is needed at the early stage of drug discovery support or if regulatory aspects are concerned. A comparison between the various systems to study the metabolism of a drug in vitro is given in Table 42.1 based on a review elsewhere (Plant 2004, see also Brandon et al. 2003).

Whereas sophisticated technologies are required for slices or organ perfusion studies (see also Chap. 35, "Distribution - In Vivo - Perfused Organs"), others can be easily performed with cells (Li 1999) or fractions commercially available. A typical preparation scheme for preparation of subcellular fractions by differential centrifugation is given in Fig. 42.3.

The biological function of metabolic transformation is to increase the water solubility of a drug. Typically, metabolism is divided into two steps

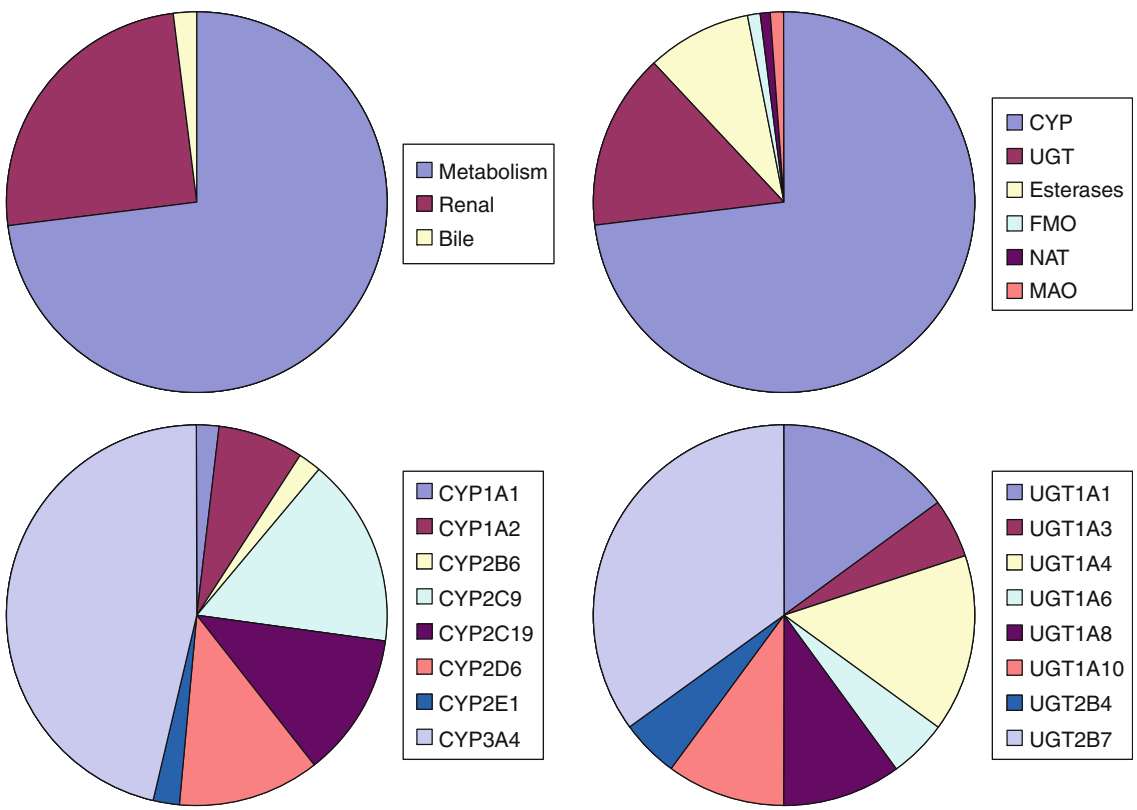

Fig. 42.4 Importance of clearance mechanism and the relative role of drug-metabolizing enzymes (Williams et al. 2004)

Table 42.2 Characteristics of cytochrome P450 isozymes (Donato and Castell 2003; Brandon et al. 2003)

Isoform	Occurrence	Major polymorphic variant alleles	Model substrates	Inhibitor	Inducer	Abundance in human liver
CYP1A1	Mainly extrahepatic	*CYP1A1*2*	7-ethoxyresorufin *O*-deethylation	alpha-Naphthoflavone (acceptable[a], inhibits also CYP3A4)	Polycyclic hydrocarbons	<1%
		*CYP1A1*3*				
CYP1A2	Liver		Phenacetin *O*-deethylation (preferred[a])	Furafylline (preferred[a])	Smoking	8–15%
					3-Methylcholanthrene	
			Caffeine N3-demethylation (acceptable[a])		Char-grilled meat	
					Rifampicin	
CYP2A6	Liver	*CYP2A6*2*	Coumarin 7-hydroxylation (preferred[a])	Coumarin (acceptable[a])	Pyrazole	5–12%
		*CYP2A6*3*			Barbiturates	
		*CYP2A6*4*				
		*CYP2A6*5*				
CYP2B1/2			Pentoxyresorufin *O*-dealkylation			
CYP2B6	Liver		(*S*)-mephenytoin *N*-demethylation (preferred[a])	Sertraline (acceptable[a], also inhibits CYP2D6)		1–5%
			Bupropion hydroxylation (acceptable[a])			
CYP2C8	Liver, Intestine		Paclitaxel 6-α-hydroxylation (preferred[a])	Glitazones (preferred[a])	Rifampicin, Barbiturates	10%
CYP2C9	Liver, Intestine	*CYP2C9*2*	(*S*)-warfarin C6-, C7 hydroxylation (preferred[a])	Sulfaphenazole (preferred[a])	Rifampicin	15–20%
		*CYP2C9*3*	Diclofenac 4′-hydroxylation (acceptable[a])		Phenobarbital	
			Tolbutamide para CH3-hydroxylation (acceptable[a])			
CYP2C18/2C19	Liver	*CYP2C19*2*	(S)-mephenytoin 4′-hydroxylation (preferred[a])	Ticlopidine (acceptable[a], also inhibits CYP2D6)	Rifampicin	<5%
		*CYP2C19*3*		Ketoconazole	Carbamazepine	
CYP2D6	Liver, Intestine, Kidney	*CYP2D6*2* × *n*	Bufuralol 1′-hydroxylation (preferred[a])	Quinidine (preferred[a])		2%
		*CYP2D6*4*	Dextromethorphan *O*-demethylation (preferred[a])	Haloperidol		
		*CYP2D6*5*	Codeine *O*-demethylation (acceptable[a])			
		*CYP2D6*10*				
		*CYP2D6*17*				
CYP2E1	Liver, Intestine, Leukocytes	*CYP2E1*2*	Chlorzoxazone 6-hydroxylation (preferred[a])	Diethyl-dithiocarbamate	Ethanol	7–11%
		*CYP2E1*3*	Lauric acid ω-1-hydroxylation (acceptable[a])	4-methyl pyrazole (acceptable[a])		
		*CYP2E1*4*				
CYP3A4	Liver, GI tract	*CYP3A4*2*	Midazolam 1′-hydroxylation (preferred[a])	Ketoconazole (preferred[a])	Rifampicin	30–40%
				Troleandomycin (preferred[a])	Barbiturates	
		*CYP3A4*3*	Testosterone 6β-hydroxylation (preferred)	Cyclosporine (acceptable[a])		
				Grapefruit juice		
CYP4A11	Liver, Kidney		Lauric acid ω-hydroxylation	17-Octadecynoic acid		

[a]Recommendation according to Tucker et al. 2001

More recent recommendations of substrates and inhibitors can be found in the EMA guidance on drug-drug interactions (EMA 2012)

Table 42.3 Characteristics of individual isozymes in the human UGT family (Coughtrie and Fisher 2003; Ritter 2000; Ohno and Nakajin 2009)

Isozyme	Probe substrates	Tissues	Reported inducers
UGTIA1	Bilirubin β-estradiol (3-gluc) 17α-ethinylestradiol (3-gluc)	Liver, small intestine, colon, stomach	3-methylcholanthrene, phenobarbital, oltipraz; phenytoin
UGTIA3	Hyodeoxycholic acid (COO-gluc)	Liver, small intestine, colon	
UGTIA4	Imipramine Trifluoperazine	Liver, small intestine, colon	
UGTIA6	Serotonin	Liver, brain, small intestine, colon, stomach	TCDD, TBHQ
UGTIA7	Benzo[a]pyrene metabolites	Stomach, colon, esophagus	
UGTIA8	Not known	Small intestine, colon, esophagus	
UGTIA9	Propofol	Liver, kidney, intestine, colon esophagus	TCDD, TBHQ
UGTIA10	Not known	Stomach, small intestine, solon, esophagus	
UGT2B4	Hyodeoxycholic acid (6-gluc)	Liver, intestine	
UGT2B7	AZT, morphine	Liver, kidney, small intestine, colon, esophagus	TBHQ
UGT2B10	Not known	Liver, kidney, intestine	
UGT2B11	Not known	Liver, prostate, mammary	
UGT2B15	(S)-oxazepam	Liver, prostate, small intestine, colon, stomach, esophagus	
UGT2B17	Dihydrotestosterone	Prostate, liver, small intestine, colon, stomach	

TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *TBHQ* *ter*-butylhydroquinone

(Lee et al. 2003): firstly, named phase I, functionalization of the molecule takes place leading to introduction or liberation of polar groups like alcohols, phenols, and amines by hydroxylation, dealkylation, heteroatom oxidation, and oxidative dehalogenation. Also, esters and amides are hydrolyzed.

Phase I metabolites, together with unchanged parent compound, are excreted via bile and urine, if sufficient solubility and/or transporter specificity is given. In a second step, conjugation reactions often increase polarity even more by glucuronidation, sulfation, or glutathione conjugation (phase II).

Enzymes responsible for these biotransformation reactions are present in many organs and tissues, the most important one in general being the liver. Under the enzymes involved in the biotransformation of drugs, the cytochrome P450 superfamily plays the most important role, especially CYP3A, followed by involvement of glucuronidases and esterases (Wrighton and Stevens 1992; Donato and Castell 2003; Kumar and Surapaneni 2001; Williams et al. 2004; Arinc 2010). The relative contribution of metabolic clearance pathways and the role of P450 isozymes to the biotransformation of drugs are shown in Fig. 42.4 (Williams et al. 2004).

Cytochrome P450s are present in the endoplasmic reticulum and therefore present in microsomal preparations. An overview on isoforms, polymorphisms, substrates, inhibitors, inducers, and occurrence of cytochrome P450s is given in Table 42.2 (Donato and Castell 2003; Brandon et al. 2003). It is noteworthy to mention that CYP3A being the isoform of highest abundance and predominantly involved in the metabolism of drugs is also highly present in the intestine (Galetin et al. 2008) and thus plays an important role in the intestinal first-pass metabolism of drugs.

As total inhibitors of the P450 enzyme family, 1-aminobenzotriazole (Lee and Slattery 1997) and proadifen (SKF525A) (Lee et al. 1998) are suitable to distinguish from non-cytochrome P450-mediated pathways. Other oxidative pathways are dependent on, e.g., flavin-containing monooxygenases (FMOs), molybdenum hydrolases (aldehyde oxidase and xanthine oxidase), amine oxidases, alcohol dehydrogenases or aldehyde dehydrogenases (Benedetti et al. 2006).

Table 42.4 Some properties of the human sulfotransferase enzyme family (Coughtrie and Fisher 2003)

SULT isoform	Probe substrates	Known drug substrates	Major sites of expression
SULT1A1	4-Nitrophenol	Acetaminophen, troglitazone, minoxidil, 4-OH tamoxifen, apomorphine	Adult liver, adult GI tract, adult platelets, placenta
SULT1A2	No selective substrate known	–	?
SULT1A3	Dopamine	Salbutamol, dobutamine	Adult GI tract, adult platelets, adult brain, placenta, fetal liver
SULT1B1	No selective substrate known	–	Adult liver, adult GI tract, fetal GI tract
SULT1C2	No selective substrate known	–	Fetal kidney, fetal lung, fetal GI tract
SULT1C4	No selective substrate known	–	Fetal kidney, fetal lung
SULT1E1	17β-Estradiol	17α-Ethinylestradiol	Fetal liver, fetal lung, fetal kidney, adult liver, endometrium
SULT2A1	Dehydroepiandrosterone	Budesonide, Dehydroepiandrosterone, Pregnenolone	Fetal adrenal, fetal liver, adult liver, adult adrenal
SULT2B1	Cholesterol (2B1b), Pregnenolone (2B1b)	–	Adult skin, prostate, placenta
SULT4A1	No selective substrate known	–	Brain

Also present in microsomes are flavin-containing monooxygenases (FMOs) involved in oxygenation of heteroatoms like nitrogen and sulfur (Lang and Kalgutkar 2003). Five different isoforms of FMOs are currently characterized. In adult human liver, FMO1 and FMO3 play an important role. Selective marker substrates for FMO1 are imipramine and orphenadrine. Methimazole inhibits FMO-dependent pathways (Wynalda et al. 2003) but also CYP2B6, CYP2C9, and CYP3A4.

Monoamine oxidases (MAOs) are present in the mitochondria and are involved in oxidation of endogenous and exogenous amines (Lang and Kalgutkar 2003).

Carbonyl reductases and alcohol and aldehyde dehydrogenases are cytosolic enzymes being involved in the oxidation of alcohols and aldehydes and in the reduction of aldehydes and ketones (Lang and Kalgutkar 2003; Garattini et al. 2008).

Uridine diphosphoglucuronosyl transferases (UGTs) are the most prominent enzymes within the phase II enzymes present in microsomes (Coughtrie and Fisher 2003). An overview on isoforms, substrates, and tissue expression is given in Table 42.3. UGT2B7, UGT1A1, and UGT1A6 are responsible for glucuronidation of two-thirds of the top 200 prescribed drugs in the United States in 2002 (Williams et al. 2004). Special attention is given to UGT1A1 with respect to phenotyping and drug interaction, since UGT1A1 is responsible for the conjugation of bilirubin (deficiencies in patients suffering of hyperbilirubinemia as Crigler-Najjar and Gilbert's diseases). UGTs are involved in the formation of *O*-, *N*-, and *S*-glucuronides. Formation of acyl glucuronides is considered to be of potential risk as they can form reactive intermediates (Shipkova et al. 2003). As for CYP3A, intestinal UGTs are discussed as potential modifiers of pharmacokinetics of drugs (Ritter 2006).

Cytosolic sulfotransferases are involved in the sulfation of endogenous compounds and drugs (Coughtrie and Fisher 2003). An overview on isoforms and their characteristics is given in Table 42.4.

Other important drug-metabolizing enzymes are microsomal and soluble epoxide hydrolases (Hassett et al. 1997; Srivastava et al. 2004; Omiecinski et al. 2000), glutathione *S*-transferases (Igarashi and Satoh 1989; Strange et al. 2001), *N*-acetyltransferases (Price-Evans 1989), and methyltransferases (Weinshilboum et al. 1999). Genetic polymorphisms of these enzymes play a role in metabolism and toxicity of drugs (Wormhoudt et al. 1999; Hengstler et al. 1998).

It becomes evident that organs other than liver, such as lung (Yost 1999; Zhang et al. 2006), kidney and intestine (Roediger and Babidge 1997; Paine and

Thummel 2003; Kaminsky and Zhang 2003; Shin et al. 2009), skin (Oesch et al. 2007), brain (Dutheil et al. 2009), and placenta (Syme et al. 2004), can also contribute to the metabolism of xenobiotics (De Kanter et al. 1999) and partly to organ specific toxicity.

Another important matrix for the metabolism of xenobiotics is blood. In addition to in vitro studies in whole blood, serum or plasma prepared from blood from humans or animals is used (Williams 1987). Under the enzymes present in blood, serum esterases have the most significant effect on drugs. These include cholinesterase (ChE), serum arylesterase (SA), carboxylesterase (CE), and red blood cell (RBC) esterases. These enzymes play a role in both activation of prodrugs and deactivation of drugs (Williams 1987). Recent examples of marketed prodrugs are adefovir, tenofovir, valganciclovir, olmesartan, parecoxib, tamiflu, famciclovir, and ximelagatran (Los et al. 1996; Bernardelli et al. 2002; Boyer-Joubert et al. 2003; Li et al. 1998; Vere-Hodge et al. 1989; Balimane et al. 2000; Clement et al. 2003; Powell et al. 1993). The stability in blood is also of special importance for the administration of peptides due to the presence of endo-, carboxy-, and aminopeptidases (Powell et al. 1993).

In addition to the potential of liberating the active drug upon absorption, orally applicable prodrugs require a sufficient stability in the gastrointestinal tract which is most importantly pH-dependent chemical stability (Balbach and Korn 2004) as well as stability against GI esterases as pepsin and pancreatin. Enzymatic or bacterial degradation of prodrugs in the GI tract can be studied in vitro, e.g., in Caco-2 cells concomitant to permeability experiments (see also Chap. 27, "Absorption: In Vitro Tests - Cell Based"), ex vivo (see also Chap. 28, "Absorption: In Vitro Tests - Non Cell Based"), or in vivo.

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42.2 In Vivo Biotransformation Studies

PURPOSE AND RATIONALE

In vitro studies can only give a limited, "mechanistic" picture of biotransformation in animals or humans. The quantitative importance of each individual metabolite needs to be assessed in vivo. Samples collected from in vivo studies give rise to comprehensive metabolite identification work (Watt et al. 2003; Clarke et al. 2001) which is also required from a regulatory point of view (Baillie et al. 2002). Due to the labor-intense nature of these studies and the need of applying radiolabeled compounds in order to get a complete picture of biotransformation, these studies are performed during preclinical and clinical phase of drug development. Of particular importance is the collection of human metabolism data from the human ADME study using radiolabeled drug (Krone et al. 2011) to identify relevant human metabolites and to compare their exposure between human and animal species used in toxicological studies and to assess their pharmacological activity.

PROCEDURE

Samples for a biotransformation investigation are typically collected concomitant to mass balance and radiokinetic studies in animal and human employing radiolabeled test compound (Iyer et al. 2001, 2003; Lantz et al. 2003; Hayakawa et al. 2003; Huskey et al. 2004; Cui et al. 2004; Cook et al. 2003). Plasma prepared from blood, urine, and feces samples serve as a basis. Additional matrices (e.g., tissues and bile) might complete the picture. Before analysis, samples, possibly after pooling, are processed in most instances to enhance sensitivity and to separate matrix components interfering with the analytical procedure, e.g., proteins. Protein precipitation is normally applied to plasma followed by a concentration step if necessary. Sample processing is mandatory for feces and tissue homogenates and is typically done by extraction with mixtures of organic solvents like acetonitrile or methanol with buffer in different ratios followed by evaporation of the extracts. Urine can be analyzed directly without processing if sample concentration is not needed.

In addition to generation of metabolite profiles of the original samples, information on glucuronic acid conjugates or sulfates (Walle et al. 1983) present as phase II metabolites can be gained by enzymatic digestions of complete samples or isolated metabolite peaks.

Urine and feces homogenates might serve as a basis for structure elucidation of metabolites by LC-MS/MS directly or—if necessary—by more detailed NMR/MS investigation after isolation of the metabolites by preparative chromatography.

EVALUATION

Samples are analyzed by a suitable chromatographic system, typically HPLC with online radiodetection. Metabolite profiles in plasma and tissues are given as percent of radioactivity present in total. Profiles in urine, bile, and feces are given as percent of the dose administered.

MODIFICATION OF THE METHOD

Different analytical methodologies adapted to the particular test compound and its metabolites are used which have to be optimized case by case and cannot be generalized. Whereas high-performance liquid chromatography (HPLC) is a method of choice for many pharmaceuticals, also other techniques like gas chromatography (GC), e.g., for profiling of steroids (Holland et al. 1986), or capillary electrophoresis (CE) might play a role. In recent years, the application of microplate scintillation counter for 96-well plates (TopCount) revolutionized the practice in offline radiodetection to enhance sensitivity and speed in analyzing low radioactivity level samples as an alternative to online radiodetection or offline radiodetection using the classical approach of liquid scintillation counting (Nassar et al. 2004b; Kiffe et al. 2003; Boernsen et al. 2000). Accelerator mass spectrometry (AMS) might serve as an offline radiodetector for samples with low radioactivity content.

CRITICAL ASSESSMENT OF THE METHOD

Despite the elaborate effort necessary for using radioisotopes, mainly carbon-14, metabolic profiling studies still play a dominant role in this field. Only quantification on the basis of radiodetection gives a reliable profile of structurally unknown metabolites formed from a given drug within a complex endogenous matrix. A quantitative comparison of metabolites by means of LC-MS/MS is only possible if the respective metabolites are known and available for calibration.

Application of hyphenated techniques, in particular LC-MS/MS, in combination to radiodetection or as a sole device in analysis of studies employing nonlabeled test compounds gives rise to rapid structural information (Watt et al. 2003; Clarke et al. 2001). However, uncertain extraction yield of metabolites has to be considered. The dominating role of LC-MS/MS is not reached by LC-NMR coupling despite of advances in this technique (Dear et al. 2000b). Other techniques applied in this context comprise improvements of hyphenated MS techniques like capillary liquid chromatography (Sandvoss et al. 2004), or ion exchange liquid chromatography-MS for charged polar molecules (Dear et al. 2000a).

In most instances, classical approaches in isolation of metabolites followed by MS and NMR analyses are still necessary to obtain the definitive structural information. Complex structures of metabolites require often multiple NMR experiments in addition to an initial one-dimensional ^{1}H NMR experiment for final structure elucidation.

Techniques applying test compounds labeled with stable isotopes are applied as well (Browne et al. 1993). However, they have not found broad acceptance in substituting radioisotopes in this field due to their restrictions in quantification of unknown metabolites.

Of particular interest is the generation of metabolite profiles across species to assess the sufficient exposure of human metabolites in animal species used in toxicological studies. Alternatives to the classical approach of a series of radioactive animal and human studies are proposed based on nonradioactive analyses (Gao et al. 2010).

Accelerator mass spectrometry (AMS) offers the possibility to perform metabolite profiling concomitant to microdosing or microtracer studies in humans applying ultralow amounts of radioactivity (Smith 2011; Garner 2000; Garner et al. 2000; Combes et al. 2003). At least for a limited number of representative samples, AMS might serve as an expensive offline radiodetector for HPLC eluates as well.

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EXAMPLE

Iyer et al. (2001) investigated the metabolism of [^{14}C] omapatrilat in humans with samples collected during a clinical study. Plasma samples were prepared from blood spiked with or without methyl acrylate to trap compound-free sulfhydryl groups, which was important for this particular compound. Samples were pooled over the 12 subjects enrolled in the study. Urine was pooled over time to give a 0–24 h pooled urine sample

representing 92% of the radioactivity excreted in urine and a 0–168 h sample. Feces were not analyzed.

For sample processing, pooled plasma samples (1 and 6 h, 2 mL) were mixed with acetonitrile (6 mL), vortexed vigorously, and centrifuged. The precipitates were again extracted with acetonitrile (2x), and the supernatants from previous extraction were combined. The pooled extracts were evaporated at room temperature under a stream of nitrogen, and the residues were reconstituted in HPLC mobile phase. A portion of the sample was used for radioactivity determination, and the remaining sample used for HPLC profiling.

Pooled 0- to 24-h and 0- to 168-h human urine samples were concentrated on an Oasis HLB 3.0-mL cartridge. The cartridge was loaded with pooled urine (3 mL) and washed sequentially with water containing 0.1% acetic acid (2 × 3 mL, pH 3.2) and methanol (2 × 3 mL). Liquid scintillation counting of the water and the methanol extracts showed quantitative recovery of radioactivity in the methanol fractions. The methanol extracts were combined, evaporated to dryness under a stream of nitrogen, and dissolved in HPLC mobile phase.

Selected urine samples were digested for cleavage of conjugates. For this, a solution of β-glucuronidase (0.2 mL, 2,104 U) in water was added to a solution of 0- to 24-h pooled human urine (1.0 mL) in 0.2 M sodium acetate buffer (1.0 mL, pH 4.8). The mixture was incubated at 37°C in a water bath for 24 h. In addition, control experiments were done in the absence of the enzyme (negative control), positive control in the presence of phenolphthalein glucuronide (1 mg), and incubation in the presence of the β−glucuronidase inhibitor 1,4-saccharolactone (10 mg). A 0.2 M glycine buffer (8.0 mL, pH 10.5) was added at the end of the incubation to the positive control. The amount of phenolphthalein generated was quantified against a phenolphthalein standard curve with a UV-visible spectrophotometer operating at 550 nm. All other samples were centrifuged for 5 min in a bench top microcentrifuge and stored at −20°C until further analysis.

Plasma and urine samples were profiled using a suitable HPLC system under specific conditions (gradient, column). Readout was done offline by fraction collection of the HPLC run followed by liquid scintillation counting. Biotransformation profiles were prepared by plotting the baseline corrected radioactivity against time after injection.

For metabolite isolation, 1.5 L of pooled urine was applied to a XAD-2 resin column first. The ethyl acetate extract obtained containing 85% of the radioactivity was applied upon evaporation to semi preparative HPLC on a Zorbax RX C18 column (9.4 × 250 mm, 5 μm) using gradient elution. Fractions obtained were further separated by isocratic elution on the semipreparative column. The metabolite fractions obtained were finally purified by preparative thin-layer chromatography. Liquid chromatography/mass spectrometry (LC/MS) and LC/MS-MS analysis was applied to the isolated metabolite fractions for structure characterization.

42.3 Perfused Organs

PURPOSE AND RATIONALE

In comparison to other in vitro systems for studying drug metabolism, metabolism studies with isolated organs allow a definitive conclusion about the contribution of a given organ to in vivo drug metabolism. Metabolism studies in perfused organs give the best correlation to the in vivo situation since all metabolic pathways and cofactors are available. In comparison to in vivo animal studies, experiments in isolated organs can be performed under precisely defined conditions such as composition of the perfusion media or perfusion rate. Since the liver is the most active organ for drug metabolism, liver perfusion studies are frequently used. Additionally, tissue preparation for perfusion studies in heart (e.g., Enser et al. 1967; Scheuer and Olson 1967), lung (e.g., Baciewicz et al. 1991; Kaneda et al. 2001), kidney (Nizet 1975; Newton and Hook 1981), brain (e.g., Thompson et al. 1968), and intestine (e.g., Pang et al. 1985; Cong et al. 2001) has been published.

Concentration of the drug and related metabolites is determined using appropriate analytical methods such as HPLC, LC-MS, or LC-MS/MS techniques. The use of a radiolabeled drug allows more easy and precise recognition of the drug and related metabolites.

PROCEDURE

It is described in Chap. 35, "Distribution - In Vivo - Perfused Organs".

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42.4 Organ Slices

PURPOSE AND RATIONALE

The slice technique was already introduced by Otto Wartburg in 1923 and commonly used in in vitro liver research until isolated hepatocytes and isolated perfused liver preparation were introduced and optimized (Krumdieck et al. 1980; Olinga et al. 1998). Previous methods of slicing using the Stadie-Riggs (Stadie and Riggs 1944), Vibratome (Smith et al. 1985), or handmade slice technique (Forster 1948) suffered on a rapid preparation of thin tissue slices of uniform thickness and dimension under conditions that minimize trauma to the live tissue (Krumdieck et al. 1980).

Since the introduction of the Krumdieck slicer (Krumdieck et al. 1980) and a new incubation system for slices (Smith et al. 1985), tissue slices are commonly used in drug metabolism and toxicity studies.

PROCEDURE

Cylindrical piece of tissue of about 12 mm in diameter is placed into the Krumdieck microtome and cut by means of a sharp punch. Slices are released into circulating buffer. The slice thickness is set by the screw-adjustable rest and is usually between 160 and 640 μm. The variability of the slice weight increases with increasing slice thickness (Krumdieck et al. 1980). Afterward, the slices were placed on the inside walls of stainless steel roller-equipped steel mesh scintillation vials containing 2 mL Waymouth's 752/1 media supplemented with 10% fetal calf serum and gentamicin (84 μg/mL). Vials were capped with elastic rubber septa (300 μM) to allow gas exchange and placed in an incubator set at 37°C with constant aeration (O_2:CO_2 = 95:5). The vials were rotated at 10 rev/min and culture media replaced every 12 h (Smith et al. 1985). Incubation of NCE is done in media in comparison to a solvent control (Dogterom 1993).

EVALUATION

Thickness and viability of the slices (e.g., morphology, ATP content) are investigated in parallel. Metabolic capacity of various enzymes is ensured by positive and negative controls during the incubation procedure. Concentration of the drug and related metabolites is determined using appropriate analytical methods such as HPLC, LC-MS, or LC-MS/MS techniques. The use of a radiolabeled drug allows more easy and precise recognition of the drug and related metabolites.

MODIFICATION OF THE METHOD

Vandenbranden et al. (1998) used additional antibiotics to reduce bacterial impurities during long-term cultivation.

The slicing technique can be adapted to various organs and allows tissues specific comparison of metabolism and toxicity within animals and humans. Stefaniak et al. (1988) used the method for metabolism studies in lung. Therefore, lung trachea or bronchiole (rat/human) was cannulated, and lungs were instilled with 1.5% (w/v) low melting agarose solution containing 0.9% (w/v) NaCl at 37°C and allowed to gel on ice. Tissue cylinders with an 8-mm diameter were cut as described from Krumdieck et al. (1980). Ruegg et al. (1987) adapted the method to investigate cell-specific toxicity in kidney cells.

CRITICAL ASSESSMENT OF THE METHOD

The use of liver slices as an in vitro tool for drug metabolism has various advantages compared to isolated hepatocytes such as faster and easier preparation, the presence of different cell types, and the maintenance of an intact tissue architecture with cell-cell and cell-matrix interactions (except the first cell layers which are always damaged because of the slicing

procedure), so that even in very good slices, normally no more than approximately 80% of the cells are intact (De Graaf et al. 2002). In general, the complete cellular machinery is available. Since mostly all organs are applicable to the slice technology, organ-selective drug toxicity or the relative contribution of the organs to the total body clearance can be investigated.

Organ slices are applicable to long-term cultures; viability up to 72 h has been described for rat liver slices (Fisher et al. 1995). On the other hand, investigation of enzyme activity revealed changes in the metabolic capacity of phase I and phase II enzymes in long-term cultures (Vandenbranden et al. 1998). De Kanter and Koster (1995) established a general method for cryopreservation of slices. Postthaw viability, especially phase I and phase II biotransformation activity, maintained for at least 4 h (De Graaf et al. 2002). Generally, cryopreservation allows a more universal use of liver slices since the method becomes independent from the availability of tissue, especially for human tissue. In addition, using slices from the same donor simplifies a comparison of compounds in the early stage of drug discovery.

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42.5 Primary Hepatocytes

PURPOSE AND RATIONALE

Isolated hepatocytes provide a convenient link between the complex architecture of the intact organ and subcellular fractions. The latter approach suffers from lack of relevance to the in vivo situation, while the former one, as close as possible to the liver in vivo, suffers from complex methodology and limited use for multiple compounds (Mandan et al. 2002; Gómez-Lechón et al. 2008).

Cryopreserved hepatocytes in suspension were successfully applied in short-term metabolism studies and as metabolizing system in mutagenicity assays (Hengstler et al. 2000b), providing qualitative metabolic information and quantitative pharmacokinetic parameters from key animal species and human at the early stage of drug discovery and drug development. Culturing hepatocytes in a sandwich configuration between collagen and Matrigel or on collagen alone have been studied and used in a broad variety of assays that requires prolong culturing such as clearance/metabolism studies, induction, inhibition, or hepatotoxicity studies (Hewitt et al. 2007).

PROCEDURE

Hepatocytes—either freshly prepared or cryopreserved—are commercially available or prepared in-house if intact liver can be accessed. Isolation of the animal hepatocytes follows after a two-step collagenase perfusion of the liver via the vena porta in situ (Seglen 1976) or via several blunt-end cannulae inserted into vessels available on the cut surface of

pieces of human liver obtained from resection. Liver cells are gently scraped out into suspension buffer and washed twice to three times by centrifugation to remove cell fragments and nonvital cells. Hepatocytes are used immediately or cryopreserved for further use according to published cryopreservation procedures (De Kanter and Koster 1995; Li 2007).

Isolation of hepatocytes according to the standard operation procedures mentioned below results generally in a viability of the hepatocytes ≥80%. Incubation with NCE is performed in various drug concentrations and incubation time point(s).

EVALUATION

Viability of the hepatocytes is usually determined by trypan blue exclusion rate. Viability over incubation time can be determined by LDH retention or albumin secretion (Gebhardt et al. 2003). For metabolism purposes, positive controls with well-known phase I and/or phase II metabolism are incubated in parallel to NCEs to assure metabolic capacity of the hepatocytes, e.g., ethoxyresorufin, ethoxycoumarin, testosterone for phase I metabolism, and 4-hydroxybiphenyl, 4-methylumbelliferone, 1-chloro-2,4-dinito-benzene, and 2-naphthol for phase II metabolism (Gebhardt et al. 2003). Concentration of the drug and related metabolites is determined using appropriate analytical methods such as HPLC, LC-MS, or LC-MS/MS techniques. The use of a radiolabeled drug in combination of LC/MS techniques allows an easier and precise recognition of the drug and related metabolites.

MODIFICATION OF THE METHOD

Since enzyme activities of hepatocytes in suspension decrease markedly with longer incubation time, hepatocytes in culture are required if longer incubation time is necessary, particularly phase II metabolism. To achieve longer incubation times, cultivation of freshly isolated or cryopreserved hepatocytes in culture on monolayer (Maslansky and Williams 1982; Wang et al. 2002; Pichard et al. 2006; Klieber et al. 2010), sandwich culture on variable matrices (e.g., Maurel (1996), Kern et al. (1997), Wang et al. (2002)), in coculture (Hengstler et al. 2000a, b; Gebhardt et al. 2003), or in bioreactors (perfusion culture system or 96 well bioreactor) (e.g., Bader et al. 1998; Wang et al. 2002; Gebhardt et al. 2003) are described. Immobilization of liver cells in alginate beats has facilitated commercialization of isolated hepatocytes (Guyomard et al. 1996; Rialland et al. 2000) but has not succeeded in a broad acceptance.

For elucidating the specific enzyme responsible for a certain metabolic step occurring in hepatocytes, specific inhibitors are useful. Selective or total inhibitors are used for cytochrome P450s in particular for CYP1A2, CYP2C9, CYP2D2, and CYP3A4 using furafylline, sulfaphenazole, quinidine, and ketoconazole and aminobenzotriazole (ABT) as total CYP inhibitor at low concentrations (1, 10, 3, 3, and 1,000 μM). Furafylline is a well-known suicide inhibitor which needs to be added prior to the incubation (Donato and Castell 2003; Newton et al. 1995; Rettie et al. 1995; Birkett et al. 1993). Also antibodies raised against specific enzymes are used for studying the involvement of these enzymes in the biotransformation. These are mainly in use for cytochrome P450s (e.g., Wang and Lu 1997) due to their commercial availability. An interesting advancement in the application of intact hepatocytes in the evaluation of metabolic stability is the incubation of the test article and hepatocytes in 100% human serum, therefore providing an experimental condition similar to humans in vivo (Li 2007). It has been reported that in vivo hepatic clearance can be predicted more accurately from data obtained with hepatocytes incubated in serum than from data obtained in the absence of serum using rat hepatocytes (Blanchard et al. 2004) and human hepatocytes (Blanchard et al. 2005). The combination of human hepatocytes and 100% human serum may represent a more physiologically relevant experimental model and eliminates the need to consider plasma protein binding and intracellular concentration for the prediction of in vivo metabolic clearance.

CRITICAL ASSESSMENT OF THE METHOD

Metabolism studies in hepatocytes might be a good compromise between perfused livers and subcellular fractions such as microsomes, since the complete cellular machinery is available. Isolation (and cultivation) of hepatocytes is still time- and lab-intensive and needs to be optimized for livers of every different animal species (De Graaf et al. 2002). Nevertheless, some pitfalls have to be taken in account:

A modulation in metabolic capacity and transport internalization of hepatocytes in suspension and in long-term cultures has been described (Hengstler et al. 2000a, b; Olinga et al. 1998). The loss of cofactors

such as NADPH are probably the reason for a stronger decrease of metabolic capacity of phase I and phase II metabolism in cryopreserved hepatocytes compared to fresh hepatocytes or liver homogenate (Hengstler et al. 2000b), whereas other groups suspect the absence of microcirculation for the downregulation of detoxification genes and loss of functional activity including a variety of P450 isoenzymes (Vinci et al. 2011). An increase of metabolism of cryopreserved hepatocytes of benzo[a]pyrene equally to freshly isolated hepatocytes was reported after Percoll centrifugation (Hengstler et al. 2000b). Hence, Percoll centrifugation is recommended before using cryopreserved hepatocytes in metabolism studies. Due to the variability of the enzyme activity with time, the maximum incubation time in suspension should be limited up to 4–8 h (Hengstler et al. 2000b; Olinga et al. 1998). Under specific culture conditions, hepatocytes retain their polarity (Swift et al. 2010).

Since fresh human liver samples are not available to many laboratories, cryopreserved hepatocytes from commercial sources are a good alternative. Various studies have shown that kinetic profiles are similar in cryopreserved and fresh hepatocytes, and CYP3A atypical kinetic phenomena are displayed in cryopreserved hepatocytes as well (Hewitt et al. 2007). Good prediction of in vitro clearance of 41 drugs by cryopreserved human hepatocytes was shown in 4 independent studies with drugs being CYP and UGT substrates (Browne et al. 1993; Hewitt et al. 2007). Pooled cryopreserved hepatocytes—similar as pooled microsomes—are available that reflects the "average donor" as well as cryopreserved hepatocytes from individuals from special subpopulations (e.g., donors with 2D6 and 2C9 polymorphic enzymes) which can be used for later screening approaches or detailed metabolism studies for regulatory documents. Since a couple of years, cryopreserved hepatocytes, which attach and form a confluent layer after cryopreservation which is used, e.g., for longer lasting induction studies are available from commercial sources as well (Pichard et al. 2006).

A direct comparison of drug clearances in freshly isolated rat hepatocyte suspensions and cultures revealed that the measured in vitro clearance for high-turnover compounds was lower for conventional monolayers compared to suspensions whereas conventional cultures gave a higher estimation for low turnover such as S-warfarin (Griffin and Houston 2005). Thus, hepatocytes in culture may be more suitable for predicting the Clint of low clearance compound (Blanchard et al. 2004).

As quality control, hepatocytes should be characterized using marker substrates for the major metabolizing enzymes in parallel to the investigations with new/unknown drugs.

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EXAMPLE

42.5.1 Isolation of Human Hepatocytes

The isolation of human hepatocytes, previously reviewed (Li 2007), is described according to Hengstler et al. (2000b).

Preparation of buffer solutions

Suspension buffer

- 620 mL glucose solution (9 g/L D-glucose)
- 100 mL KH buffer (60 g/L NaCl, 1.75 g/L KCl, 1.6 g/L KH_2PO_4 adjusted to pH 7.6 with NaOH)
- 100 mL HEPES buffer (60 g/L HEPES adjusted to pH 7.6, without carbogen equilibration)
- 150 mL amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagines, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1 g/L L-histidine, 1 g/L L-glutamic acid, 1 g/L L-glycin, 0.4 g/L L-isoleucine, 0.8 g/L L-leucine, 1.3 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.8 g/L L-valine. Dissolve the amino acids that cannot be dissolved at neutral pH by addition of 10 N NaOH and thereafter adjust pH to 7.6 by 37% HCL).
- 5 mL insulin solution (2 g/L insulin dissolved in 1 N NaOH, adjusted to pH 7.6 by 1 N HCl)
- 8 mL $CaCl_2$ solution (19 g/L $CaCL_2 \times 2H_2O$)
- 4 mL $MgSO_4$ (24.6 g/L $MgSO_4 \times 7H_2O$)
- 2 g BSA/L; dissolved in the mixture of the abovementioned solutions

Buffer A

- 498 mL washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjusted to pH 7.4 with 4 N NaOH)

- 2 mL EGTA solution (47.5 g EGTA/L; dissolved by addition of NaOH, adjusted to pH 7.6 by HCL)

Buffer B
- 498 mL washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjusted to pH 7.4 with 4 N NaOH) Collagenase buffer (Buffer C)
- (3.9 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 0.7 g/L $CaCl_2 \times 2\ H_2O$, adjusted to pH 7.2 with 4 N NaOH), prewarm to 37°C before use
- Dissolve 100 mg collagenase[1] in 200 mL of buffer C immediately before perfusion

Human liver samples, e.g., from liver resection or nontransplantable organs should be immediately transferred into ice-cold suspension buffer (resected tissue can be stored in ice-cold suspension buffer for at least 4 h). Liver samples of approximately 100 g should be cut off in such a way that they only present one cut surface. Perfuse with buffer A for 20 min at 37°C (3 drops/s). Perfusion should be performed by several blunt-end cannulae inserted into vessels of the cut surface. The number of the cannulae depends on the number of large vessels available on the cut surface. Perfuse with buffer B for additional 20 min at 37°C. Thereafter, perfuse with collagenase buffer for 30 min in a circulating way at 37°C. Transfer the tissue into a large Petri dish with suspension buffer; scrape liver cells gently out with spatula. Filter the liver cell suspension through gauze and centrifuge for 5 min at 50×*g*. Wash twice with suspension buffer, centrifuge again, and resuspend the final pellet in 30 mL suspension buffer. Determine trypan blue exclusion rate after a 1:1 dilution of the hepatocyte suspension with trypan blue solution (4 g/L trypan blue). A similar procedure is described for the isolation of rat hepatocytes (Hengstler et al. 2000b).

A standard operation procedure for the isolation of rat hepatocytes is published from Gebhardt et al. (2003).

42.5.2 Cryopreservation

The cryopreservation of hepatocytes is described according to Hengstler et al. (2000b).

[1]Selection of an adequate batch of collagenase is the key critical step for successful isolation of human hepatocytes. Whereas the majority of all collagenases allow successful isolation of rat hepatocytes, selection of a good batch for human liver is more critical. Mostly, the collagenase concentration has to be optimized for an individual batch of collagenase.

Adjust hepatocytes to 3 Mio cell/mL in suspension buffer in an Erlenmeyer flask. Incubate for 30 min at 37°C during gentle shaking and carbogen equilibration. Determine the volume (original volume) and centrifuge the suspension for 5 min at 50×*g* at 4°C. All further steps are performed at 4°C. Discard a volume equal to two-thirds of the original volume from the supernatant and resuspend the cell pellet in the remaining suspension buffer (one-third of the original volume) by shaking gently. Add ice-cold suspension buffer containing 12% (v/v) of DMSO to the cell suspension up to 50% of the original volume, resulting in a DMSO concentration of 4% and approximately 6 Mio/mL hepatocytes. After 5 min on ice, add suspension buffer containing 16% (v/v) of DMSO up to the original volume of the cell suspension, resulting in a DMSO concentration of 10% and 3 Mio/mL hepatocytes. After 5 min in ice, transfer the hepatocyte suspension to cold cryovials with 1.5 mL/vial. Start the freezing program within 5 min. The time period between the second addition of DMSO and initiation of the cryopreservation should not exceed 10 min. The freezing procedure should be performed as follows (can be performed by any computer-controlled freezing machine):
- Cooling in 10 min down to 0°C
- 8 min at 0°C
- In 4 min down to −8°C
- In 0.1 min down to −28°C
- In 2 min down to −33°C
- In 2 min up to −28°C
- In 16 min down to –60°C
- In 4 min down to −100°C

The temperature in the chamber and in one cryovial should be monitored by a chart record to control whether crystallization heat was sufficiently compensated. Transfer the cryovials into liquid nitrogen immediately after the freezing program has been finished.

42.5.3 Thawing

Thawing of hepatocytes is described according to Hengstler et al. (2000b).

Thaw the frozen hepatocytes quickly by gentle shaking in a 37°C water bath. The hepatocytes should thaw but not become warm. Transfer the hepatocyte suspension into an ice-cold Erlenmeyer flask immediately after

thawing and dilute DMSO gradually by the addition of ice-cold not carbogen-equilibrated suspension buffer, 0.5-, 1-, 2-, and 3-fold of the volume of the thawed hepatocyte suspension. Suspension buffer should be added dropwise, and hepatocytes should be on ice for 3 min before the next dilution step takes place. After centrifugation at $50\times g$ for 5 min at 4°C and resuspension in 10 mL suspension buffer, the hepatocytes can be purified by Percoll centrifugation.

42.5.4 Percoll Centrifugation

The Percoll centrifugation of hepatocytes is described according to Hengstler et al. (2000b).

Add the following into an ice-cold 50-mL Falcon tube:

- 10 mL of hepatocytes in suspension buffer (max. 20 Mio cells)
- 22 mL of suspension buffer (without carbogen equilibration)
- 18 mL Percoll solution

Mix gently and centrifuge at $250\times g$ for 20 min at 4°C. The pellet contains intact hepatocytes. Resuspend the cell pellet in suspension buffer and wash twice in suspension buffer (centrifugation at $50\times g$ for 5 min at 4°C).

42.5.5 Incubation of NCEs with Hepatocytes in Suspension

Transfer 1 mL of suspension buffer containing 1 Mio hepatocytes (fresh or cryopreserved) into glass vials (the diameter of the hepatocyte suspensions in the glass vial should be at least twice its height). Transfer the glass vials into a shaking water bath or in an air-conditioned rotating incubator (37°C, approximately 40 rpm). Add NCE in the appropriate concentration and incubate up to 2 h (Gebhardt et al. 2003), 4 h (Olinga et al. 1998), or 8 h (Hengstler et al. 2000b), respectively. Organic solvent should be minimized as much ($\leq$0.2% of DMSO or $\leq$1% methanol or acetonitrile) as possible to circumvent inhibitory effects of the solvents (Busby et al. 1999). The reaction is stopped by adding ice-cold acetonitrile and a sharp centrifugation step afterward. The supernatant is directly applied to HPLC or LC/MS analytics for quantification of the remaining compound and related metabolites. Note, activities of xenobiotic-metabolizing enzymes decrease within the incubation time (Hengstler et al. 2000b).

42.6 Homogenates

PURPOSE AND RATIONALE

Metabolism studies in tissue homogenate are probably one of the oldest methods and already published as early as in 1930s (Potter and Elvehjem 1936). Drug metabolism studies in homogenate are usually used to investigate potential species-species difference early in the drug metabolism research in view of selecting the appropriate species for toxicology studies. In comparison to subcellular fractions, e.g., S9 or microsomes, tissue homogenate contains most of the enzymes and sometimes cofactors necessary for function.

PROCEDURE

The tissue is homogenized at low temperature in buffer media using a mincer or a mixer such as a Waring blender or Ultra-Turrax followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle to produce a uniform suspension, which is immediately used or deep-frozen until use. Incubation with NCE is performed in various drug and cofactor concentrations with different incubation time point(s) and extraction procedures.

EVALUATION

Concentration and structure characterization of the drug and related metabolites are usually determined with HPLC, LC/MS, or LC/MS/MS techniques. The use of a radiolabeled drug in combination with LC/MS techniques allows an easier and more precise recognition of the drug and related metabolites.

MODIFICATION OF THE METHOD

Numerous applications using liver homogenates are published with variable drug and cofactor concentration, incubation time points, and workup procedures.

Kinetic but also dynamic responses are investigated in various other tissue homogenates such as lung (e.g., Zhang et al. 1996; Manautou et al. 1992), kidney (e.g., Gergel et al. 1992; Knudsen et al. 1996), and brain (Yagen et al. 1991; Aragon et al. 1992; Hornykiewicz 2002).

CRITICAL ASSESSMENT OF THE METHOD

Due to interindividual variation of the animals and differences in the enzyme panel of each individual, a quantitative comparison of two different compounds seems to be difficult. The same is true for reproducibility. Determination of the protein concentration should be performed and kept constant in the incubation.

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EXAMPLE

Tissues were immediately frozen on dry ice after sacrifice until use. The tissue is homogenized at 0–4°C in a fivefold volume (tissue wt/buffer vol) of 0.1 M potassium phosphate buffer containing 5% sucrose, pH 7.4 using a 15 s spin in a Waring blender followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle, to produce uniform suspension. The suspension was immediately used or deep-frozen until use (Norton et al. 1992). Incubation with NCE is usually performed in 1 mL suspension and at a drug concentration of 1 μM or 10 μM and glucose-6-phosphate dehydrogenase, glucose-6-phosphate, 0.4 mM TPN, and $MgCl_2$ (3.5 U/mL, 1 mM, 0.4 mM, and 10 mM, respectively) for 30 min at 37°C (Norton et al. 1992). Reaction was stopped by acidifying, and the drug and related metabolites are extracted with organic solvent.

According to Norton et al. (1992), the drug BW1370U87 was incubated in 1 mL suspension and at a drug concentration of 1 or 10 μM and glucose-6-phosphate dehydrogenase, glucose-6-phosphate, 0.4 mM TPN, and $MgCl_2$ (3.5 U/mL, 1 mM, 0.4 mM, and 10 mM, respectively) as cofactors for 30 min at 37°C. Reaction was stopped by adding 0.1 mL of 1 N HCl, and the drug and related metabolites are extracted with methyl-3°-butyl ether. Iley et al. (1999) demonstrated the biotransformation of various tertiary amidomethyl ester prodrugs at a concentration of 200 μM in 300 μL liver homogenate using the following cofactor concentrations: 6.25 mM glucose-6-phosphate, 1.25 mM $NADP^+$, 6 mM $MgCl_2$, and 2.5 U/mL glucose-6-phosphate dehydrogenase at 37°C. The reaction was stopped at various time points by adding ten volumes of acetonitrile (ice-cold) and centrifuged. 3-Methylcholanthrene-induced rat liver homogenates were used to study the metabolism of (+)-trans-benzo[a]pyrene at a concentration of 20 μM and various volumes of rat homogenate at 37°C (Sindhu and Kikkawa 1995). The incubation medium consisted of 50 mM Tris–HCl, pH 7.4 containing 150 mM KCl, 5 mM $MgCl_2$, and 0.5 mM NADPH in a total volume of 1 mL. After various time points, the reaction was stopped by adding H_2O-saturated ethyl acetate. For additional application, see also Zhang et al. (1996), Otsuka et al. (1996), Guillouzo (1995), Poon et al. (1995), or Lan et al. (1989).

42.7 9,000*g* Fractions

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations, the procedures described here focus on liver 9,000*g* fractions exemplarily. 9,000*g* supernatants (S9 fractions) contain both microsomal and cytosolic proteins in a significant lower concentration compared to microsomes based on total protein content. 9,000*g* fractions are commercially available and more easily to prepare than microsomes since 9,000*g* centrifugation is an intermediate step in the preparation of microsomes (Fig. 42.3). In combination with the high storage stability, S9 fractions also give rise to high-throughput applications using S9 fractions. 9,000*g* liver fractions from a variety of species are available from various commercial suppliers.

PROCEDURE

The 9,000*g* fraction might either be of commercial source or prepared individually according to literature (Testa and Jenner 1976; Wrighton and Stevens 1992; Jones et al. 1998; Linget and du Vignaud 1999; Li 2004). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation at a definite protein concentration (usually 2 mg/mL). The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or an NADPH-generating system consisting of $NADP^+$, glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethyl sulfoxide most often as solubilizer (final concentration <0.5%). Incubation is carried out at 37°C over a definite incubation time (usually up to 1 or 2 h). To stop the reaction, ice-cold acetonitrile or aqueous trifluoroacetic acid containing a definite amount of internal standard is added in equal amounts or up to three volumes depending on the sensitivities of the analytics used. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g., LC-MS/MS and HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

MODIFICATION OF THE METHOD

Depending on the matter of interest in use of 9,000*g* fractions, modifications in use of cofactors are necessary. With respect to microsomal proteins, an NADPH-regenerating system has to be present to cover cytochrome P450-dependent metabolism, UDPGA as a cofactor for glucuronidation in combination with alamethicin as a modifier. Indeed these cofactors are in use in most instances as by Wittman et al. (2000, 2001), Rajanikanth et al. (2003), Epperly et al. (2001), Mae et al. (2000), and Hewawasam et al. (2002). Incubation time and substrate concentrations are highly subject to modifications.

Besides applications in studying the metabolism of compounds, the S9 liver fractions of human or aroclor-induced rat are in use as "metabolic activation" of the Ames test for mutagenicity of chemicals, in most instances without addition of phase II cofactors (Maron and Ames 1983). S9 fractions are also used for activation in reporter gene assays (Sumida et al. 2001).

CRITICAL ASSESSMENT OF THE METHOD

Due to the presence of both microsomal and cytosolic enzymes present in 9,000*g* fractions, these preparations offer the advantage of the most complete picture of biotransformation compared to other subcellular fractions (Plant 2004; Brandon et al. 2003) if cofactors in the appropriate concentrations are added. On the other hand, metabolically active enzyme concentration related to total protein concentration is significantly lower compared to microsomal preparations causing lower turnover of a substrate in general. Because of these characteristics, the 9,000*g* fraction is applied to a lesser extent in accelerated-throughput applications for screening of metabolic stability compared to microsomes.

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9,000*g* fractions because organic

solvents deactivate cytochrome P450 isozymes and other concentration dependently (Busby et al. 1999; Easterbrook et al. 2001). Solvent influences are lowest for methanol and acetonitrile (<10% inhibition at 0.3% solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

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EXAMPLE

A standard procedure is taken from Mandagere et al. (2002): Substrates are incubated at 37°C and pH7.4. The reaction mixture consists of 4 mL of a 5 mg protein/mL suspension of liver S9 (equivalent to approximately 1 mg/mL of microsomal protein in the final reaction mixture), 4 mL of an NADPH-generating cofactor (6.4 mM glucose-6-phosphate, 1.1 mM NADP, and 1.3 mM MgSO4), 0.32 mL of glucose-6-phosphate dehydrogenase, 7.58 mL of 0.1 N K2HPO4, and 0.1 mL of substrate (6–8 μg/mL) such that the concentration was 10 μM in the final reaction volume of 16 mL. Aliquots are taken at 1, 3, 5, 10, 15, 30, and 60 min and snap frozen for deactivation of enzymes. Samples are stored at −70°C prior to analysis. Note that no cofactors for phase II metabolism were applied in the protocol.

42.8 Microsomes

PURPOSE AND RATIONALE

Liver microsomes are a widely used test system in studying metabolic stability and profiles both of large number of compounds in the early part of the value chain during drug discovery and development (Brandon et al. 2003). Commercial availability and the ease of preparation (Testa and Jenner 1976; Raucy and Laskor 1991; Wrighton and Stevens 1992; Jones et al. 1998; Linget and du Vignaud 1999; Li 2004) of the microsomal liver fraction in combination with the capability for automation of the incubation give rise to accelerated-throughput applications. High storage stability (Meier et al. 1983; Pearce et al. 1996; Yamazaki et al. 1997) also provides the possibility to generate a human liver bank (Von Bahr et al. 1980) in order to study the huge interindividual differences in enzyme activities especially in the human population. The latter is applied in the correlation analysis to study metabolic pathways in the context of enzyme typology (Beaune et al. 1986).

PROCEDURE

Procedures in use are numerous and depend strongly on the objective of the application. Microsomes from almost all organs of interest can be applied from commercial source or prepared separately from organ pieces. A comprehensive overview on preparation of subcellular fractions is shown in Fig. 42.3 (Ekins et al. 1999). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for and used in incubation at a definitive protein concentration (usually up to 1 mg/mL). The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or an NADPH-generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethyl sulfoxide most often as solubilizer (up to 0.5% final concentration). Incubation is carried out at 37°C over a defined incubation time (usually up to 1 h). To stop the reaction, ice-cold acetonitrile or aqueous trifluoroacetic acid containing a definitive amount of internal standard is added in equal amounts or higher, depending on the sensitivity of the analytics used. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g., LC-MS/MS and HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of full enzyme kinetics (V_{max}, K_m) is possible using various time points and drug concentrations.

MODIFICATION OF THE METHOD

Numerous modifications are in place at each lab regarding incubation conditions like substrate and protein concentration, incubation time, cofactor use and concentration, sequence of the different pipetting steps, etc. (e.g., Kling et al. 2003; Mitsuya et al. 2000; Diana et al. 1995). These modifications depend on the purpose of the method, e.g., as a high-throughput screen in early drug discovery or as a tool to characterize a development compound in vitro for regulatory files. For screening purposes, a high and low turnover compound should be added as quality control in each run. Besides these modifications on the biological part of the assay, specific protocols on pipetting robots and conditions used for evaluation by LC-MS/MS also in combination with the instrumental equipment available are applied which are typically not published. Besides LC-MS/MS, also LC-UV (Shearer et al. 2002; Stratford et al. 1999) and capillary electrophoresis (Clohs et al. 2002) are described.

Typically the reaction is started by addition of NADPH (Plobeck et al. 2000; Stratford et al. 1999) or the NADPH-regenerating system (Bloomer et al. 2001; Wei et al. 2000).

MacKenzie et al. (2002) added 5 μM $MnCl_2$ in addition to $MgCl_2$.

Linget and du Vignaud (1999) incubated in the presence of 3% bovine serum albumin to assist dissolution of compounds with poor solubility.

For extrapolation of in vitro metabolism data to in vivo, more detailed in vitro investigations are necessary as a first step. In this either enzyme kinetics is determined to calculate K_m and V_{max} and finally the intrinsic metabolic clearance as the quotient of both (Houston 1994a, b; Iwatsubo et al. 1996). Alternatively, the in vitro $t_{1/2}$ method is used (Obach et al. 1997; Obach and Reed-Hagem 2002; Jones and Houston 2004).

In order to cover glucuronidation reactions in incubations in microsomal fractions, several modifications have been applied in order to optimize conditions. These comprise longer incubation times than necessary for oxidative reactions by cytochrome P450s, and use of modifiers, both to overcome the latency in activity due to the diffusional barriers of the endoplasmatic reticulum (Coughtrie and Fisher 2003; Csala et al. 2004). Modifiers used are detergents or the pore-forming peptide alamethicin (Fisher et al. 2000). Also disruption of cells by sonication is applied (Ethell et al. 1998).

For elucidating the specific enzyme responsible for a certain metabolic step occurring in liver microsomes, the addition of enzyme-specific inhibitors is useful. If suicide inhibitors are used, preincubation of the inhibitor is needed. Selective or total inhibitors are used for cytochrome P450s in particular (Donato and Castell 2003; Newton et al. 1995; Rettie et al. 1995; Birkett et al. 1993). Also antibodies raised against

specific enzymes are used for studying the involvement of these enzymes in the biotransformation. These are mainly in use for cytochrome P450s (e.g., Wang and Lu 1997) due to their commercial availability.

CRITICAL ASSESSMENT OF THE METHOD

The high batch-to-batch variability of enzyme activities affords usage of pooled microsomes if metabolic stability has to be screened. Individual donors are used only for specific applications like correlation analysis. Since no cytosolic enzymes are present, the screening in liver microsomes might sometimes lead to misleading results if compared to in vivo data. On the other hand, the enzyme activities are higher in microsomal preparations than in 9,000*g* fractions giving rise to higher metabolic turnover (Brandon et al. 2003). However, this might lead also to overestimation of metabolic instability in microsomes compared to systems like hepatocytes or liver slices (Sidelmann et al. 1996).

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9,000*g* fractions because organic solvents deactivate cytochrome P450 isozymes and other concentration dependently (Busby et al. 1999; Easterbrook et al. 2001). Solvent influences are lowest for methanol and acetonitrile (<10% inhibition at 0.3% solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used. Published applications of microsomal stability tests use DMSO amounts of up to 1% which clearly can lead to enzyme-specific inhibition.

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EXAMPLES

42.8.1 Example 1: High-Throughput Application for Metabolic Stability Tests

Di et al. (2003) described in detail an automated application on a Packard Multiprobe robot for high-throughput screening. The following final concentrations are applied:

1.3 mM NADPH
3.3 mM glucose-6-phosphate

3.3 mM $MgCl_2$
0.4 U/mL glucose-6-phosphate dehydrogenase
1 mM EDTA
88 mM phosphate buffer, pH 7.4
0.5 mg/mL microsomal protein
1 μM test compound
0.2% DMSO

The incubation is performed in 96 multititer plates for 0 and 5 min. The pipetting steps for the 5-min incubation plate are:

1. 990 μL of dilution buffer was added to each well of the 2-mL deep well dilution plate.
2. 10 μL of sample stock (0.5 mM) was added to the corresponding well of the dilution plate and mixed by aspiration/dispensation three times at high speed to form the diluted sample.
3. 100 μL of NADPH cofactor (10.938 mL of phosphate buffer, 1.609 mL of regenerating solution A, and 0.322 mL of solution B) was added to each well of the incubation plate.
4. 50 μL of each diluted sample was added to the 1-mL deep well incubation plate in duplicate and warmed at 37°C for 10 min.
5. 100 μL of the prewarmed microsomes (see previous section) was added to each well of the incubation plate.
6. The plate was incubated off the robot at 37°C, 70 rpm for 5 min on a shaker (Armalab, Bethesda, MD).
7. At the end of the incubation, the plate was immediately returned to the Packard robot, and 500 μL of cold acetonitrile was added to each well. The plate was gently mixed on a vortexer (VWR, So. Plainfield, NJ).

Subsequently, precipitated protein is removed by centrifugation.

The supernatants obtained are analyzed by LC-MS/MS with compound-specific methods.

A general generic LC comprises of application of a trapping cartridge to improve efficiency and peak shape and subsequent elution of the trapped components with a high amount of organic modifier. The following analytical conditions are used:

Column: Keystone Aquasil C18 10 × 2.1 mm, 5 μm, guard cartridge
Mobile phase: Loading = 0.1% formic acid/water, elution = 0.1% formic acid (95% acetonitrile/water)
Flow rate: optimization = 0.3 mL/min, 0.8 min, loading = 3.0 mL/min, 5 s, flash/analysis = 1.0 mL/min, 0.5 min
Gradient: isocratic
Injection volume: 20 μL
Detection: electrospray (positive or negative mode), multiple reaction monitoring

A backup generic LC method is in place for compounds failed by method 1 using the conditions as follows:

Column: Keystone Aquasil C18 50 × 2.1 mm, 5 μm, column
Mobile phase: A = 0.1% formic acid/water, B = 0.1% formic acid/acetonitrile
Flow rate: 0.8 mL/min
Gradient: 0.1 min/10%B, 2.5 min/90%B, 4.0 min/90% B, 4.1 min/10%B, 5.5 min/10%B
Injection volume: 20 μL
Detection: atmospheric pressure chemical ionization (positive or negative mode), selected ion mode

Final results are given as percent remaining after incubation by dividing peak area of parent compound in the 5-min sample by the peak area of the time 0-min sample, multiplied by 100.

42.8.2 Example 2: Enzyme Mapping Studies by Use of P450 Isozyme Selective Inhibitors

An example for applying specific inhibitors for cytochrome P450 isozymes in incubations with human liver microsomes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Human liver microsomes from three patients were used to optimize the conditions of perazine metabolism. On the basis of the obtained results, perazine metabolism in liver microsomes was studied in respect of the linear dependence of product formation on the time and concentrations of protein and substrate. Microsomal protein, 500 μg, was resuspended in 500 μL of 20 mM Tris/HCl buffer (pH = 7.4). For inhibition studies, 25 μM perazine was chosen as a therapeutic concentration in the liver, which did not saturate the enzyme. Perazine was incubated with liver microsomes and the specific CYP inhibitors: 2 μM a-naphthoflavone (a CYP1A2 inhibitor), 10 μM sulfaphenazole (a CYP2C9 inhibitor), 5 μM ticlopidine (a CYP2C19 inhibitor), 10 μM quinidine (a CYP2D6 inhibitor), 200 μM DDC (a CYP2A6 + CYP2E1 inhibitor), and 2 μM ketoconazole (a CYP3A4 inhibitor). After 3-min preincubation at 37°C, the reaction was initiated by adding NADPH to a final concentration of 1 mM. After 15-min incubation, the reaction

was stopped by adding 200 µL of methanol. Perazine and its metabolites were analyzed by HPLC. The reduction of formation of the two main metabolites formed by *N*-demethylation and sulfoxidation was determined in comparison to control incubations without inhibitor.

42.9 Cytosol

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations, the procedures described here focus on liver cytosol exemplarily. Liver cytosol fraction contains soluble phase I and phase II enzymes which play an important role in drug metabolism (Brandon et al. 2003). These are alcohol and aldehyde dehydrogenases, epoxide hydrolases, sulfotransferases, glutathione *S*-transferase, *N*-acetyl transferases, and methyl transferases. Therefore, in cytosolic preparations, these biotransformation steps can be studied. Cytosolic fractions are commercially available or easy to prepare, alternatively.

PROCEDURE

In a typical procedure to study sulfation reactions (Tabrett and Coughtrie 2003), the reaction mixtures consist of a 6.25 mM potassium phosphate buffer (pH 7.4), containing 60 µM adenosine 3′-phosphate 5′-phosphosulfate (PAPS) as a cofactor, 75 µg liver cytosolic protein, and 0.125–100 µM substrate (4-nitrophenol in the example cited here). Reaction is started by the addition of PAPS and incubated for 10 min at 37°C. PAPS not utilized in the reaction is precipitated with 200 µL 0.1 M barium acetate, 200 µL 0.1 M barium hydroxide, and 200 µL 0.1 M zinc sulfate and centrifuged for 5 min at 14,000*g*.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g., LC-MS/MS and HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

MODIFICATION OF THE METHOD

Conditions for cytosolic incubations depend on the aim of the assay, e.g., to cover specific enzymatic activity present in the cytosol. For this purpose, it is essential to fortify the incubation medium with the specific cofactor for the reaction—if needed (Ekins et al. 1999). β-Nicotinamide adenine dinucleotide (NAD) is needed for alcohol and aldehyde dehydrogenases, flavin adenine dinucleotide (FAD) for polyamine oxidase, and β-nicotinamide adenine dinucleotide phosphate (NADPH) for dihydropyrimidine dehydrogenase. Phase II reactions depend on PAPS (sulfotransferases), glutathione (glutathione *S*-transferases, Raney et al. 1992; Slone et al. 1995), acetyl-coenzyme A (*N*-acetyltransferases), or *S*-adenosylmethionine (methyltransferases). NADPH as a cofactor has to be added if cytosolic reductases are the aim of interest (Inaba and Kovacs 1989).

Obach et al. (2004) set up a method for inhibition studies in cytosol using phthalazine as a substrate of aldehyde oxidase. In this system, 0.05 mg protein/mL was used in 25 mM potassium phosphate buffer pH7.4 containing 0.1 mM ethylenediaminetetraacetic acid. Incubation is terminated after 2.5 min.

Chen et al. (1999, 2003) used cytosol prepared from various sections of the human intestine to study the occurrence and distribution of sulfotransferases in the gastrointestinal tract. They fortified the cytosol with PAPS. They utilized the sulfuryl group transfer from p-nitrophenol sulfate to PAPS to generate PAPS for measurement of the phenol sulfotransferase activity by measurement of the colored product p-nitrophenol. Cytosolic incubation were stopped by addition of Tris buffer, pH 8.7.

Antibodies against SULT can be incorporated into the assay (Lewis et al. 2000; Thomas et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

The conduct of stability/profiling studies in liver cytosol is most often fine-tuned to the specific biotransformation reaction which is intended to be studied (Favetta et al. 2000; Frandsen and Alexander 2000; Long et al. 2001). A generalized procedure, e.g., with fortification of the cytosol with all relevant cofactors to cover the entire cytosolic enzyme activities, is uncommon. This might also be due to the fact that utilization of cytosol as a screening tool for metabolic stability is rather rare (Linget and du Vignaud 1999) and may only come into consideration if an in vitro-in vivo correlation cannot be established on the basis of microsomal tests. On the other

hand, the enzyme activities of NAT, SULT, and GST are higher in cytosol preparations than in 9,000*g* fractions, giving rise to better cover the metabolites derived by these routes (Brandon et al. 2003).

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EXAMPLE

In an automated assay developed by Linget and du Vignaud (1999), incubations were performed using 2.5 μM substrate and cytosolic protein concentrations between 0.26 and 2.6 mg/mL. Incubations were done on a 215 Gilson liquid handler. The assay was set up for the screening of glutathione adducts. Therefore, 1,2 epoxy-3-(*p*-nitrophenoxy) propane was used as a model substrate, and 1 mM glutathione was added. Substrate was pipetted as a solution in 0.1 M Tris HCL buffer containing 5 mM of magnesium chloride and 3% bovine serum albumin. The BSA was added to assist dissolution of compounds with poor solubility. Incubations are done on a vibrating device. Samples are taken after incubation times of 0, 1, 2, 3, 4, and 5 min. At each of these time points, an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening. Half-life and rate were determined for intra- and interassay variability showing standard deviations of less than 5%.

42.10 Recombinant Enzymes

PURPOSE AND RATIONALE

Recombinant enzymes are used to elucidate the enzymes involved in certain biotransformation steps and to determine the relative contribution if more than one enzyme is involved (Rodrigues 1999; Friedberg et al. 1999; Brandon et al. 2003; Donato et al. 2003). This is particularly of importance for metabolic steps which are catalyzed by polymorphic enzymes. In combination with correlation analyses from data obtained with individual human liver microsomes and inhibition experiments in human hepatocytes and/or liver microsomes employing either specific chemical inhibitors or

antibodies, a solid statement on the enzymes involved in the metabolism of a drug can be obtained in many instances.

PROCEDURE

Experimental conditions are similar to those applied in human liver microsomal incubations. In case of human P450 isozymes, microsomal protein is derived from CYP-transfected insect cells (Supersomes™, Baculosomes™) or bacteria (Bactosomes™). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or an NADPH-generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethyl sulfoxide most often as solubilizer in as low as possible amounts. Incubation is carried out at 37°C over incubation time of up to about 1 h. To stop the reaction, ice-cold acetonitrile or aqueous trifluoroacetic acid containing a definite amount of internal standard is added in equal amounts or higher depending on the sensitivity of the analytics used. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g., LC-MS/MS and HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

MODIFICATION OF THE METHOD

Commercially available (BD Gentest, Invitrogen, Cypex) are a wide variety of cytochrome P450 isozymes including allelic variants expressed in insect or mammalian cells. They comprise of uridine diphosphoglucuronosyl transferases (UGTs), flavin monooxygenases (FMOs), monoamine oxidases (MAOs), microsomal epoxide hydrolases, arylamine N-acetyltransferases (NATs), glutathione transferases (GSTs), and sulfotransferases (SULTs). According to the isozyme to be studied, specific cofactors have to be incorporated: an NADPH-regenerating system for P450 isozymes and FMOs, uridine diphosphoglucuronic acid (UDPGA) for UGTs in combination with alamethicin as a modifier to assist membrane transport (Ethell et al. 2003; Kuehl and Murphy 2003), acetyl-coenzyme A for the cytosolic *N*-acetyl transferases, glutathione for GSTs, and PAPS for SULTs. If radiolabeled cofactors are available ([^{3}H]GST, [^{14}C]UDPGA), their use facilitates in some instances the detection of the metabolites formed even from nonlabeled substrates.

CRITICAL ASSESSMENT OF THE METHOD

Besides expression in insect cells and bacteria (*Escherichia coli*), a variety of other expression systems are used like mammalian cells, V79 hamster cells, and systems in yeast (Friedberg et al. 1999 and references cited therein). High expression level and/or high yields in producing cells are achieved in baculovirus and *E. coli*. Therefore, these systems are nowadays in broad application in the industrial environment, also because of their commercial availability.

Supersomes are available containing cDNA-expressed cytochrome b5 or not. Since insect cell microsomes do not contain significant amounts of cytochrome b5, the incorporation of this enzyme increases the respective cytochrome P450 activity.

The availability of allelic variants gives rise to genotyping studies with recombinant enzymes (Coller et al. 2002).

Organic solvents deactivate cytochrome P450 isozymes and other concentration dependently (Busby et al. 1999). Moreover, this effect is different between the isoforms and depends also on the solvent used. Solvent influences are lowest for methanol and acetonitrile (<10% inhibition at 0.3% solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

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EXAMPLE

42.10.1 Example 1

The example for applying cDNA-expressed P450 isozymes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Microsomes from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (Supersomes^{TM}) were obtained from Gentest. All the Supersomes^{TM} were coexpressed with human P450 reductase.

CYP2E1 was additionally coexpressed with the human cytochrome b-5. Microsomal protein, 500 μg, was resuspended in 500 μL of 20 mM Tris/HCl buffer (pH = 7.4). To study perazine metabolism in Supersomes^{TM}, the incubations were carried out at the neuroleptic concentration of 750 μM (3 K_m) allowing to achieve the velocity of reaction of about V_{max} to show the maximum ability of cDNA-expressed enzyme to metabolize perazine. After 3-min preincubation at 37°C, the reaction was initiated by adding NADPH to a final concentration of 0.1 mM. After 2 h incubation, the reaction was stopped by adding 200 μL of methanol. Perazine and its metabolites were analyzed by HPLC.

42.10.2 Example 2

A fully automated assay for the determination of the intrinsic metabolic clearance in cytochrome P450 isozymes has been developed by McGinnity et al. (2000) using the major drug-metabolizing human hepatic cytochrome P450s (CYP1A2, -2C9, -2C19, -2D6, and -3A4) coexpressed functionally in *E. coli* with human NADPH-P450 reductase, to predict the CYP isoform(s) involved in the oxidative metabolism of NCEs.

CYP CL_{int} determination assays were performed by a robotic sample processor (RSP) (Genesis RSP 150; Tecan, Reading, UK). The primary stock of all probe substrates was prepared manually in dimethyl sulfoxide or acetonitrile at 100-fold final incubation concentration. The final concentration of organic solvent in the incubation was 1% v/v. At this concentration, dimethyl sulfoxide has been shown to reduce the activities of CYP2C9/19. All substrates were incubated at 3 μM except tolbutamide (CL_{int} calculated by determining V_{max} and K_m), ibuprofen (10 μM), and testosterone (10 μM). The RSP was programmed to add chilled quenching solvent (100 μL of acetonitrile) to 96-well refrigerated blocks, and compound stocks were then prediluted in 100 mM potassium phosphate buffer, pH 7.4. *E. coli* membranes and microsomes prepared from baculovirus coexpressing individual CYPs and NADPH reductase were added to incubation tubes (100 pmol of CYP/mL final concentration)

located in a 96-well heated block (37°C). An aliquot was removed to produce a 0-min time point, and the assay was initiated via addition of NADPH (1 mM final concentration). Aliquots (50 μL) were removed at 5, 10, 15, and 20 min and quenched in acetonitrile, frozen for 1 h at −20°C, and afterward centrifuged at 3,500 rpm for 20 min. The supernatants were removed and transferred into HPLC vials using the RSP. Test compounds used for the validation of the method (diltiazem, testosterone, dextromethorphan, propranolol, metoprolol, diazepam, tolbutamide, ibuprofen, verapamil, omeprazole) were analyzed by HPLC under compound-specific conditions.

42.11 Blood, Plasma, and Serum

PURPOSE AND RATIONALE

Mainly due to esterase activities, many drugs reveal degradation in blood (Williams 1987). In some instances, namely, if a prodrug concept (Huryn et al. 2004) is applied, e.g., for enhancing the absorption of a drug, cleavage of the ester prodrug in blood is intended for liberation of the pharmacologically active compound. Therefore, stability studies in blood are important. In most instances, these studies are performed in plasma or serum derived from human and/or animal blood. However, also red blood cells are accountable for metabolic activity.

PROCEDURE

In a typical procedure, the drug to be investigated is spiked to serum or plasma in a concentration of 25 μM and subsequently incubated at 37°C over a time of up to several hours (typically 5 min to 1 h). After addition of acetonitrile, denatured proteins are removed, and the supernatant is analyzed appropriately.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g., LC-MS/MS and HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds.

MODIFICATION OF THE METHOD

It can be appropriate to coincubate the compound of interest in the presence of inhibitors of serum esterases, e.g., sodium fluoride, physostigmin, or ecothiophate iodide (Chien and Tang-Lieu 1990; Quon et al. 1993). In case of carboxy- or aminopeptidase cleavage of peptides, specific peptidase inhibitors like amastatin, bestatin, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, or ethylenediaminetetraacetic acid (EDTA) are useful (Lee and Stavchansky 1995).

An esterase-like activity of human serum albumin (HSA) might also contribute to the serum instability of esters which can be studied in buffer preparations containing albumin (Ohta et al. 1987).

Quon et al. (1985) investigated the stability of esmolol in blood, plasma, red blood cells, and purified enzymes (human serum pseudocholinesterase, human and dog serum albumin, acetyl choline esterase, carbonic anhydrases A and B, and human hemoglobin). Udata et al. (1999) studied the hydrolysis of propranolol ester prodrugs in purified acetylcholine esterase.

Some authors reported the use of serum or plasma diluted to 80% with buffer or less instead of the native matrix (Di-Stefano et al. 2001; Mahfouz et al. 1999; Scriba 1993).

CRITICAL ASSESSMENT OF THE METHOD

In general, serum as well as plasma reflects the enzymatic status in blood in a similar manner. However, it may become relevant that the coagulation cascade is suppressed during preparation of plasma by addition of citrate, heparin, or EDTA, whereas in serum, those enzymes are present in nonphysiological concentrations. Since these enzymes, e.g., thrombin, reveal proteolytic activity (Lafleur et al. 2001), this has to be considered if studying the stability of peptides.

Stability tests in plasma and serum are fixed component in the validation procedures of bioanalytical assays (see also Chap. II.P of this book: Chaps. 31 "Bioanalytical Assays LC-MS/MS", 32 "Bioanalytical Assays:RIA/EIA", and 33 "Bioanalytical Assays - Toxicokinetics").

Besides modifications of time and concentrations, incubations in serum or plasma appear to be rather simple and reliable in predicting the in vivo relevance of the in vitro data.

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EXAMPLE

An example for an automated stability test in plasma is described by Linget and du Vignaud (1999). Incubations are performed on a 215 Gilson liquid handler. Incubation was done at substrate concentrations of 50 μM on 96 deep well plates. Each incubation tube contained 375 μL of a 200 μM test compound solution (in 0.1 M Tris buffer with 3% BSA, added to assist dissolution of compounds with poor solubility) and 1125 μL of plasma. Samples are taken after incubation times of 0, 1, 2, 3, 4, and 5 min. At each of these time points, an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening.

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Pharmacogenomics in Drug Metabolizing Enzymes and Transporters

43

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43.1 Pharmacogenomics of Drug Transporters

43.1.1 Introduction

The role of membrane transporters for the pharmacokinetic (PK) and pharmacodynamic (PD) properties of a drug is becoming evident in many aspects. Membrane transporters are major determinants for the absorption, distribution, and elimination of many drugs or at least of their more hydrophilic metabolites. Thereby, the overall effect of transporters is thoughtlessly underestimated, if only the systemic concentration of a drug is monitored clinically. The role of drug transporters on cellular and tissue distribution, especially with regard to the brain, the heart, the liver, the kidney, and other peripheral tissues seems to be of much higher importance. Unfortunately, the clinical investigation of tissue distribution phenomena requires the development of new techniques like PET (positron emission tomography), but already today, there is a clinical example of ^{11}C-verapamil crossing the blood-brain barrier in the presence of a strong P-gp inhibitor (Eyal et al. 2010, pp. 579–585). Another impressive example is a significant and growing body of data showing a correlation between long-term response to imatinib chemotherapy in CML patients with functional OCT1 activity in the cancer cells (Engler et al. 2011, pp. 608–611) (imatinib is described as substrate of OCT1). More examples like this, illustrating the importance of transporters for cellular or tissue exposure, will be experienced in the near future with the development of PET-labeled transporter probe substrates and approaches of personalized medicine. The understanding of the role of pharmacogenetics in drug metabolism expanded greatly in the 1990s. This is mainly

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due to technological improvements in gene scanning and gene variant identification. The number of variant alleles identified for genes coding for drug metabolizing enzymes (DMEs) considerably increased in the early 2000s, and continues to increase. The clinical consequences – or at least genotyping-phenotyping relationships – of DME polymorphisms have not been demonstrated for all variants. Only those DME allele variants will be mentioned for which significant changes in enzyme activity have been found using probe drugs.

Two large superfamilies, the SLC (solute carrier) family and the ABC (ATP-binding cassette) family, have been classified. Presently, 378 different members belong to the SLC family classified in 51 subfamilies (http://www.bioparadigms.org). Most of the SLC members are uptake transporters mediating the transport of nutrients, vitamins, and other endo- or xenobiotics into the cell. The ABC transporter family consists of 49 members divided into 7 subfamilies (Dean et al. 2001, pp. 1156–1166). All ABC transporters share a conserved catalytic domain for ATP hydrolysis and couple the unidirectional efflux of a substrate to the hydrolysis of ATP. By this energy-dependent efflux mechanism, the ABC transporter can protect the cell from toxic compounds. For this reason, ABC transporters are highly discussed as one resistance mechanism of cancer cells to chemotherapeutics. From these, altogether 427 transporter proteins seven and nine transporters are selected by the ITC consortium White Paper (including FDA representatives) (Giacomini et al. 2010, pp. 215–236) and the EMA drug-drug interaction draft guidance (April 2010), respectively, as most relevant in the drug development process today. OATP1B1, OATP1B3, OAT1, OAT3, and OCT2 are uptake transporters from the liver and the kidney, identified by both groups, while the biliary uptake transporter OCT1 is requested by the EMA only. In addition to the well-established efflux transporters P-gp (ABCB1) and BCRP (ABCG2), the EMA has identified BSEP as a source of clinical pharmacodynamic interactions. In recent years, only a handful of single-nucleotide polymorphisms (SNPs) in transporter proteins are identified as clinically relevant, but due to the emerging interest in personalized medicine, the total number is expected to increase very fast. Thousands of SNPs are listed in the NCBI-SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) and with a focus on drugs at the Pharmacogenetics Knowledge Base (http://www.pharmgkb.org/). With the next-generation sequencing projects of about 1,000 complete genomes, our knowledge will expand dramatically in the near future (Kaiser 2008, p. 395; Siva 2008, p. 256). This part will focus on clinically relevant SNPs in the 7/9 drug transporters only; for further insights in the numerous SNPs of the transporters, it will be referred to original publications and reviews.

43.1.1.1 SLC Transporter Family

OATP1B1

OATP1B1 (SLCO1B1) is predominantly expressed in the basolateral membrane of human hepatocytes. Interestingly, no directly related isoform of human OATP1B1 (and also OATP1B3) is expressed in mouse or rat, questioning the significance of these preclinical species for substrates of these transporters – nevertheless, a broad substrate overlap between the species was observed. Besides endogenous substances like bile acids, thyroid hormones, and bilirubin conjugates, many highly prescribed drugs are identified as substrates of OATP1B1 with statins like atorvastatin, rosuvastatin, pitavastatin, and pravastatin as most relevant ones today. Furthermore, cardiovascular drugs from the sartane family, anticancer drugs, antibiotics, HIV protease inhibitors, some toxins, and more are identified as substrates in vitro. A comprehensive overview of OATP1B1 substrates, inhibitors, and also the importance of the transport protein for the hepatic uptake of anions is summarized in Niemi et al. (2011, pp. 157–181).

Genotyping

About 41 nonsynonymous variants are described for the SLCO1B1 gene today (Niemi et al. 2011, pp. 157–181), and for some of them, a decreased activity was proven in vitro (Tirona et al. 2001, pp. 35669–35675). The common SNP c.521T > C, also known as, *5 allele has become very prominent due to pronounced clinical effects described later. In vitro, a reduced membrane expression of the protein was predicted. The c388A > G variant is another prominent SNP found in the gene of OATP1B1, and it is remarkable that c388A > G and c.521T > C together form four well-known haplotypes *1A (c388A – c521T), *1B (c388G – c521T), *5 (c388A – c521C), and *15 (c388G – c521C). The functional consequences of these haplotypes have been studied extensively with mixed results, maybe due to different expression systems and/or probe substrates, but *15 and *5 are clearly identified as haplotypes with

reduced activity in vitro (Iwai et al. 2004, pp. 749–757; Kameyama et al. 2005, pp. 513–522; Nozawa et al. 2005, pp. 434–439;. Ho et al 2006a, pp. 1077–1084, 2006b, pp. 1793–1806; Katz et al. 2006, pp. 186–196; Tsuda-Tsukimoto et al. 2006, pp. 2646–2656; Deng et al. 2008, pp. 424–433). Haplotype *1B seems to be correlated with some increased activity of the transporter, but results are very controversial (reviewed in Niemi et al. (2011), pp. 157–181; Deng et al. (2008), pp. 424–433). More SNPs also leading to a stop codon or located in the promoter region of the SLCO1B1 gene were identified with very low incidence. The combined frequency of the low activity *5 and *15 haplotypes is 15–20% in Europeans and 10–15% in Asians.

Clinical Effects

Many studies with, to some extent, unexpected results have demonstrated the clinical relevance of SNPs in the SLCO1B1 gene and are comprehensively summarized in Niemi et al. (2011), pp. 157–181, Deng et al. (2008), pp. 424–433. Most importantly, the c521T > C variant leads to a remarkable increase in the AUC of statins like rosuvastatin (1.7-fold), pravastatin (1.9-fold), atorvastatin (2.1-fold), and simvastatin acid (3.1-fold), while the most hydrophobic statin fluvastatin was not significantly affected (1.2-fold) (Pasanen et al. 2006, pp. 873–879; Niemi et al. 2006, pp. 356–366). Due to these results, it was speculated that carriers of the *5 and *15 SNP might be on higher risk for statin-induced myopathy, a concentration-dependent skeletal muscle toxicity and a rare, but serious adverse reaction in statin therapy. In a remarkable genome-wide association study, this was confirmed for simvastatin but only for the highest dose of 80 mg/day (Pasanen et al. 2006, pp. 873–879). A second study confirmed the higher risk of the c521T > C SNP also for milder forms of simvastatin, atorvastatin, and pravastatin induced adverse events (Voora et al. 2009, pp. 1609–1616). In 2009, even a dose adjustment for statin therapy according to a SNP analysis in patients was suggested (Niemi 2010, pp. 130–133). Furthermore, there are some good indications that the c521T > C SNP also effects the AUCs of meglitinide like repaglinide and nateglinide clinically, although it is not proven in vitro that these drugs are OATP1B1 substrates (Niemi et al. 2005, pp. 468–478; Zhang et al. 2006, pp. 567–572; Kalliokoski et al. 2008a, pp. 937–942, 2008b, pp. 311–321, 2008c, pp. 818–825).

OATP1B3

OATP1B3 (SLCO1B3) is also expressed nearly exclusively in the basolateral membrane of human hepatocytes. OATP1B1 and OATP1B3 are homolog proteins and share a broad overlap of substrates with differences in the transport kinetics. Some specific substrates for OATP1B3, especially the octapeptide cholecystokinin, were identified. As OATP1B1 and OATP1B3 are the main liver-specific anion transporters for many drugs, it will be very useful to evaluate to which proportion of OATP1B1 and OATP1B3 a drug is transported into the liver to predict pharmacokinetic properties and possible interactions.

Genotyping and Clinical Effects

In vitro data on SNPs in SLCO1B3 are limited and partly contradicting. Experimental results about localization and transport function were substrate and cell line dependent (Letschert et al. 2004, pp. 441–452). In vivo data from clinical trials are also rare and do not show conclusive results to demonstrate the impact of haplotypes of OATP1B3 clinically. In a study with 92 adult Caucasian cancer patients, six variants of the SLCO1B3 gene (334T > G (*2), 439A > G (*3), 699G > A (*4), 767G > C (*5), 1559A > C (*6), and 1679T > C (*7)) were analyzed and did not show a correlation with the docetaxel clearance (Baker et al. 2009, pp. 155–163). In a second study with the related taxane paclitaxel, the SNPs 334T > G and 699G > A analyzed in a cohort of 90 cancer patients were also not correlated with a changed plasma clearance (Smith et al. 2007, pp. 76–82).

OAT1 and OAT3

OAT1 (SLC22A6) and OAT3 (SLC22A8) are expressed at the basolateral membrane of the kidney tubular cells and are responsible for the uptake of mostly hydrophilic small organic anions into the organ as the first step of active renal secretion. OAT1 and OAT3 share partly overlapping substrate specificities for endogenous substrates and drugs like captopril, olmesartan, furosemide, several β-lactam antibiotics, antivirals, and NSAIDs. Furthermore, OAT3 shares some substrates with the hepatic OATP family like pravastatin and rosuvastatin. Properties of the members of the OAT family are comprehensively reviewed (VanWert et al. 2010, pp. 1–71; Burckhardt and Burckhardt 2011, pp. 29–104).

Genotyping

Several nonsynonymous SNPs were described in the OAT1 gene, comprehensively reviewed in Srimaroeng et al. (2008, pp. 889–935). For the 149G > A (R50H), in vitro transport was unaltered for p-aminohippurate, ochratoxin A, and methothrexate (Fujita et al. 2005, pp. 201–209), while affinities for adefovir, cidofovir, and tenofovir were significantly higher. Clinical consequences are not described until today. The SNP 1361G > A (R454Q) identified in African-Americans leads to a nonfunctional transporter in vitro, but surprisingly adefovir clearance was not altered clinically (Fujita et al. 2005, pp. 201–209). Several nonsynonymous SNPs were identified in the coding region of the SLC22A8 gene, encoding OAT3 (Nishizato et al. 2003, pp. 554–565; Erdman et al. 2006, pp. F905–F912; Urban et al. 2006, pp. 223–230). In Japanese, 1166C > T (A389V) had no remarkable influence on the pravastatin clearance compared to wild type (Nishizato et al. 2003, pp. 554–565). Others SNPs like 913A > T (I305F) showed altered transport of probe substrates estrone-3-sulfate and/or cimetidine in vitro (Erdman et al. 2006, pp. F905–F912). Three SNPs were identified as loss of function mutations (445C > A, R149S; 715C > T, Q239X; and 779T > G, I260R) with a possible impact on the excretion of drugs, while clinical data are missing until today.

OCT1 and OCT2

OCT1 (SLC22A1) and OCT2 (SLC22A2) organic cation transporters are predominantly expressed at the basolateral membrane of the liver and the kidney, respectively. The proteins are also expressed in many other cells and tissues (reviewed by Koepsell et al. (2007, pp. 1227–1251)). OCT1 and OCT2 mediate the uptake of organic cations into hepatocytes and kidney proximal tubular cells, respectively, thereby mediating the first step of hepatic and renal metabolism and/or secretion. Again, substrate specificity of the two related transporters is highly overlapping with some distinct differences. Endogenous substrates for OCTs are monoamine neurotransmitters, choline, creatinine, and guanidine, while the most prominent pharmaceutical transported by OCTs is the antidiabetic drug metformin. Metformin response is thought to be influenced by the uptake into the liver by OCT1, while OCT2 is involved in the active renal secretion of the drug. In addition, the histamine H2 antagonist cimetidine, some antivirals, and the antiarrhythmic quinidine are described as substrates for OCTs.

Genotyping and Clinical Effects

Due to the high clinical variability of metformin response and the rare, but severe occurrence of lactic acidosis during metformin treatment, genetic variabilities in SLC22A1 and SLC22A2 genes are highly discussed as an obvious explanation. Numerous studies were performed with partly contradictory data summarized comprehensively in a review by Zolk et al. (Zolk 2011).

By in vitro models, six common naturally occurring SNPs in the SLC22A1 gene were shown to translate into proteins with reduced or eliminated functions (61R > C, 189S > L, 220G > V, 401G > S, 420del, and 465G > R) (Shu et al. 2007, pp. 1422–1431). Some or all of these SNPs were analyzed in three clinical studies in healthy volunteers and patients with mixed results. In the first report by Shu et al. (2007, pp. 1422–1431) with healthy volunteers, the glucose response of metformin was lowered for individuals carrying one of the four alleles with reduced function (61R > C, 401G > S, 420del, 465G > R). This effect could not be confirmed in additional studies with patients. Zhou et al. (2009, pp. 1434–1439) analyzed the two most common SNPs (61R > C, 420del) with no effect on metformin response, and in two additional studies, the six SNPs were not associated with a change in metformin response (Shikata et al. 2007, pp. 117–122; Becker et al. 2009, pp. 242–247). To complicate the story even more, Gambineri et al. observed in a prospective study that carriers of the four SNPs (61R > C, 401G > S, 420del, 465G > R) showed no change in metformin response regarding glucose tolerance, but an impaired insulin response (Gambineri et al. 2010, pp. E204–E208). Many SNPs, but mostly with very low frequencies, were identified in the SLC22A2 gene. Only the SNP c808G > T (270A > S) with a frequency of about 15% was analyzed clinically in several studies with slightly different results summarized in Zolk et al. (Zolk 2011). This SNP influenced the pharmacokinetics of metformin clearance by about 40% reduction of the clearance but only in homozygote carriers.

43.1.1.2 ABC Transporter Family

BCRP (ABCG2)

The BCRP (breast cancer resistance protein) is an efflux transporter and expressed in numerous tissues and cell lines. At the apical membrane of the small intestine and at the blood-brain barrier, the protein limits the oral bioavailability and penetration of drugs into the brain, respectively. This efflux function of the transporter is also described at the blood testis/placental tissue barrier. In the sinusoidal membrane of hepatocytes and the apical membrane of kidney tubular cells, the transport protein is responsible for the active secretion of drugs into bile and urine, respectively. Therefore, the transporter influences pharmacokinetic properties of substrates at the absorption and excretion level. The protein was first discovered in a breast cancer cell line as the major factor mediating drug resistance and has then been identified in many cell lines derived from malignant tissues of various origins. The BCRP protein is characterized by a very broad substrate specificity for a variety of anticancer drugs but also several nonchemotherapeutics like the statins rosuvastatin and pitavastatin, antibiotics like ciprofloxacin and other drugs like cimetidine, leflunomide, sulfasalazine, and glyburide. Poguntke et al. give an extensive review of in vitro and in vivo knowledge regarding the BCRP protein (Poguntke et al. 2010, pp. 1363–1384).

Genotyping and Clinical Effects

Numerous SNPs have been identified in the ABCG2 gene. At least one SNP c.421C > A, with a common allele frequency of 10–15%, 25–30% and 0–5% in the Caucasian, Asian, and African-American population, respectively, is related to a reduced function of the transporter by a decreased expression rate. Therefore, the SNP should affect pharmacokinetic properties of ABCG2 substrates, and indeed the AUC of HMG-CoA reductase inhibitors was markedly increased by 144% for rosuvastatin, 111% for simvastatin lactone, and 72% for atorvastatin, while, e.g., non-BCRP substrate pravastatin was unaffected (Keskitalo et al. 2009a, pp. 1617–1624, 2009b, pp. 197–203). Sulfasalazine, a BCRP substrate with a very low intestinal absorption and oral bioavailability, showed a dramatic 110-fold increase in AUC in ABCG2 knockout mice (Zaher et al. 2006, pp. 55–61), while clinical studies were contradictory. Urquart et al. documented a 2.5-fold increase in sulfasalazine AUC in human subjects with the c.34GG/c.421CC genotype indicating an influence on sulfasalazine plasma exposure (Urquhart et al. 2008, pp. 439–448). A second study with 36 healthy volunteers failed to reproduce this result (Adkison et al. 2010, pp. 1046–1062).

P-gp (MDR1, ABCB1)

Like BCRP, P-gp is an efflux transporter and expressed in numerous tissues and cell lines with a very similar expression pattern and function at the apical membrane of the small intestine, at the blood-brain barrier, at the sinusoidal membrane of hepatocytes, and at the apical membrane of kidney tubular cells. P-gp is also characterized by a very broad substrate specificity for a variety of anticancer drugs, but also several nonchemotherapeutics from many different indication areas, like digoxin, quinidine, verapamil, cyclosporine, atorvastatin, levofloxacin, loperamide, and many more.

Genotyping and Clinical Effects

About 100 SNPs have been identified in the coding region and many more in the complete gene. A silent polymorphism 3435C > T, sometimes in combination with other SNPs (like 2677G > T/A and 1236G > T), was analyzed comprehensively in many clinical studies with the probe substrates digoxin and talinolol and furthermore with various drugs from different therapeutic areas (protease inhibitors, immunosuppressants, antihistamines, anticonvulsants, and cytostatics) summarized in Cascorbi (2011, pp. 261–283). For all compounds, positive and negative correlations were observed. Thus today, no particular SNP in the gene is identified as a predictor of individual pharmacokinetics of P-gp substrates.

BSEP (ABCB11)

The bile salt efflux pump (BSEP) is expressed solely in the canalicular membrane of human hepatocytes and is responsible for the hepatic excretion of bile salts and thereby a major driver for the enterohepatic circulation of bile salts. BSEP is not described as a typical drug transporter today, but the inhibition of BSEP by drugs or endogenous compounds like steroid metabolites leads to an acquired form of liver diseases, namely,

drug-induced cholestasis and intrahepatic cholestasis of pregnancy, respectively. A common genetic variant of BSEP (c1131T > C) is identified as a susceptibility marker for acquired cholestasis comprehensively reviewed (Stieger and Geier 2011, pp. 411–425; Pauli-Magnus et al. 2010, pp. 147–159).

43.1.1.3 Recommendations for All Drug Transporters

It is becoming preclinical practice that especially for BCS and/or BDDCS class 3 and 4 drugs, the substrate specificity of drug transporters is analyzed for the drug itself and active metabolites. Even the pharmacokinetic behavior of BCS/BDDCS class 2 compounds is partly influenced by transporters (Wu and Benet 2005, pp. 11–23). If a drug is identified as substrate of a specific transporter, it is highly recommended to analyze subjects in clinical studies for the SNPs identified in the genes from phase I to phase III clinical trials. It is very likely that patients will benefit from this in many aspects, not only from a safety perspective, but also in the mind-set of personalized medicine. Special populations will be identified which will, e.g., profit more from a medication, are nonresponders, are on a higher or lower risk, or need dose adjustments. This becomes even more obvious as it is expected that the impact of drug transporters on cellular and tissue distribution (at the target site!) is of much higher importance than on "simple" pharmacokinetic properties. As SNP analysis today by high-throughput methods is fast and inexpensive, it should be considered to analyze the SNPs of all identified drug transporters on a routine basis in selected clinical trials – safety, tolerability, and effectiveness of new drugs will benefit.

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43.2 Pharmacogenomics in Drug Metabolizing Enzymes

The understanding of the role of pharmacogenetics in drug metabolism expanded greatly in the 1990s. This is mainly due to technological improvements in gene scanning and gene variant identification. The number of variant alleles identified for genes coding for drug metabolizing enzymes (DMEs) considerably increased in the early 2000s, and continues to increase. The clinical consequences – or at least genotyping-phenotyping relationships – of DME polymorphisms have not been demonstrated for all variants. In the following text, only those DME allele variants will be mentioned for which significant changes in enzyme activity have been found using probe drugs. Complete information on CYPs alleles can be found at www.imm.ki.se/CYPalleles and Phase I and Phase II DMEs at www.pharmgkb.org/index.jsp.

43.2.1 Phase I Enzymes

43.2.1.1 CYP1A2

PURPOSE AND RATIONALE

CYP1A2 is involved significantly in the metabolism of several drugs (imipramine, clozapine, fluvoxamine, olanzapine, theophylline, acetaminophen, propranolol, tacrine) as well as in diet components (methylxanthines), endogenous substrates (estrogens), numerous aryl, aromatic and heterocyclic amines, and polycyclic aromatic hydrocarbons. It is inducible, notably by cigarette smoking, diet habits such as consumption of cruciferous vegetables (e.g., broccoli, watercress, collard greens, Brussels sprouts, mustard) and of charbroiled meats, some drugs (omeprazole, phenytoin, rifampicin), and is a target enzyme for the development of some cancers. Up to now, at least 23 CYP1A2 alleles have been detected. Probe drugs for CYP1A2 phenotyping are caffeine and theophylline. For safety concerns and drug availability, the preferred probe is caffeine. Caffeine 3-demethylation is mediated by CYP1A2 and accounts for 80% of caffeine clearance. Caffeine is also a probe drug for N-acetyltransferase and xanthine oxidase (Kalow and Tang 1993).

PROCEDURE

Phenotyping: A fixed or weight-adjusted dose of caffeine (solution, tablet, coffee) ranging from 1 to 3 mg/kg is administered. Diet requirements have to be respected (stable xanthine-free diet avoiding beverages like coffee, tea, cola, chocolate, no food component with CYP1A2-inducing properties) during the test period. As smoking is known to induce CYP1A2, control of stable smoking status is mandatory.

There are two commonly used and robust methods for phenotyping. The first one measures caffeine (1,3,7-methylxanthine) and its N-demethylated metabolite 1,7-dimethylxanthine (paraxanthine) in a plasma or saliva sample collected within 5–7 h post-caffeine dosing (Fuhr and Rost 1994). The second one uses the assay of the metabolites 1-methylurate (1U), 1-methylxanthine (1X), and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1,7-dimethylurate (17U) levels in urine collected at least for 8 h post-dosing (Campbell et al. 1987; Rostami-Hodjegan et al. 1996).

Commonly used methods for caffeine and metabolite(s) assay in plasma or urine involve an extraction step followed by HPLC with UV detection (Krul and Hageman 1998; Rasmussen and Bosen 1996; Schreiber-Deturmeny and Bruguerolle 1996). Urine needs to be acidified (pH 3.0–3.5) before sample freezing.

Genotyping: Reduced activity has been reported for CYP1A2*1C and CYP1A2*1F alleles in smoking subjects. Induction of CYP1A2 activity has been associated with these alleles, but the effect of CYP1A2*1F mutation on CYP1A2 activity has not been confirmed (Nordmark et al. 2002). In Caucasians, frequency of the CYP1A2*1C and CYP1A2*1F variants is about 1% and 33%, respectively (Sachse et al. 2003).

EVALUATION

Metabolic ratios (MRs) used are plasma 17X/137X and urinary (1U + 1X + AFMU)/17U.

In controlled conditions, in nonsmoking young and elderly subjects, intraindividual and interindividual variability in 17X/137X MR was about 17% and 47%, respectively, with no effect of age (Simon et al. 2003). A 70-fold range in MR has been observed in smoking and nonsmoking female Caucasian subjects using the urinary MR (Nordmark et al. 1999). Up to 200-fold differences were found using the urinary test. Lower variability is expected using the plasma caffeine test.

Higher CYP1A2 activity in men versus women has been reported, though inconsistently, and in children. Higher MR is usually observed in smokers versus nonsmokers, when population sample size is large. Pregnancy and oral contraceptives intake were found to decrease CYP1A2 activity (Abernathy and Todd 1985; Caubet et al. 2004; Kalow and Tang 1993). CYP1A2 activity was found lower in colorectal patients versus controls (Sachse et al. 2003).

Large variability in CYP1A2 activity explains that its distribution has been described unimodal, bimodal, or trimodal. Poor metabolizers (characterized with a MR <0.12) have been identified in a Chinese population and represented about 5% of the population tested, whereas PMs could represent 5–10% of Caucasian populations and 14% in Japanese population (Ou-Yang et al. 2000).

CRITICAL ASSESSMENT OF THE METHOD

Numerous studies have shown good correlation between the 17X/137X plasma MR and caffeine systemic clearance, and plasma MR is considered more

robust than the urinary one, since this last one can be affected by the effect of urinary flow on metabolite renal clearances.

Currently, no relationship between CYP1A2 genotype characteristics and CYP1A2 activity, as assessed by the caffeine test, has been usually found. Some associations have been found in specific genetic and environmental conditions (Han et al. 2001). Non-well-controlled conditions for urine sample collection, the effects (induction) linked to environmental factors, may overcome the role of CYP1A2 polymorphism, which can explain the paucity of clear associations between CYP1A2 genotyping and phenotyping.

Further investigations are needed to characterize the effect of variants (SNPs, haplotypes) on CYP1A2 activity.

MODIFICATIONS OF THE METHOD

Recent drug assay development involved LC-MS methods (Caubet et al. 2004; Kanazawa et al. 2000). A less practical breath test, using ^{13}C or ^{14}C labeled caffeine, can also be used (Kalow and Tang 1991).

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43.2.1.2 CYP2C9

PURPOSE AND RATIONALE

CYP2C9 is involved significantly in the hydroxylation of about 16% of drugs (Schwarz 2003), including drugs with narrow therapeutic index such as anticoagulants (warfarin, acenocoumarol, phenprocoumon active S-enantiomers), and anticonvulsivants (phenytoin, hexobarbital), as well as numerous antidiabetic agents (i.e., tolbutamide, glibenclamide, glipizide), antihypertensive drugs (losartan, irbesartan), nonsteroidal

anti-inflammatory agents (i.e., diclofenac, ibuprofen, celecoxib), and diuretic (torsemide) and anti-rheumatoid agents (leflunomide).

A couple of CYP2C9 variants – mainly CYP2C9*2 and CYP2C9*3 – code for in vivo decreased activity, and two – CYP2C9*6 and CYP2C9*15 – have been reported to be associated with no activity. In Caucasian populations, CYP2C9*2 and CYP2C9*3 are encountered in 20–25% of subjects, while these genotypes have been found in less than 5% of East Asian subjects (Rosemary and Adithan 2007).

Probe drugs used for CYP2C9 phenotyping are tolbutamide, warfarin, phenytoin, and losartan. Diclofenac, flurbiprofen, Phenprocoumon, and torsemide have also been used. For safety concerns, the current preferred probe is tolbutamide, despite some risk of hypoglycemia.

PROCEDURE

Phenotyping: The method measures tolbutamide, its CYP2C9-formed 4′-hydroxylated metabolite, and the subsequent carboxytolbutamide metabolite of hydroxytolbutamide, formed by dehydrogenase enzymes. The urinary excretion of these two metabolites represented more than 85% dose of administered tolbutamide (Veronese et al. 1990, 1993).

Subjects receive a single oral 500 mg tolbutamide tablet in usual Phase I standard controlled conditions, with care to be paid to blood glucose. Urine is collected from drug intake to 8 or 24 h post-dosing.

The assay of tolbutamide and its metabolites is usually performed using HPLC and UV or fluorescence detection (Csillag et al. 1989; Veronese et al. 1990; Kirchheiner et al. 2002a; Hansen and Brosen 1999).

Genotyping: About two-third of Caucasian subjects express the wild genotype C9*1/*1. C9*1/*2 and C9*1/*3 heterozygote variants are expressed in 15–25% and 7–16% of Caucasian subjects, whereas the frequency of other variants is lower: 0.5–2.5%, 1–3%, and <1–1.5% for C9*2/*2, C9*2/*3, and C9*3/*3 variants, respectively (Scordo et al. 2001; Lee et al. 2002a; Schwarz 2003). More than 95% of Afro-American subjects express the wild genotype C9*1/*1 (Lee et al. 2002b). In Asian populations, CYP2C9*1/*3 is expressed in 2–8% subjects, but CYP2C9*2 is absent or extremely rare (Rosemary and Adithan 2007; Schwarz 2003; Xie et al. 2002). Overall, it has been estimated that 0.2–1% and 2–3% of Caucasian and Asian population could be qualified as poor metabolizers (PMs), respectively (Meyer 2000).

EVALUATION

The urinary metabolic ratio (MR, hydroxytolbutamide + carboxytolbutamide)/tolbutamide is generally used. There is a large interindividual variability in MRs in subjects with the same genotype. Different studies performed with different probe drugs (Yasar et al. 2002a; Kirchheiner et al. 2002b, 2003b; Lee et al. 2002c; Miners and Birkett 1998; Morin et al. 2004), highlighted that a PM status could be given to subjects who are homozygous for CYP2C9*3, or expressing CYP2C9*2/*3 variant, but intermediate situations – from extensive to slow metabolizer status – may vary not only among different allele combinations but also with the probe drug used.

Oral contraceptives were found to inhibit CYP2C9 activity using losartan for phenotyping (Sandberg et al. 2004).

CRITICAL ASSESSMENT OF THE METHOD

The tolbutamide test has the most convincing ability to discriminate between genotype variants and pharmacokinetics. There could be an analytical issue linked to the urine assay precision, as the urinary concentrations of the parent drug are very low in comparison with those of its metabolites.

To date, the CYP2C9*3 variant has been the only one found influencing significantly drug pharmacodynamics for warfarin, acenocoumarol (Sandberg et al. 2004; Morin et al. 2004; Versuyft et al. 2003), glipizide, and glyburide (Kirchheiner et al. 2002b) or drug side effects (Sevilla-Mantilla et al. 2004). Unconstant results were found regarding tolbutamide effects (Kirchheiner et al. 2002a; Shong et al. 2002). For anticoagulants, the possession of CYP2C9*2 and CYP2C9*3 variants was associated with decreased warfarin dose requirement in patients and an increased risk of adverse events such as bleeding (Daly and King 2003). An African-American subject with only the CYP2C9*6 variant exhibited serious phenytoin side effects associated with a marked impaired elimination of the drug (Kidd et al. 2001).

The variability of CYP2C9 activity observed among ethnic groups cannot be explained with our current knowledge on CYP2C9 variant alleles distribution (Xie et al. 2002).

MODIFICATIONS OF THE METHOD

Losartan (25 mg dose) has been proposed as a safer alternative to tolbutamide. The determination of losartan/E3174 (oxidized metabolite) ratio in 0–8 h urine or in plasma at 6 h post-dosing have been proposed (Yasar et al. 2002b; Sekino et al. 2003). However, in a comparative study in 16 subjects, a better correlation between genotyping and phenotyping was found with tolbutamide, as compared to losartan or flurbiprofen, though there was no subject with the C9*2/*3 or C9*3/*3 variants (Lee et al. 2003).

Recently, a 125 mg tolbutamide dose has been validated, with proposal of the use of just one blood sample collected 24 h post-dosing. Its safer use needs the drug to be assayed using LC-MS/MS methodology (Jetter et al. 2004).

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43.2.1.3 CYP2C19

PURPOSE AND RATIONALE

CYP2C19 contributes to the metabolism of about 8% of drugs (Rogers et al. 2002), including *S*-mephenytoin, proton pump inhibitors (omeprazole, lansoprazole, pantoprazole), tricyclic antidepressants

(amitriptyline, imipramine, clomipramine, citalopram), benzodiazepines (diazepam, flunitrazepam), torsemide, fluvastatin, and proguanil. Two main variants – CYP2C19*2 and CYP2C19*3 – are coding for in vivo nil activity, as well as for CYP2C19*4, *5, *6, *7, and *8 variants. About 15–20% Asians, 4–7% Black Africans, and 3% Caucasians are PMs (Scordo et al. 2004).

Probe drugs used for CYP2C19 phenotyping are mephenytoin, omeprazole, and proguanil. The most currently used probe drug is omeprazole.

PROCEDURE

Phenotyping: The method measures omeprazole and its CYP2C19-formed 5-hydroxylated metabolite in plasma.

Subjects receive a single oral 20 or 40 mg omeprazole capsule in usual Phase I standard controlled conditions. Plasma can be collected from drug intake up to 24 h post-dosing, or only one plasma sample is collected at 2 or 3 h post-dosing.

The assay of omeprazole and its metabolite is usually performed using HPLC and UV detection (Lagerstrom and Persson 1984; Ieri 1996; Yim et al. 2001; Tybring et al. 1997) or LC-MS/MS assay (Kanazawa et al. 2002).

Genotyping: The two alleles CYP2C19*2 and CYP2C19*3 account for quite all PMs in Asians (>99%) and Black Africans, but defective alleles have not been fully characterized in 10–15% Caucasians. The CYP2C19*2 allele is the most frequent in Asian populations (30% in Chinese), as well as in Black Africans (about 17%) and in Caucasians (about 15%) (Xie et al. 2001). The CYP2C19*3 accounts for about 25% of inactive forms in Orientals and is extremely rare in Caucasians (Scordo et al. 2004; Rosemary and Adithan 2007).

EVALUATION

The AUC or plasma ratio of omeprazole to 5-hydroxyomeprazole is used.

As expected, homozygous PM subjects have lower metabolic activity as compared to heterozygous PM subjects, and potential interethnic difference has been noticed within a genotype (Yin et al. 2004).

Decreased CYP2C19 activity has been observed with oral contraceptives containing ethinylestradiol (Tamminga et al. 1999; Laine et al. 2000).

CRITICAL ASSESSMENT OF THE METHOD

Omeprazole hydroxylation rate correlates with S-mephenytoin hydroxylation rate, which was initially the CYP2C19 probe drug (Andersson et al. 1990; Chang et al. 1995; Balian et al. 1995). The alternate pathway – conversion of omeprazole to its sulfone derivative –, which is mediated via CYP3A4, does not influence the CYP2C19 pathway of omeprazole (Balian et al. 1995).

Time-dependent kinetics of omeprazole limits its use for phenotyping during chronic therapy (Gafni et al. 2001). CYP2C19 phenotyping with omeprazole may be affected by age, liver disease, and omeprazole therapy (Kimura et al. 1999).

Interethnic differences observed with different CYP2C19 substrates for subjects with same genotype have been attributed to differences in substrate specificity or enzyme isoforms (Bertilsson et al. 1992). The clearance of omeprazole is higher in Caucasian extensive metabolizers (EMs) than in Oriental EMs, due to a higher proportion of heterozygous EMs in this latter population (Ishizaki et al. 1994).

MODIFICATIONS OF THE METHOD

It has been proposed to use omeprazole for both CYP2C19 and CYP3A4 phenotyping (Gonzalez et al. 2003).

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43.2.1.4 CYP2D6

PURPOSE AND RATIONALE

CYP2D6 is involved significantly in the metabolism of drugs mainly used in CNS (antidepressants, i.e., imipramine, paroxetine, citalopram; neuroleptics, i.e., haloperidol, risperidone) or cardiovascular (β-adrenoceptor blockers, i.e., metoprolol; antiarrhythmics, i.e., propafenone, flecainide) disorders. Significant interethnic and interindividual intraethnic differences in CYP2D6 activity have been found: 5–10% Caucasians, 6–8% Afro-Americans, and only 1% Asians have reduced CYP2D6 activity, and exhibit the PM phenotype. Expression of CYP2D6 has been shown to be polymorphic with up to now more than 80 genetic variants detected for the encoding gene, with more than 15 encoding for inactive enzyme. Probe drugs for CYP2D6 phenotyping are dextromethorphan, debrisoquin, sparteine, and metoprolol. For safety concerns and drug availability, the preferred probe is dextromethorphan (Schmid et al. 1985).

PROCEDURE

Phenotyping: The method measures dextromethorphan DM and its O-demethylated metabolite, dextrorphan DX, which is formed by CYP2D6. DM and DX, and other metabolites, are excreted in urine, mainly as glucuronide conjugates.

Subjects receive a single oral 10 to 30 mg dextromethorphan (generally hydrobromide salt syrup) dose. Urine is collected from drug intake to 8 h post-dosing. Other collection times (0–6, 0–10, 0–12, or 0–24 h) can be used, but short collection intervals might lead to increased intrasubject variability.

Urine is first hydrolyzed with β-glucuronidase. Then, different methods can be used involving DM and DX extraction, followed either by HPLC and fluorescence detection (Chladek et al. 1999; Hoskins et al. 1997) or capillary gas chromatography (Wu et al. 2003).

Genotyping: The incidence of alleles coding for inactive enzymes varies between populations: three "population specific" alleles are CYP2D6*4 in Caucasians, *10 in Asians, and *17 in Africans (Bertilsson et al. 2002). CYP2D6*3, *4, *5, *6 are the main inactive alleles producing the PM phenotype in Caucasians with CYP2D6*4 most commonly associated with the PM phenotype. By far the most frequent null allele – not encoding a functional protein product – is CYP2D6*4 with a frequency of 20–25% in Caucasians (Zanger et al. 2004). The frequency of the *17 allele – associated with decreased enzyme activity – is high in Black Africans and in Black Americans but practically absent in Caucasian populations (Bapiro et al. 2002; Gaedigk et al. 2002; Zanger et al. 2004). Four potential subgroups – ultrarapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) – have been defined based on the genotype-phenotype relationships.

In Caucasian subjects, it has been recommended for "routine test" to genotype for alleles *1, *3, *4, *5, *6 which allow to detect 86–100% of poor metabolizers

(Sachse et al. 1997). To assign correct phenotype in nearly 100% subjects, *9 and *10 variants should also be determined.

EVALUATION

Subjects with a DM/DX metabolic ratio (MR) >0.3 are poor metabolizers. Subjects with DM/DX <0.03 are extensive metabolizers. Those with $0.03 < MR < 0.3$ are intermediate metabolizers.

No difference or slightly higher CYP2D6 activity in females has been found when comparing to male subjects (Hägg et al. 2001; McCune et al. 2001).

Relationship between phenotyping and genotyping is investigated by plotting log MRs versus CYP2D6 allele combinations (Chou et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

The method is widely used due to easy and safe administration. High intrasubject variability limits the test for discriminating between EMs and UMs (Zanger et al. 2004).

The method is not appropriate in patients with renal impairment, due to reduced renal excretion of DM glucuronide metabolites. Sparteine has been recommended as a probe for this population and to discriminate between the four phenotypes UM, EM, IM, and PM. The DM/DX MR does not allow for consistent differentiation between CYP2D6 extensive metabolizers with one or two active alleles.

MODIFICATIONS OF THE METHOD

Assays have been developed to determine DM and DX in plasma or saliva (Bolden et al. 2002; Hu et al. 1998; Chladek et al. 2000; Härtter et al. 1996). The use of saliva or plasma for CYP2D6 phenotyping has been developed for subject convenience, or for the development of single point methods to be easily incorporated in the "cocktail methods." Good correlation between metabolic ratios calculated from plasma, saliva samples, and those obtained from urine has been observed.

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43.2.1.5 CYP3A

PURPOSE AND RATIONALE

CYP3A is the predominant P450 subfamily (CYP3A4, CYP3A5, CYP3A7, CYP3A43) in the human liver and contributes significantly to the metabolism of many (at least 50%) drugs in numerous therapeutic classes. CYP3A4 is the major P450 present notably and predominantly in the liver and small intestine, and interindividual variability in the level of its expression is very high – 20-fold or more – (Shimada et al. 1994). CYP3A5 shares rather similar tissue distribution with CYP3A4, but it is preferentially expressed in the lung. It represents generally a few percentage of total CYP3A as compared to CYP3A4 (exceptions are esophagus and prostate, specific for CYP3A5, and kidney in which CYP3A5 is predominantly expressed). CYP3A4 and CYP3A5 exhibit overlapping substrate specificity, and there is currently no specific CYP3A5 probe drug. CYP3A7 is primarily the major fetal CYP3A enzyme.

Most of drugs biotransformed with CYP3A are also P-glycoprotein substrates (noticeable exceptions are midazolam and nifedipine). CYP3A and P-glycoprotein contribute substantially to the first-pass elimination of highly cleared CYP3A substrates when orally administered. However, CYP3A4 and P-glycoprotein activities are not coordinately regulated in the liver and in the intestine (von Richter et al. 2004).

Currently, 40 and 24 alleles have been identified for CYP3A4 and CYP3A5, respectively. Expression of CYP3A5 varies greatly among individuals (Lamba et al. 2002).

Due to multiple confounding factors, such as those involved in endogenous expression of CYP3A regulatory factors, numerous exogenous factors (environment, diet), the interplay between CYP3A and transporters in regulating drug disposition, the establishment of consistent relationships between CYP3A genotype and phenotype is actually a challenge (Wilkinson 2004). Currently, the value of CYP3A genotyping in drug development is far from being clinically useful.

The most used and validated probe drugs for CYP3A phenotyping are midazolam and ^{14}C-erythromycin (Watkins 1994). Alfentanyl, alprazolam, dapsone, dextromethorphan, lidocaine, nifedipine, omeprazole, quinine, and verapamil have also been used but less frequently, and CYP3A specificity for some of them has been questioned. The "endogenous" 6β-hydroxycortisol test (measurement of 6β-hydroxycortisol: cortisol ratio in urine) is only useful for detecting CYP3A induction and may be influenced by renal CYP3A activity.

Due to intraindividual differences in liver and intestinal CYP3A activity, phenotyping test results are related to the probe drug route of administration.

PROCEDURE

Phenotyping: *Midazolam test*: Midazolam is primarily metabolized to 1′-hydroxymidazolam by CYP3A. It is rapidly and completely absorbed after oral administration (Gorski et al. 1998). It is the probe of choice to assess intestinal and hepatic or hepatic CYP3A activities only, after oral (Thummel et al. 1996) or intravenous administration, respectively.

Oral test doses are 2, 5, or 7.5 mg (as a solution). IV doses are 0.015, 0.025, or 0.05 mg/kg, or 1 or 2 mg per subject, as a 2- to 30-min infusion.

Blood samples are collected over a 6-h period. Numerous GC, GC/MS, HPLC/UV, or LC/MS methods have been developed for plasma midazolam assay (see Lepper et al. (2004) and Frison et al. (2001)).

^{14}C-erythromycin breath test or ERMBT: CYP3A4 catalyzes the N-desmethylation of [^{14}C N-methyl] erythromycin. The test consists of the measurement of a single breath expired $^{14}CO_2$ collection obtained at 20 min following the IV administration of a 0.03 mg dose of ^{14}C-erythromycin (2–4 μCi administered) (Watkins 1994). This test is used for assessing hepatic CYP3A activity.

Genotyping: Allelic CYP3A4 gene variants are rare. No impact of the presence of the most common CYP3A4*1B mutation (with a frequency ranging from 0% in Chinese and Japanese to 45% in Afro-Americans) on midazolam, erythromycin, or nifedipine clearance has been evidenced. Most significant mutations are observed for CYP3A5 and CYP3A7. Further information on polymorphic expression of CYP3A5 and CYP3A7 can be found in the review by Lamba et al. (2002).

EVALUATION

A complete pharmacokinetic profile is required to assess midazolam clearance and is therefore more invasive than the ERMBT; however, the latter requires specific logistics for radiolabeled material use.

The midazolam or ERMBT phenotype tests are used for dose individualizing of narrow therapeutic index CYP3A-metabolized drugs such as anticancer agents. The ratio 1′-hydroxymidazolam/midazolam has generally been found not useful for phenotyping.

Within a population of similar demographic and health characteristics, a fourfold to sixfold range in the metabolic clearance of a CYP3A-drug substrate is usual, with common individual outliers exhibiting high or low activity (Lamba et al. 2002).

CRITICAL ASSESSMENT OF THE METHOD

Midazolam clearance has been found to correlate with hepatic CYP3A levels (Thummel et al. 1994) as well as ERMBT results (Lown et al. 1992). However weak, inconstant or lack of correlations between midazolam and ERMBT test results have been observed, which could be explained by binding to different CYP3A active sites. In addition, contrary to the midazolam test, the ERMBT does not capture CYP3A5 activity.

An ethnic difference – that could be drug specific – in CYP3A4 activity has been observed for few CYP3A4 substrates (alprazolam, nifedipine), with a lower clearance in Asians than in Caucasians (Xie et al. 2001).

CYP3A4 and CYP3A5 genotyping tests could not explain sufficiently the interindividual variability observed in midazolam pharmacokinetics (Eap et al. 2004a).

MODIFICATIONS OF THE METHOD

The combined use of IV midazolam and oral ^{15}N-midazolam or of the ERMBT and oral midazolam tests have been proposed to assess simultaneously the contributions of liver and intestine in CYP3A activity (Gorski et al. 1998; McCrea et al. 1999). The administration of orally given midazolam followed by an intravenous administration has also been validated (Lee et al. 2002c). A low oral 75 µg oral dose has recently been proposed, but it needs validation on a large scale (Eap et al. 2004b).

Modifications of the ERMBT have been described to improve its predictability in drug clearance estimations in cancer patients (Rivory et al. 2000).

A single blood sample for midazolam assay at 4 h post-dose has been reported as good estimator for IV or oral midazolam clearance determination (Lin et al. 2001).

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43.2.1.6 Other CYPs

This section summarizes succinctly the current knowledge on some other CYPs, the role of which in drug metabolism and the impact of genetics on it have been more recently investigated as compared to other CYPs.

CYP2A6

CYP2A6, primarily expressed in the liver, is the major CYP (the sole at usual low concentrations) involved in nicotine oxidation, and is also involved in the metabolism of carcinogen or procarcinogen compounds (such as nitrosamines and aflatoxins). A couple of drugs is metabolized by CYP2A6: chlormethiazole, coumarin, disulfiram, halothane, valproic acid, and others (Oscarson 2001). CYP2A6 PMs are less than 1 in Caucasians but up to 20% in Orientals (Oscarson 2001; Raunio et al. 2001; Xu et al. 2002). The most "in vivo deficient" alleles for PM status are CYP2A6*2 and CYP2A6*4, rather common in Orientals (15% in Chinese, 20% in Japanese). The important role of CYP2A6 in nicotine metabolism was shown in an epidemiological study revealing that the CYP2A6 genotype was a major determinant for smoking behavior and susceptibility to tobacco-related lung cancer (Fujieda et al. 2004).

Phenotyping has been performed in some countries with coumarin (not available in all countries), despite some limitations with data accuracy obtained with the analytical methods used (Pelkonen et al. 2000; Cok et al. 2001). The test assesses the amount of 7-hydroxycoumari (free and conjugated) in urine after ingestion of 2–5 mg coumarin by the subjects. Nicotine has also been used as the probe drug for CYP2A6 in vivo activity testing. Recent investigations using pilocarpine as probe demonstrated that PM status was associated with two inactive CYP2A6 alleles, CYP2A6*4A, CYP2A6*7, CYP2A6*9, or CYP2A6*10 (Endo et al. 2008).

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CYP2B6

CYP2B6 has been estimated to represent 1–10% of the total hepatic CYP content. It catalyzes bupropion hydroxylation, and S-mephenytoin N-demethylation and is involved in the metabolism of cyclophosphamide, ifosfamide, mianserin, efavirenz, artemisinin, and propofol (Turpeinen et al. 2006). CYP2B6*6 has been associated with reduced bupropion clearance in vitro (Hesse et al. 2004) but not in vivo; whereas, a moderate clearance increase was observed with CYP2B6*4 (Kirchheiner et al. 2003b). Multiple gene polymorphisms have resulted in phenotypic null alleles (Lang et al. 2004). Pharmacokinetics of the anti-HIV drug efavirenz has been associated with CYP2B6-G516T polymorphism (Saitoh et al 2007).

Bupropion (150 mg dose) has been proposed for phenotyping, but it is recommended to administer body weight-adjusted doses (Faucette et al. 2000). Efavirenz may also be a valuable probe for CYP2B6 (Ward et al. 2003).

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CYP2C8

CYP2C8 is involved in the metabolism of arachidonic acid, all-trans retinoic acid, paclitaxel, amiodarone, amodiaquine, repaglinide, rosiglitazone, torsemide, troglitazone, and zopiclone. Most of these drugs are also metabolized by CYP3A4. Recently, the potential contribution of CYP2C8 to the metabolism of NSAIDs in addition to the well-known CYP2C9 role has been highlighted for ibuprofen (Garcia-Martin et al. 2004). The CYP2C8*3 allele (present in 13% and 2% of Caucasians and Afro-American subjects, respectively) has been shown in vitro deficient for paclitaxel and arachidonic acid metabolism (Dai et al. 2001; Bahadur et al. 2002). For the antidiabetic repaglinide, unexpected in vivo lower exposure was observed in subjects with CYP2C8*1/*3 genotype, without any pharmacological consequences (Niemi et al. 2003). For ibuprofen, reduced clearance of the R(-) enantiomer was related to CYP2C8*3 allele, and reduced clearance of the S(+) enantiomer was influenced by CYP2C8*3 and CYP2C9*3 alleles. In subjects homozygous or double heterozygous for these variants (8% of 130 subjects evaluated), the clearances of ibuprofen were only 7–27% of the clearances observed in subjects with no CYP mutations. A strong association between CYP2C8*3 and CYP2C9*2 occurrence has been characterized in a large Swedish population, highlighting linkage between CYP2C8 and CYP2C9 polymorphisms (Yasar et al. 2002c).

Further in vitro/in vivo investigations are needed to assess the relationship between CYP2C8 (and CYP2C9) polymorphisms and drug metabolic clearance, in order to address the clinical relevance of CYP2C8 genotyping.

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CYP2E1

CYP2E1, an ethanol-inducible CYP, activates some procarcinogens like nitrosamines, is involved in the metabolism of endogenous substrates (steroids, bile acids), alcohols, xanthines, volatile chemicals (toluene, benzene, halocarbons) but of few drugs (chlorzoxazone, etoposide, dapsone, high-dose acetominophen) (Lieber 1997). Seven alleles, 13 genetic mutations have been described, but no genotyping-phenotyping relationships have been well established to date. Based on safe use and CYP selectivity (though CYP1A1, CYP1A2 have been found involved in its biotransformation in vitro), chlorzoxazone is the only in vivo probe drug to phenotype CYP2E1 activity toward assessment of its 6-hydroxylation (Ono et al. 1995; Lucas et al. 1999; Ernstgard et al. 2004). Due to dose-dependent metabolism, the dose should be preferably administered on a milligram/kilogram basis (10 mg/kg rather than the common 250 or 500 mg doses). Relatively low intraindividual variability in chlorzoxazone metabolism has been observed. Measurement can be done in urine or in plasma, after enzymatic hydrolysis of

6-chlorzoxazone glucuronide, using HPLC and UV detection or LC/MS/MS methods (Frye and Stiff 1996; Frye et al. 1998; Scoot et al. 1999). The use of plasma metabolite ratio determined with only one plasma sample – at 2 h post-dosing – has been recently validated.

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43.2.2 Phase II Enzymes

With the exception of N-acetyltransferases (detailed below), there are few deficiencies in Phase II drug metabolism enzymes that have resulted in clinically significant effects. Each Phase II enzyme class is most often a superfamily of enzymes, and usually there is large interindividual and interethnic variability in drug conjugations, and overlapping substrate specificity exists for numerous isoenzymes. Despite the crucial role of conjugation enzymes in xenobiotic metabolism, the functional significance of enzyme polymorphism is only known for few substrates. Therefore, with the exception of the caffeine and TPMT tests (see below), no probe test drug has been yet investigated for in vivo phenotyping and validated to assess phenotyping-genotyping relationships. Nevertheless, some important aspects of enzyme polymorphism on the pharmacokinetics of drugs with narrow therapeutic index are summarized below.

43.2.2.1 N-Acetyltransferases

PURPOSE AND RATIONALE

N-acetyl transferases Type I (NAT1) and Type II (NAT2) catalyze N- and O-acetylation reactions involved in the metabolism of drugs containing arylamino, hydroxyl, sulfhydryl groups and hydrazine structure, and also in environmental carcinogens (such as those present in tobacco smoke, or in diet such as charcoal-broiled food) (Weber and Hein 1985). Pending on the drug, and on the interplays between CYPs and N-acetylases (and other Phase II conjugation enzymes) in xenobiotic metabolism, the impact of subject status "poor acetylator" or "rapid acetylator" on drug activity and/or toxicity may vary, and then is drug specific. NAT1 and NAT2 exhibit a high degree (81%) of amino-acid sequence homology and also share common substrates (Meisel 2002), but coding genes loci are regulated independently. Main NAT2 drug substrates are isoniazid, sulfonamides, procainamide, hydralazine, acebutolol, aminogluthetimide, and dapsone.

Para-aminosalicylic and para-minobenzoic acids are considered specific substrates for human NAT1, and sulfamethazine, isoniazid, procainamide, and dapsone are considered specific substrates for human NAT2 (Butcher et al. 2002). NAT1 is considered as ubiquitously distributed in the body, whereas NAT2 is expressed in the liver and the intestinal mucosa.

Polymorphic N-acetylation was first described for isoniazid in the 1950s and is the first example of interindividual pharmacogenetic variability. Until 2007, about 30 and more than 50 variant alleles have been described for NAT1 and NAT2, respectively. At http://N-acetyltransferasenomenclature.louisville.edu overviews on the NAT alleles can be found. The presence of some NAT1 variants, as well as NAT2 variants, has been linked to increased susceptibility to some cancers (notably bladder and colon cancers), and NAT2 polymorphism associated with some drug-induced diseases such as lupus erythematosus (hydralazine, procainamide), Stevens-Johnson or Lyell syndromes (sulfonamides).

Significant interethnic and geographic differences in NAT2 activity have been found. Slow acetylators represent 40–70% Caucasians and 10–20% Asians. High acetylation capacity has been reported in 5% Caucasians (Meyer and Zanger 1997).

Probe drugs for NAT-1 phenotyping is PAS, and for NAT-2 phenotyping are caffeine, sulfamethazine, procainamide, and isoniazid or dapsone. In vivo testing for NAT2 has been proved to be useful for drug monitoring to avoid potential side effects generally observed in slow metabolizers (the exception was the anticancer agent amonafide, with myelotoxicity observed in rapid acetylators). The most used test to identify rapid and slow acetylators is the caffeine test, which is described thereafter, though the N-acetylation step takes place after the N-desmethylation of caffeine by CYP1A2 followed by the biotransformation into an unstable intermediate.

PROCEDURE

Phenotyping: Caffeine is metabolized by CYP1A2, NAT2, and xanthine oxidases. The methods could involve the measurement of 5-acetyl-formylamino-3-methyluracil (AFMU), 5-acetyl-amino-3-methyluracil (AAMU, degradation product of AFMU), 1-methylxanthine (1MX), and 1-methyluric acid (1MU) in 0–8, 0–12, 0–24 h urine of subjects orally given 200 mg or 2–3 mg/kg caffeine after a xanthine-free regimen. The common metabolic ratio used is AFMU/1MX, but the AFMU/(AFMU + 1MX + 1MU) is more discriminating (Relling et al. 1992; Rostami-Hodjegan et al. 1996) and should be used when xanthine-oxidase inhibitors may be present (Fuchs et al. 1999). Other ratios such as AFMU/(1MX + 1MU), or AAMU/1MX, AAMU/(AAMU + 1MX + 1MU) have been validated (Tang et al. 1991; Nyeki et al. 2002).

The most common methods to assay caffeine and its metabolite in urine used HPLC with UV detection (Grant et al. 1984; Krul and Hageman 1998) or mass spectrometry (Baud-Camus et al. 2001).

Genotyping: Mutations of NAT2*5, NAT2*6, NAT2*7, NAT2*14, and NAT2*17 alleles are associated with a slow acetylation phenotype for homozygous subjects (Butcher et al. 2002).

There are large differences among ethnic groups regarding alleles' frequency. High frequency (>28%) of NAT2*5 alleles has been observed in Caucasians and Africans, and of NAT2*7 in Asians (>10%) and of NAT2*14 in Africans (>8%), this last one being <1% in Caucasians and Asians (Meyer and Zanger 1997).

EVALUATION

Caffeine test: Subjects with a AFMU/1MX ratio <0.55 or a AFMU/(AFMU + 1MX + 1MU) ratio <0.26 are slow acetylators (Fuchs et al. 1999). Higher activity has been observed in black as compared to white subjects (Relling et al. 1992), and a gender effect has generally not been observed (Kashuba et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

Depending on the probe drug used and on the experimental method, two or three acetylator types can be described: slow, intermediate, and rapid, the intermediate one being not always distinguished from the rapid one. Phenotype distribution has been considered as a continuous variable (Meisel 2002). Due to slow postnatal maturation of the acetylation enzymatic systems, the acetylation status is evolving in newborns and infants and depends on the probe drug used (Rane 1999).

Good relationships between genotyping and phenotyping tests have been reported (Meisel et al. 1997; Kita et al. 2001).

The urinary caffeine test is not based on assays of specific substrates and products of NAT2 ("including" other metabolism pathways involving at least xanthine-oxidases) and is affected by diet habits, xanthine-oxidase inhibitors such as allopurinol (Fuchs et al. 1999), or other drugs (Klebovitch et al. 1995). NAT activities are affected by anti-inflammatory drugs. Of note, acetominophen is an inhibitor of NAT2 in vivo (Rothen et al. 1998).

Discordances between caffeine and dapsone phenotyping data and between NAT2 phenotyping status and genotyping have been observed in acutely ill patients infected with HIV (O'Neil et al. 2000), which may be due partly to nondetection of rare NAT2 alleles (Alfirevic et al. 2003).

MODIFICATIONS OF THE METHOD

Some recent references for other used NAT2 phenotyping tests can be found for dapsone in Alfirevic et al. (2003), O'Neil et al. (2000), Queiroz et al. (1997), for sulfamethazine in Hadasova et al. (1996) and Meisel et al. (1997), and for procainamide in Okumura et al. (1997) and Mongey et al. (1999).

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43.2.2.2 Uridine Diphosphate Glucuronosyltransferases

Glucuronidation is a potent detoxification pathway. The uridine diphosphate glucuronosyltransferases (UGTs) are involved in the biotransformation of endogenous substances (bilirubin, biliary acids, steroid hormones) and numerous drugs and carcinogens. Currently, 20 functional UGTs have been characterized with activity mainly expressed in the liver and GI tract. There are three subfamilies: UGT1A, UGT2A, and UGT2B, with distinct but broad overlapping substrate specificity existing for the different isoforms of each family. UGT1A1 is the most abundant UGT in the liver. Human diseases related to deficient UGT1A1 alleles are the well-characterized inherited unconjugated hyperbilirubinemias, including the Gilbert's syndrome which affects 6–12% of Caucasian subjects. Exhaustive reviews on roles, tissue patterns of expression, and pharmacogenomics of UGTs can be found in papers from Tukey and Strassburg (2000), Fischer et al. (2001), Guillemette (2003), and Wells et al. (2004).

A decreased clearance has been observed for some drugs metabolized by glucuronidation in patients with Gilbert's syndrome. A clinically significant impact of UGT polymorphism has to date is only demonstrated for some anticancer agents: clearly for irinotecan and with contradictory results for flavopiridol (Zhai et al. 2003). UGT1A1 and UGT1A9 are involved in the glucuronidation of the active metabolite SN-38 of irinotecan. The presence of the deficient UGT1A1*28 variant (most frequent variant as compared to UGT1A9 variants) has been clinically linked to a decrease in SN-38 glucuronidation rate and to an increased occurrence of serious side effects, mainly severe diarrhea and neutropenia (Ando et al. 1998; Innocenti et al. 2004; Iyer et al. 2002; Paoluzzi et al. 2004). Variants of UGT1A7 were reported to affect SN-38 glucuronidation but only in vitro (Villeneuve et al. 2003). Other factors, such as polymorphism in drug transporter P-glycoprotein and renal excretion, may play a role in the complex disposition pattern of irinotecan.

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43.2.2.3 Methyltransferases

There are at least four enzymes catalyzing S-, N-, and O-methylation using S-adenosylmethionine, but only thiopurine methyltransferase (TPMT) polymorphism has been found to have important clinical consequences. To date, no endogenous substrate of TPMT is known. TPMT is involved in the metabolism of mercaptopurine, azathioprine, and thioguanine, narrow therapeutic index drugs in use for treatment of patients with neoplasia or autoimmune disease, or of transplant recipients. About 0.3% of Caucasian subjects have no detectable enzyme activity and 10% intermediate activity (McLeod and Evans 2001). Four alleles TPMT*2, TPMT*3A, TPMT*3B, and TPMT*3C account for 80–95% of Caucasians with intermediate or low enzyme activities. Patients with low inherent TPMT activity are at great risk for severe potentially life-threatening myelosuppressive toxicity with treatment by the above-mentioned drugs, whereas subjects with very high activity might be underdosed (Zhou 2006). Patients with two nonfunctional variant TPMT alleles should receive 5–10% of drug standard doses. TPMT genotyping has proved its usefulness in individualizing mercaptopurine dose in patients and can replace the phenotyping test: measurement of the erythrocyte enzyme activity, based on the in vitro conversion of 6-mercaptopurine to 6-methylmercaptopurine or 6-thioguanine to 6-methylthioguanine (Innocenti et al. 2000; Evans 2004). A cutoff concentration of 45.5 nmol thioguanine/gHb.h^{-1} for this TPMT phenotyping test has been proposed for assessing the need of the genotyping test (Wusk et al. 2004).

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43.2.2.4 Glutathione S-Transferases and Sulfotransferases

Glutathione and sulfatation conjugations are important pathways for generally detoxifying endogenous substrates and xenobiotics (Commandeur et al. 1995). However, some produced metabolites (i.e., mercapturic acids, O-sulfo conjugates) are toxic by different mechanisms, often by reaction with DNA and other cellular nucleophils.

Eight classes of glutathione-S-transferases (GSTs) have been described. The role of the glutathione pathway and the impact of enzyme polymorphism have been highlighted for detoxification and some disease susceptibility, and routine phenotyping of some GSTs exists for clinical safety measurement, but currently there is not yet evidence of genotyping or phenotyping usefulness for drug dosage adjustment (Hayes and Strange 2000; Tetlow et al. 2004). GSTs are involved in the detoxification of chemotherapeutics, including platinum derivatives. Polymorphisms in the GSTP1 genotype might become a powerful tool to predict oxaliplatin-induced cumulative neuropathy (Lecomte et al. 2006).

Soluble sulfotransferases are involved in the sulfonation of endogenous substrates (notably steroids, neurotransmitters, eicosanoids) and numerous xenobiotics (i.e., acetaminophen, organic-platin anticancer agents). The presence of some sulfotransferases variants could be associated with some cancer risk. Phenotyping tests have been developed for some forms (SULT1A, SULT1A3) by measuring platelet sulfotransferase activity (Glatt and Meinl 2004).

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Physicochemical Properties

44

Heiko Tietgen and Michael Walden

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PURPOSE AND RATIONALE

Characterization of physicochemical properties attained strong interest in the pharmaceutical research area and is now a standard method. One of the key challenges is to develop a pharmaceutical active ingredient into a drug, which combines biological activity with an appropriate physicochemical profile. Poor solubility in aqueous media is one of the major hurdles in the drug development process. Many promising drug candidates have failed simply due to inadequate solubility. The impact of solubility in drug discovery on biological assays, dosing and formulation, and intestinal absorption is discussed by Kerns et al. (2008).

A poor solubility can be overcome if a compound has an appropriate high permeability through a cell membrane. Passive permeability through a membrane has been correlated with the lipophilicity of a compound in many cases (Lipinski et al. 1997). Generally speaking, high lipophilicity will ease passive cell permeation but may result in poorly soluble compounds. To find here a compromise belongs to the art of drug discovery.

Another important physicochemical parameter is pKa, which describes the ionization state of a compound at a given pH. The ionization state of a compound in the different components of the gastrointestinal system (stomach, jejunum, ileum, and colon) is crucial for the understanding of drug absorption (Dressman et al. 1998). Ionized compounds have in general better solubility, but passive permeation through the membrane is limited (Comer and Tam 2001).

All these parameters have been long investigated in drug discovery but were part of the later stage of the

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process. Nowadays, the characterization of physicochemical properties moved also in the lead-finding phase of drug discovery (Kibbey et al. 2001). Thus, screening assays had to be developed able to give comparably accurate data and a throughput to characterize hundreds of compounds a day (Kerns et al. 2008).

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44.1 Solubility Assays

PURPOSE AND RATIONALE

Solubility assays gain still growing attention in drug discovery because many pharmaceutical active compounds can be adjusted to in vivo testing just with cosolvents. Furthermore, in vitro assays may also lead to false results simply for precipitation of a compound in the assay media. Solubility assays vary in one main point: they are performed from either solids or stock solutions. A nomenclature has been established in the literature, which tries to distinguish between these methods. Determinations from stock solutions are often called "kinetic solubility," whereas "thermodynamic solubility" stands for solubility of solids (Kerns 2001). Thermodynamic solubility takes the crystal lattice forces into account. Batch-to-batch variations and polymorphism of investigated compounds are neglected in early stage of discovery but may become a key focus in later stage. Ritonavir is a prominent example (Law et al. 2001).

Stock solutions—already prepared by central logistic centers—have the advantage of standard start conditions, which are additionally similar to conditions found in biological assays. They allow a high throughput because the time delaying sample preparation is already done.

44.1.1 Determination of Solubility by Hyphenated HPLC Methods

PURPOSE AND RATIONALE

The range of solubility assays has to be <0.001 mg/ml up to >10 mg/ml in some cases. In vivo tests (orally or intravenous) need sufficient solubility of a compound in physiological or buffered solution. "True" data is also needed in compound optimization, where solubility is one of the parameters to be improved. Classification in poor, medium, or good solubility is often not sufficient. Hyphenated HPLC methods have been a long tradition in quantification and characterization of compounds in analytical sciences. Many reviews and handbooks cover this field (Unger 1994; Lunn and Schmuff 1997). The detection of the compounds is done with UV, mass spectroscopy, and light scattering or by nitrogen detection. In addition to the quantification of the solubility, impurities and side products are detected. Furthermore, buffer instability can be determined.

PROCEDURE

As described in the general introduction for solubility assays, there is a difference between kinetic and thermodynamic solubility. Thermodynamic solubility needs solid material of good quality, whereas kinetic solubility relies on organic stock solutions. The sample preparation is therefore different (Kibbey et al. 2001).

The procedure for determining solubility follows standard quantification methods with hyphenated HPLC. 0.5–1 mg of solid is weighed in a microvial, and 250 µl of buffer is poured over the solid. The vial is shaken for 24 h at 25°C. After shaking, the suspension is centrifuged and the supernatant is filtered. An aliquot (1–10 µl) of the supernatant is injected in a HPLC system. Eluents are generally acetonitrile and water containing 0.5% trifluoric acid or 0.5% formic acid. Both eluents are mixed via gradient device from 5% acetonitrile to 95% acetonitrile content. Separation is obtained on a reversed-phase column. The same aliquot of a standard is injected in a following run if one

point calibration is done (Pan et al. 2001). Better accuracy but longer evaluation time is needed if a five-point calibration is done covering a range from 0.1 µg/ml up to 2,000 µg/ml. Gradient time is set either to have higher throughput (fast gradient) or better chromatographic resolution.

The quantification is done via a calibration curve where the compound is weighed as standard and dissolved in an appropriate organic solvent (in most cases acetonitrile/water). The throughput of this method is 20 compounds a day.

If the standard stock solution is delivered from a centralized stock solution, the throughput can be increased significantly. Solubility obtained from organic stock solutions leads to kinetic solubility data (see also Sect. 44.1.2).

An aliquot of 30 µl is transferred in 250 µl buffer. The solution is shaken for 24 h at 25°C. If precipitation occurs, the sample is centrifuged and filtrated. The following procedure is the same as for the solubility from solids described above. The achieved throughput is mainly limited by the gradient time. Pan et al. (2001) investigated the effect of filtration on the quantification of solubility. They recommend polytetrafluoroethylene (PTFE) as the filter material of choice. In their compound set, they found 98% recovery after filtration.

Quantification of the compounds is done via different detection modes such as:
UV or photodiode array (PDA)
Mass spectroscopy (MS)
Nitrogen detection (CLND)
Light scattering (ELS)
or a combination of them (Guttman et al. 2004). Evaluation of the compound structure before quantification is necessary because not all compounds have, for instance, a chromophore (UV detection) or nitrogen (CLND).

EVALUATION

High-throughput methods use one-point calibration. The quantification of the solubility (c_{sol}) is done via the peak areas under the curves (AUC) of standard and measurement. For one-point calibration,

$$c_{sol} = \frac{AUC_{sol}}{AUC_{st}} * c_{st}.$$

Identification of the compound is done via either the retention time or the mass of the peak, which is to be quantified. If the chromatographic resolution is not good enough to separate impurities or degradation product, the measurement has to be repeated with longer gradient times or longer reversed columns.

CRITICAL ASSESSMENT OF THE METHOD

In the early stage of drug discovery, the main variance of solubility data is in most cases linked to batch-to-batch variations of a synthesized compound. Most batch-to-batch variations are related with different side-product profile or purity, and differences in the crystallinity. In extreme cases, oily, amorphous, or crystalline batches may be obtained for one compound. These effects superimpose errors coming from the experimental conditions.

Very poor soluble and very good soluble compounds will give an AUC far different from the standard injection. This may leave the linear range of the calibration. They are quantified therefore far more accurately with 5-point calibration, which covers a wide concentration range. Very good soluble compounds should be diluted to reach the linear range of the AUC/$c_{analyte}$ curve.

Very poor soluble compounds are in most cases lipophilic. They tend to stick to the used filter systems or used plastic devices, which leads to compound loss. Thus, very poor soluble compounds may be underestimated due to the sample preparation (Pan et al. 2001).

Each detection system has its own limitations. Compounds with no chromophore are not UV active for instance and may be quantified with ELS. A short analysis of the compound class is therefore needed to prepare and interpret the solubility experiments. Direct quantification of the sample without any external calibration is possible if a nitrogen detector is used. Throughput is higher and a standard injection is not necessary.

If a compound with high solubility exceeds the buffer capacity, it may change the pH of the buffer system. This is not realized without any pH control, which could be difficult to control in a standardized and miniaturized environment. This methodology fails for compounds without nitrogen or with nitrogen-nitrogen double bonds.

MODIFICATIONS OF THE METHOD

To achieve high-quality data with low sample amount, a commercial pSOL instrument (pION, Woburn, MA)

can be used. It delivers with a pH metric approach a solubility pH profile with 100 µg of solid compound. The throughput is 4–6 compounds a day.

TREGA describes a protocol for pH-adjusted measurements (TREGA). They need very accurate data for their idea of an computational program, simulating drug absorption in the gastrointestinal system. Five times 30–50 mg of compound are weighed in 10-ml vials. The vials are filled with 8 ml of buffer covering a pH range between 2 and 8. The vials are shaken for 8 h and the pH is controlled. If necessary, the pH is adjusted. The pH adjustment is done with HCl and NaOH. After pH adjustment, the compounds are shaken for additional 2 h. The pH adjustment is done until saturation solubility is achieved and the pH is stable. The experiment is tedious and reduces the throughput to a couple of compounds a day.

Kerns et al. (2008) reported a method where solid compounds are weighed into a Mini-UniPrep™ filter chamber. 0.45 aqueous buffer is added and the chamber is shaken for 24 h at room temperature. After automatic filtration, the supernatants are directly injected in a HPLC instrument. The throughput is reported to be 50 compounds a day per HPLC instrument.

An enhancement of this method is reported by Zhou et al. (2007). The so-called high-throughput equilibrium solubility (HT-Eq sol) assay starts from DMSO stock solutions but dries the compounds by evaporation. This avoids the time-limiting step of manual weigh-in, but results in the loss of the crystalline form. A comparable technique was used in the PASS assay Alsenz et al. (2007); here, the compound is handled as a suspension in heptane to achieve automated liquid handling. After drying the compound, the crystal lattice is not changed and the solubility measurement can start in a very small scale (96-well PCR plates). The readout is done by a fast UPLC method, resulting in a total throughput of 600 measurements per week.

Dressman reviewed the methodologies used for simulating biofluids. These methodologies focus on the solubility behavior and dissolution rate in solvents, which mimic the fluids in the gastrointestinal system like Fessif and Fassif. These dissolution tests are invented for better prediction of in vivo performance of drug products. They are barely comparable with solubilities in buffered aqueous media and have a limited throughput of a couple of compounds a day.

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44.1.2 High-Throughput Solubility Assays

PURPOSE AND RATIONALE

In vitro testing of drug candidates and combinatorial chemistry lead to an increase of lipophilic compounds with poor solubility (Lipinski et al. 1997). Poor solubility itself may lead to poor oral availability of a potential drug. The growing demand for solubility data in lead phase of drug discovery is answered by a variety of simple solubility assays, which allow the classification of a compound without real quantification (see Chapter "Solubility Assays"). One of the easiest ways to detect saturation in a solvent is the

turbidity of the solution if precipitation occurs. The turbidity caused by precipitation of a poorly soluble compound can be detected by a couple of detection methods (Van de Hulst 1981; Hongve and Akesson 1998). Lipinsky describes the first methodology, which uses UV as detection method and is able to screen hundreds of compounds a day with one instrument.

PROCEDURE

Lipinsky dissolved compounds in DMSO at a concentration of 10 μg/ml. Complete dissolution is controlled by eye. One microliter of this solution is added into a cuvette containing 2.5 ml pH 7 phosphate buffer. Mixing of the system is controlled via an integrated mixing device. The temperature is kept constant between 22°C and 25°C. Stepwise one-microliter portions are added to the mixing chamber. After each step, an equilibrium time of 5 min is allowed before turbidity is analyzed. These steps are repeated up to 14 times, covering a range of <5 to >65 μg/ml. If precipitation occurs, the addition of compound is stopped after further two consecutive additions. The volume percent aqueous DMSO does not exceed 0.67%.

EVALUATION

Precipitated particles lead to an increase in UV absorbance due to light scattering. Lipinsky used a diode array UV (Hewlett Packard HP8452) at 600–820 nm for their experiment. UV absorbance (y-axis) vs. μl DMSO plots (x-axis) is used to detect the precipitation point. A strong increase in the slope of the curve indicates precipitation. Precipitation defines the maximum solubility level in this experiment. The method allows a classification between poor, moderate, and good soluble compounds. Poorly soluble compounds have in this scheme a solubility less than 10 μg/ml, whereas good solubility is defined as solubility higher than 65 μg/ml.

CRITICAL ASSESSMENT OF THE METHOD

The method has been used and modified by different authors (Bevan 2000; Green et al. 2004) and is today a standard solubility assay in industry. Advantages are the simplicity of the experiment and the low sample consumption. Impurities of the samples (which are common in the early drug discovery) are not detected and could lead to false-positives if the mixture has a better solubility as the pure material. False-negatives are observed if impurities have lower solubility as main material. Growing DMSO content alters the solubilizing properties of the aqueous media. Up to 5% DMSO content is seen as appropriate for a solubility ranking. It does reflect also the situation in many biological assays where the DMSO content is up to 5% (Lipinski et al. 1997). However, the quality of the data allows a ranking of compounds in poor/medium/good soluble compounds.

The method does underestimate compounds with slow dissolution rate. To minimize the risk of a wrong assessment, control measurements with other methods (preferably HPLC methods, see Sect. 44.1.1) are recommended. This could be done with one or two members of a compound class.

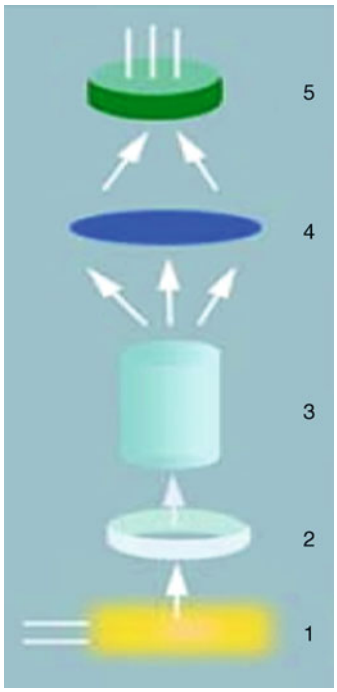
MODIFICATIONS OF THE METHOD

Bevan introduced a high-throughput alteration of the method. He used 10 mM DMSO stock solutions for a precipitation assay. In many biological assays, 10-mM DMSO stock solutions are the standard stock solutions from which aliquots are added to the various assays. 10-mM DMSO stock solutions are readily available, and the sample preparation can be minimized.

The assay itself is performed in a 96-well plate. DMSO stock solution is diluted 20-fold in PBS (0.01 M, pH 7.4). The diluted solution is again serially diluted 10 times with 5% DMSO/95% PBS across the 96-well plate in order to keep 5% DMSO content stable. The solubility assay is performed with two replicates for each compound; thus, eight compounds can be measured per plate. Detection of the precipitation is done with a helium/neon laser nephelometer (BMG Labtechnologies, Offenburg, Germany). The instrument lases at 632.8 nm, whereas the laser beam passes the well in a vertical and concentric path. The instrument detects just linear light from the source. A clear solution gives no signal (Sketch 44.1).

Precipitation leads to light scattering which is detected. The following solubility classifications are used: poor solubility (<10 mg/ml), medium solubility (10–100 mg/ml), and good solubility (>100 mg/ml).

The method can be faster if the liquid handling is done by an automated system. Many vendors have integrated nephelometer in their systems. An example is shown by Dehring et al. (2004). Here a Tecan platform was used together with the nephelometer.



Sketch 44.1 Principle of nephelometric detection

Consequent time optimization from liquid handling to data evaluation by adapted software leads to a total measurement time from protocol setup to data analysis from 6 to7 h for 96 compounds.

The methodology is now also adopted for 384-well plate format (Green et al. 2004). The data obtained by this method have good correlation with data obtained for 96-well plate format. The batch-to-batch variability is 5%. The quality of the well plates has a high influence on the obtained data. Even microscopic scratches may pretend precipitation. Pan et al. (2001) investigated different well plate types. His recommendation is to use Costar clear bottom white plates. Clear polypropylene Cliniplate (LabSystems) has also good quality but provided lesser signal enhancement.

Fligge and Schuler integrated an automated 384-well kinetic solubility method by nephelometry into the early validation LCMS process used for new compounds. Most pharmaceutical companies use a fast LCMS run for purity determination of a freshly synthesized compound delivered by their stock room as a DMSO solution. Here no additional sample logistic for the solubility measurement is necessary; the solubility value to rank a compound is available together with a purity report.

Avdeef introduced an alternative approach to measure kinetic solubility. Aliquots of DMSO stock solutions are pipetted robotically in incubation well plate, which contains an aqueous buffer. The concentration of the test compound should be between 50 and 150 μM in order to keep the DMSO content below 0.5%. After a time of incubation, the plate is filtered and the solved compound is quantified with a UV plate reader. The method is fast and robust and reported to be reliable (Kerns and Chen). Two hundred to 300 compounds can be measured a day. Additionally, pH-solubility profiles can be set up easily (Kibbey et al. 2001).

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44.2 Determination of pKa

PURPOSE AND RATIONALE

The pKa is the dissociation constant for a given medium. It is a parameter which indicates the ionization state at a given pH and describes the acidity of a compound in a special medium. In drug discovery, it is a valuable parameter for SARs but also helps in the interpretation of pH-solubility profiles. For an acid:

$$\mathrm{pKa} = -\log 10\ \mathrm{Ka}\ (\mathrm{Ka\ is\ the\ ionization\ constant})$$

$$\mathrm{HA} = \mathrm{H}^+ + \mathrm{A}^-\ \ \mathrm{Ka} = [\mathrm{H}^+][\mathrm{A}^-]/[\mathrm{HA}]$$

$$\mathrm{pKa} = \mathrm{pH} + \log[\mathrm{HA}]/[\mathrm{A}^-]$$

In practice, the pKa is the pH where 50% of the compound is ionized.

Molecules can have more than 1 pKa depending on the number of ionizable centers. In pharmaceutical research, the ionization state between pH 2 (stomach) and 8 (pH in colon) is most interesting. A review by Taylor covers the effects of the different protonization/ionization forms of a molecule on pharmacokinetic and pharmacodynamic properties. Molecules are less soluble in aqueous media but more permeable through membranes in their neutral form.

Today most pKas are determined by classical titration with UV (Albert) or potentiometric (Sirius GLpKa) detection. These methods could be coupled with multiwavelength spectrophotometer detection (Sirius, D-PAS). Tam et al. (1998) compared both methodologies and found good correlation between them.

PROCEDURE

The classical potentiometric approach (Sirius, GLpKa) can be coupled with multiwavelength detection, thus gaining enhanced sensitivity with decreased sample consumption.

Multiwavelength spectrophotometer detection is based on the change of the ionization state of a molecule. A change of the ionization state leads to a change of the electron density, which directly leads to an increase or decrease of UV absorbance – monitored by spectroscopic detection. The titration is performed with KOH or HCl across a pH range of 1.8–12.2. HCL and KOH solutions are prepared for the titration according Avdeef. The aqueous medium for compound solutions can be chosen individually. An ionic strength of 0.15 potassium chloride is recommended. The D-PAS uses an automated titration system coupled with fiber optic dip probe, a UV light source, and a photodiode detector. Spectral changes, which arise during the titration, are captured at concentrations down to 10^{-5} to 10^{-6} M.

The titration intervals can be set individually via the instrument software. The compound is solved in the measurement cell at a concentration of 10–100 μM, whereby the pH adjustment by automated titration of the system has an accuracy of 0.02 pH units. Tam suggests in their report titration steps of 0.1 pH units and to perform 25–35 pH readings and absorption spectra measurements during each titration.

Poorly soluble compounds can be measured with cosolvents as methanol, octanol, or DMSO at different concentrations. Their true water solubility can be extrapolated by the Yasuda-Shedlovsky method.

EVALUATION

The instrument software computes the operational pH readings to the pH value by a multiparameter equation reported by Avdeef. The pKa values are calculated from the pH values and the absorption data of different wavelengths. The calculation is done by target factor analysis (Allen et al. 1998).

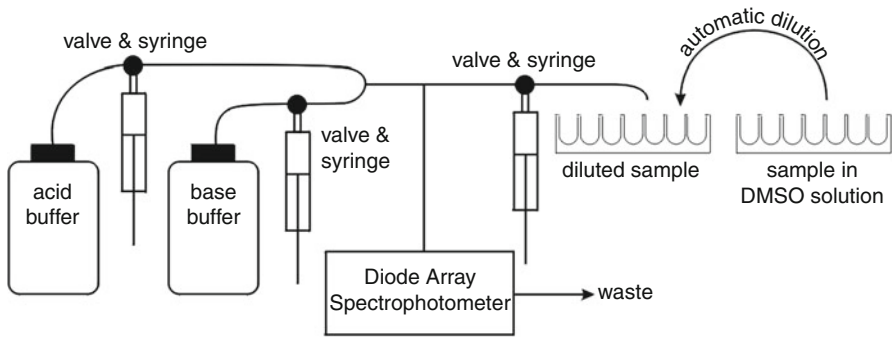
The Yasuda-Shedlovsky method pKa + log $[H_2O]$ = (A/ε) + B is used for the extrapolation of molecules with poor aqueous solubility measured within aqueous buffer containing different percentages of organic cosolvent. ε is the dielectric constant of the cosolvent mixture, $[H_2O]$ for the molar concentration of water, whereas A and B represent slope and intercept of the plot. pKa + $[H_2O]$ is plotted against 1/ε.

CRITICAL ASSESSMENT OF THE METHOD

The method shows very good correlation to pKas obtained by classical titration (Allen et al. 1998) or the literature (Lide and Frederikse 1996). Weak UV absorbance also limits the determination of pKas far away from a chromophore. A range of three bonds is considered tolerable (Tam et al. 1998). Poor UV absorbance may be enhanced by rise of the analyte concentration. If poor solubility of a compound may prevent the compound concentration to increase, then the use of cosolvents is recommended.

Furthermore, it has to be considered that side products or hydrolysis of the analyte in the pH profile can

Sketch 44.2 High-throughput pKa determination



give rise to false-positives. Thus, a quality control step before is recommended.

MODIFICATIONS OF THE METHOD

Comer and Tam (2001) developed a high-throughput pKa assay (SGA profiler, Sirius Analytical) which is able to measure the pKas of up to 200 compounds between pH2 and pH12. Compounds are dissolved in DMSO to give 10-mM stock solutions. Five to 20 µl of the stock solution is transferred in a deep well plate and is diluted with 2 ml of water. Up to 96 compounds can be measured per plate. The dilution is injected in a flow pH gradient, which is produced by mixing two buffer solutions containing mixtures of weak bases and acids. These mixtures have low absorbance in the UV above 250 nm. The flow gradient passes a diode array spectrometer covering 280–800 nm (Sketch 44.2).

Evaluation is done with the instrument software. The software calculates the change in UV absorbance at 30 different wavelengths in order to calculate the pKa. Since the pH gradient of the combined mixtures is linear over a wide range, the pH is a linear function of time. The pKas are calculated from the change of UV absorbance at multiple wavelengths as a function of time.

Up to 70% of all samples can be taken into account for a measurement, since most compounds in drug discovery have a chromophor and/or are nitrogen containing. The sample consumption is very low. Poor solubility of an analyte in the used buffer system is major hurdle of the method. If a compound precipitates during the measurement, dramatic UV changes are observed. If the compound is not soluble in water at all, it will precipitate in the mother plate. Thus, no UV change will be observed due to lack of compound. This will lead to false-negative results.

In order to measure a wider range of compounds, the traditional pH titrimetric methods (Jander and Blasius 1995) are still of good use. In the pH metric method, the sample is titrated in a pH range of choice with acid or base. The titration is monitored with a pH electrode. The pKas can be calculated from the shape of the titration curve. In order to get good resolution, small pH intervals are titrated, thus limiting the throughput of the method and leading to an increase in sample consumption. For validation and reference measurement this method is still the method of choice.

Small sample consumption combined with data of high accuracy can be obtained with capillary electrophoresis. Miller et al. (2002) demonstrated that pH-dependent capillary electrophoresis can be used for the titration of pKas. The work based on earlier contributions of Gluck et al. Analogous to the pH metric method, capillary electrophoresis is performed at a variety of different pHs. The mobility of the sample is measured and can be plotted in a pH/mobility curve. pKas are derived from the shape of the curve. Jia showed that capillary electrophoresis can be used in medium throughput for a broad range of pharmaceuticals.

Early works from Kibbey have been meanwhile customized to give commercially available equipment for high-throughput pKa measurements by CE (e.g., pKa Pro™, Advanced Analytical Technology). These systems have the advantage of standardized buffer systems – once the main problem for reliable pKa values by CE (Rafols et al. 2008). Henchoz et al. (2009) give a good summary of the methodology.

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44.3 Lipophilicity

PURPOSE AND RATIONALE

Lipophilicity of a compound is associated with many physicochemical and physiological properties, e.g., permeability through a membrane. Lipophilicity is described in most cases as partition between two phases (hydrophilic and hydrophobic). Hansch was one of the first who examined the relevance of partition coefficients for the explanation of structural activity relationships.

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents, classically octanol and water.

In the case n-octanol and water:

$$P_{ow} = c_{n-octanol}/c_{water}$$

The partition coefficient itself is a constant. It is defined as the ratio of concentration of compound in aqueous phase to the concentration in an immiscible solvent, *as the neutral molecule*. The partition coefficient (P) therefore is the quotient of two concentrations and is usually given in the form of its logarithm to base 10 (logP). The logP will vary according to the conditions under which it is measured and the choice of partitioning solvent.

However, logD is the log distribution coefficient at a particular pH. It does take into account the equilibrium between ionized and not ionized species with the two solvents:

$$\text{Distribution Coefficient, D} = [\text{Unionized}]_{(o)}/[\text{Unionized}]_{(aq)} + [\text{Ionized}]_{(aq)}$$

$$\text{LogD} = \log_{10}(\text{Distribution Coefficient})$$

LogD at pH 7.4 is often quoted to give an indication of the lipophilicity of a drug at the pH of blood plasma. There are meanwhile 100,000 logP and logD values available, thus making it a valuable tool for comparison.

44.3.1 Lipophilicity by Octanol/Aqueous Shake Flask

PURPOSE AND RATIONALE

In order to determine a partition coefficient, equilibrium between all interacting components of the system must be achieved, and the concentrations of the substances dissolved in the two phases must be determined. Octanol and water are standard solvents for the equilibrium experiments. They are readily available and allow equilibrium experiments with good repeatability and reproducibility.

PROCEDURE

Two large stock bottles (2 l) of high-purity analytical grade n-octanol or water are saturated with a sufficient quantity of the other solvent. Both solvents are shaken for 24 h on a mechanical shaker. They are allowed to stand for 2 days to permit the phases to separate. In order to determine logP, a buffer system is chosen which has >2 pKa units above the pKa of the compound if the compound is a base. For acidic compound, the pKa has to be <2 pKa units below. Neutral compounds can be examined in water.

In order to determine $\log D_{7.4}$, appropriate phosphate buffer has to be chosen. After shaking for 4 h, two equal volumes of the phases are placed in a centrifugation tube. An aliquot of the test compound is added. The centrifuge tube is rotated 100 rotations in 5 min 180° about the transverse axis. In order to separate the phases, the samples are centrifuged at room temperature. They are standing for at least 1 h to ensure phase separation and constant temperature. An aliquot of each solvent compartment is then quantified with an appropriate method. Examples of analytical methods are:

Photometric methods
Gas chromatography
High-performance liquid chromatography

EVALUATION

For the determination of the partition coefficient, it is necessary to determine the concentrations of the test substance in both phases. The total quantity of substance present in both phases should be calculated and compared with the quantity of the substance originally introduced.

The logP and logD values are calculated from the ratio of their presence in the two components:

$$P_{ow} = c_{n-octanol}/c_{water}$$

CRITICAL ASSESSMENT OF THE METHOD

The measuring range of the method is determined by the limit of detection of the analytical procedure. This should permit the assessment of values of log P_{ow} in the range of −2 to 4 (occasionally when conditions apply, this range may be extended to log P_{ow} up to 5) when the concentration of the solute in either phase is not more than 0.01 mol/l.

The test temperature should be kept constant (±1°C) and lie in the range of 20–25°C. The method is restricted to samples of high purity and where plenty of compounds are available.

The throughput of the methods is limited due to the sample preparation and tedious experimental setup. It gives high-quality data, which can be used for cross validation of methods and reference data.

MODIFICATIONS OF THE METHOD

Hitzel reported a high-throughput variation of the method. They adopted the shake flask experiment to well plate format. Octanol, buffer, and compounds are transferred in the well plate by a liquid-handling system (Beckman Biomek 2000). The plate is sealed and shaken on reciprocal shaker for 30 min. The plate is centrifuged afterward, and aliquots of the aqueous and organic phase are analyzed via fast-gradient high-throughput chromatography. The correlation of the method with manual shake flask experiments in Eppendorf vials was excellent according to the authors.

Dohta et al. (2007) describe a promising attempt to increase the throughput of logD measurements by automated sampling which turned out to be difficult due to octanol contamination of the water phase. Nevertheless, they got good correlation to literature data and a significantly increased throughput.

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44.3.2 Lipophilicity by Partition Chromatography

PURPOSE AND RATIONALE

Partition P of a compound between octanol phase (lipophilic) and water (hydrophilic) phase is one of the most commonly used parameters to express lipophilicity. The logarithmic value of P logP is the most common lipophilicity parameter. Strictly spoken, logP is the partition parameter of the neutral compound. Thus, in most cases, the logD is the measured parameter of choice (partition of neutral and charged molecule between buffered solution at a given pH and octanol). Besides shake flask experiments (see Sect. 44.3.1), chromatography is one of the major methods to determine lipophilicity data. Chromatography is readily available and easy to use. Nevertheless, one gets no exact partition coefficient, but you can find good correlation with shake flask measurements. The throughput of chromatographic methods is far higher than that of the traditional shake flask experiments (Valko et al. 1997).

PROCEDURE

The partition of the compound between stationary phase (in most cases octadecane bonded on silica) and mobile phase is used as surrogate parameter for lipophilicity. The retention time of the analyte correlates with the lipophilicity of the compound.

In order to get direct octanol-water partitioning, it is essential to immobilize octanol at the stationary phase (Mirrlees et al. 1976; Unger et al. 1978). The coating is achieved with repeated injections of pure octanol with a buffered eluent saturated with octanol. The octanol-coated columns have lengths of 1 cm (for logD 0–4.5) and 25 cm (for logD 0–1.5). The mobile phase is octanol-saturated 10-mM phosphate/KCl buffer (pH 7.4). Saturation of the buffer with octanol is achieved by shaking 1 l of octanol with 20 ml of octanol. The resulting octanol-saturated buffer is filtered. For high logD determination, up to 30% methanol is added.

20 µl of a 10-mM compound solution in DMSO is injected in the HPLC. The 1-cm column is used for high logD with a flow rate of 10 ml/min, whereas the compounds with low lipophilicity (logD < 1.5) are characterized with the 25-cm column at a flow rate of 2 ml/min. In order to avoid octanol loss on the column due to the DMSO, the direction of injections is reversed with every sample.

EVALUATION

The capacity value k' (retention time – column dead time) is used for the calculation of the logD. For this purpose, a standard set of compounds with known $\log D_{7.4}$ is used for calibration of the system.

$\log D_{7.4}$ for the standards is plotted against logk'. The linear equation

$$\log D_{7.4} = a \log k' + b$$

is used for the determination of $\log D_{7.4}$. In order to determine logP, a buffer system is chosen which has >2 pKa units above the pKa of the compound if the compound is a base. For acidic compound, the pKa has to be <2 pKa units below.

CRITICAL ASSESSMENT OF THE METHOD

The method gives high-quality data with very good correlation with traditional shake flask (Slater et al. 1994)-derived logDs. The method is not suited for compounds with very low lipophilicity logD < -1 because for them no retention on the stationary phase is achieved. The other limitation is compounds with a lipophilicity >4.5. Due to the long retention time, the peaks become not detectable any more. Furthermore, the method is limited to compounds with chromophores. The throughput of the method is about 20–40 compounds a day.

MODIFICATIONS OF THE METHOD

An isocratic octanol-saturated 0.02 MOPS buffer in water is used from Minick et al. for chromatographic extrapolation of logD. They use an octyl (C8)-bonded stationary phase and vary the methanol content of the buffer. The methanol free log k' is derived via extrapolation. This methodology is well suited for

compounds with high lipophilicity but also needs a chromophore due to the UV detection of the instrument setup.

Lombardo et al. use also different octanol concentrations in their isocratic method. They use a LC-ABZ column (Pagliara et al. 1995). This method is capable of covering a wide lipophilicity range with good correlation with $\log P_{ow}$. Alternative detection methods such as mass spectrometry, ELS, or nitrogen detector are in most cases not appropriate due to the octanol-saturated aqueous media. An alternative is the use of standard HPLC reversed-phase gradient systems (Valko et al. 1997). These systems do not represent true octanol-water partitions, but are also good lipophilicity parameters by themselves.

Valko et al. have developed chromatographic methods, which are based on established reversed-phase methods with acetonitrile water gradients. The lipophilicity is characterized as a so-called chromatographic hydrophobicity index (CHI), which approximates the percentage of acetonitrile necessary for equal distribution between mobile and stationary phase. The method is able to provide hundreds of CHIs parallel to the quality control of compounds via RP/HPLC.

The next chapter will focus on alternative partition coefficients to P_{ow}.

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Clinical Pharmacokinetic Studies

45

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45.1 Introduction

Drug efficacy and response are a function of drug concentration over time. In clinical pharmacokinetic studies, aspects of drug absorption, distribution, metabolism, and excretion over time are assessed. In the early clinical development, the pharmacokinetics of a drug is studied in healthy subjects followed by studies in patient population(s) with the aim to find the relevant dose in the target population(s). Particular pharmacokinetic studies in special populations assess the necessity of a dose adjustment from the planned/established clinical dose for patients.

In the following chapters, typical clinical pharmacokinetic studies are presented with results and conclusions, along with special emphasis on the individual study rationale, objective, design, and evaluation technique. In addition, a critical assessment of the method, as well as potential modifications and alternatives, is presented.

For the purposes of simplicity, the description of each study is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety (and pharmacodynamic) parameters were also studied.

The protocols were developed with consideration of the current good clinical practices and conducted in compliance with the protocol that had received prior independent ethics board approval.

The principles and practices concerning the clinical conduct with particular emphasis on ethical aspects are stated in guidelines (International Conference on Harmonization E6 R1 2002; International Conference on Harmonization E8 1998). These principles have their origins in "The Declaration of Helsinki" (The Declaration of Helsinki 2008).

Design and conduct of the clinical studies are presented in the examples below; all were in conformance with these principles. Subjects were included only after an informed consent was given. All studies were part of a sound clinical development plan of the sponsor.

The protocols were subject to critical review, and it was assured that the information they contain is consistent with the actual risk-benefit evaluation of the investigational product. The respective internal review boards of the sponsor had approved them before finalization.

Assays used for bioanalytical measurements were validated, as the complete evaluation, assessment, and reporting of these clinical pharmacokinetic studies followed international and scientific quality standards.

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45.2 Exploratory Assessment of Drug Dose Linearity/Proportionality and its Use for Study Optimization

PURPOSE AND RATIONALE

The first tolerability studies in early clinical development always provide pharmacokinetic (PK) data over a considerable dose range. Especially the explorative first-in-man study with escalating single doses or an explorative proof-of-principle study with escalating multiple doses provides a valuable basis for an exploratory assessment of dose linearity/proportionality of drugs in humans. In addition, such an assessment can directly help within the same study to optimize the dose selection and dose progression. Already in this early phase of the development, these data are going to support exposure-response relationships and thus a potential submission (US FDA Guidance for Industry Exposure-Response Relationships 2003; ICH E4 1994).

Information on dose linearity/proportionality in humans is required during later development if the dosage form is to be modified (EU CPMP 1999), and especially if several strengths are in use (US FDA Guidance for Industry Bioavailability and Bioequivalence Studies for Orally Administered Drugs 2003) or bioequivalence has to be supported (US FDA Bioequivalence Guidance 2006; EU CPMP 2010).

A priori however, animal data can provide hints with respect to the likelihood of observing a nonlinear relationship of the administered dose and

the resulting exposure as reflected by PK parameters. But even if doses were compared on a "per kg" basis, animal studies might be misleading due to the different physiology and extrinsic factors in animals and man.

Dose linearity/proportionality typically is assessed based on exposure-related PK parameters measured in the systemic circulation (blood), like the AUC for a specified time interval, or sometimes also based on the peak values (C_{max}), but can also be estimated based on PK parameters which are expected to be dose independent, like clearance (CL), (terminal) half-Life ($t_{½z}$), or volume of distribution (V). And for a drug, which is mainly eliminated via the renal route, this assessment of dose proportionality can also be based on the amounts of drug excreted in urine over a specified time or extrapolated to infinity. In both cases, it is important to use an assay with a sensitivity sufficient to quantify the amounts of drug completely.

PROCEDURE

The design of an exploratory assessment of dose linearity/proportionality during the conduct of a first-in-man study for candidate drug (XYZ1234) is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are in the main focus.

45.2.1 Protocol Outline

Single oral dose-escalation study, to study safety, tolerability, pharmacokinetics, and pharmacodynamics of XYZ1234 in healthy male subjects (first-in-man study).

45.2.1.1 Secondary Objective

One of the secondary objectives of the study was to determine the pharmacokinetics (PK) of ascending single oral doses of XYZ1234.

45.2.1.2 Study Design

The study had a single-center, double-blind, placebo-controlled, randomized, single oral dose-escalation study design. Eight healthy male volunteers per dose step were to be randomized and treated with up to two of the following escalating dose steps: 10-, 25-, 50-, 100-, 200-, 300-, and 400-mg XYZ1234, or placebo, with a washout period of at least 2 weeks between the treatments. Six subjects in each dose step were randomized to XYZ1234 and two subjects to placebo.

Starting with the lowest dose, each of the subsequent doses was administered only if the preceding dose was safe and well tolerated. The decision to proceed to the next higher dose was based on the full range of safety parameters and pharmacokinetic data.

Eligible subjects entered the study unit the evening before study drug administration and were to be assessed for their baseline characteristics on the morning of the day of drug administration. After each oral dose, subjects remained in the study unit for 48 hours (h).

45.2.1.3 Inclusion Criteria

Healthy male volunteers, aged between 18 and 45 years, inclusive.

45.2.1.4 Treatments

In this study, a clear, colorless solution of varying concentrations of active substance XYZ1234 was used to provide the same dosing volume for the solution in each dose step. The escalating, double-blind doses of XYZ1234 oral solutions were administered at doses of 10, 25, 50, 100, 200, 300, and 400 mg or placebo.

The decision to proceed to the next higher dose was taken based on the full range of safety parameters and the bioanalytical data (with pharmacokinetic parameters for the last-but-one dose step, which had to be available and acceptable for the next planned dose step, based on an adequate extrapolation). This could result in the planned higher dose step(s) being skipped or, conversely, to additionally include a dose below the starting dose of 10 mg. The doses to be administered might have to be modified (decreased or interim steps be defined) as needed according to the ongoing benefit/risk assessment during the conduct of the study. The dose progression was to be continued until the maximum tolerated dose had been attained, or if derived from bioanalytical data, other findings were to be expected which prohibited further dose increases (e.g., prohibitive nonlinearity or the AUC being too large compared to the AUC from toxicology).

45.2.1.5 Pharmacokinetic Data

Concentrations of XYZ1234 in urine and plasma were determined and used for calculation of the PK parameters.

If feasible and adequate, an explorative investigation on potential metabolites in plasma and urine was to be performed.

EVALUATION

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and pharmacokinetic parameters calculated.

Individual plasma concentrations of XYZ1234 were tabulated together with standard descriptive statistics. Individual and median profiles were presented graphically.

45.2.1.6 PK Parameters

If possible, the following PK parameters were to be determined based on plasma concentrations of XYZ1234 using noncompartmental or, if adequate, compartmental procedures: at least maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration-time curve from time of drug administration to t hours ($AUC_{(0–t)}$), area under the concentration-time curve from time of drug administration extrapolated to infinity (AUC_{inf}), terminal elimination half-life ($t_{½z}$), mean (residence) time (M[R]T), as well as relative total oral clearance (CL/f) and relative volume of distribution during the terminal phase (Vz/f).

For urine data, the individual and mean fractional and cumulative urinary excretions of XYZ1234 were calculated; the cumulative excretion profiles were represented graphically; fractional and total urinary excretion ($Ae_{(t2–t1)}$; $Ae_{(0–48\ h)}$), urinary recovery (% of administered dose), and renal as well as nonrenal clearance (CL_R, CL_{NR}) were determined.

Explorative dose proportionality was to be investigated in parallel to the progress of the dose escalation using dose-normalized values (on a dose per body weight basis) for AUC, C_{max}, and Ae [characterized by an additional suffix "$_{norm}$"] and/or by adequate regression analyses of all parameters. Predictions for the next dose steps were to be derived based on these findings.

CRITICAL ASSESSMENT OF THE METHOD

As rationale for selection of the dose range, doses providing a pharmacological effect (active doses) and doses being at the upper dose limit (NOAEL doses) were used. The dose progression was to be continued until the MTD was attained or—derived from bioanalytical data—until other prohibitive findings (prohibitive nonlinearity, area under the plasma concentration-time curve (AUC) exceeding that AUC from the toxicological studies) arose.

The described evaluation provides a tool, also called "online PK," which allows adjusting the dose in this "first-in-man" study on a very flexible basis. Consequently, already in the study protocol, this flexible dose scheme is described. The main prerequisite is, besides an adjustable dosing form, an immediate shipping and evaluation of the bioanalytical samples.

On an explorative basis, the relationship of concentrations resulting from the different doses can be conveniently studied using an evaluation as described in this chapter. Typically, this evaluation is part of the first study in humans (which is always a single-dose study) but could also be applied for early multiple-dose studies in analogy.

As mentioned earlier, this type of supportive study provides explorative data related to dose linearity/proportionality. In the context of the described study, these data are then used to predict the exposure for further planned dose steps, (inside) and especially outside the dose range investigated so far.

At least, if a notable nonlinear effect is seen by this explorative evaluation, then a more elaborate study will need to be performed.

MODIFICATIONS OF THE METHOD

Typically, a dose-proportional increase of exposure (AUC) cannot be expected if a concentration-dependent mechanism of distribution and elimination exists. Especially the renal clearance of a drug quite often is concentration dependent if a strong binding to blood constituents (proteins, cells) plays a major role. In those cases, it is recommendable to use free fractions of the drug instead, in order to find a parameter independent from the total concentrations, which then might be predictive for dose proportionality.

If a pivotal investigation of dose linearity/proportionality becomes necessary, a dedicated study has to be conducted. It typically will be a study where the subjects get at least three different doses in a randomized, intraindividual crossover. The dose steps should cover the clinical recommended dose range and—if possible—should also include the maximum tolerated dose; they ideally should increase in a geometric progression.

Table 45.1 Synopsis of key PK parameters: AUC_{inf}, C_{max} (including dose normalization to 100 mg), and the terminal half-life

		AUC_{inf} [µg*h/mL]				C_{max} [µg/mL]				$t_{1/2z}$ [h]		
Dose Step (mg)	N	Mean	CV %	Median	Med/100 mg	Mean	CV %	Median	Med/100 mg	Mean	CV %	Median
10	4	0.056	70.9	0.054	0.535	0.034	65.7	0.035	0.350	1.3	69.5	0.8
25	6	0.208	57.7	0.199	0.794	0.109	49.7	0.111	0.442	2.1	51.2	1.8
50	5	0.353	61.7	0.426	0.852	0.107	60.4	0.116	0.232	4.2	74.6	2.4
100	5	1.129	47.3	1.278	1.278	0.252	55.0	0.223	0.223	4.9	44.1	4.0
200	5	2.840	94.8	1.430	0.715	0.424	47.1	0.347	0.174	14.0	82.6	8.1
300	6	4.828	56.4	4.544	1.515	0.472	50.9	0.448	0.149	12.2	52.6	11.0
400	6	4.486	65.1	4.495	1.124	0.434	89.7	0.272	0.068	7.4	39.0	7.1

However, this registration study should at best be performed when the therapeutic dose range is clearly established, and the final drug formulation is available.

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US FDA (2006) Bioequivalence guidance, November 2006

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under *Procedure* is presented below. It should be noted that in this specific example study for the first application in man, only a liquid formulation was available, which—due to low solubility of the drug—generated notable inter- (and intra-) individual variability.

45.2.1.7 Results

Only at the higher 200- and 300-mg doses, XYZ1234 could be detected in plasma 48 h after administration. Following the lower doses of 10 and 25 mg, the last quantifiable concentration was detected after 3 and 4 h, respectively. The quantification-limit of the analytical method might have impeded somewhat the reliability of the PK parameters obtained for the two lowest doses (10 and 25 mg). For example, the low values of the calculated $t_{1/2z}$ for these two doses (see Table 45.1) probably resulted from not having quantifiable concentrations at time points following 3 and 4 h p.a., with the consequence of having smaller $AUC_{inf,\ norm}$ values for the lower doses (see Fig. 45.1) and a further increase up to the 300-mg dose. This is also the reason why in this case linearity was not studied with the usual statistics (bioequivalence criteria for a dose-2/dose-1 ratio). AUC_{inf} nevertheless exhibited, in average, a dose proportionality; the 95 % confidence range includes the origin of the coordinates (see Fig. 45.2).

Total relative plasma clearance (CL/f) was found to decrease for the reasons mentioned above, leveling off at the 100-mg dose.

C_{max_norm} gradually declined to the 400-mg dose. C_{max} does not always increase linearly with dose; however, this finding might indicate that the rate of oral absorption is declining with increasing doses. AUC_{inf_norm} declined at the 400-mg dose; however, the median values for the nonnormalized AUC_{inf} were almost identical for the 300- and 400-mg doses (see Table 45.1). This might indicate that the extent of absorption is maximal and is leveling off at the 300-mg dose.

Comparing the data from the seven dose groups synoptically, it can be seen that the dose-normalized exposure [AUC_{inf}] shows much less differences for the medians than the dose-normalized peak values [Cmax] (see Table 45.1). The variability [CV %] is greater than 50 % for nearly all of these observations.

As described, the assessment of the dose linearity/proportionality is hampered in this case by the fact that for the lower doses the profile is not complete assessable, that obviously at the highest dose the resorption is limited, and that the used suboptimal formulation

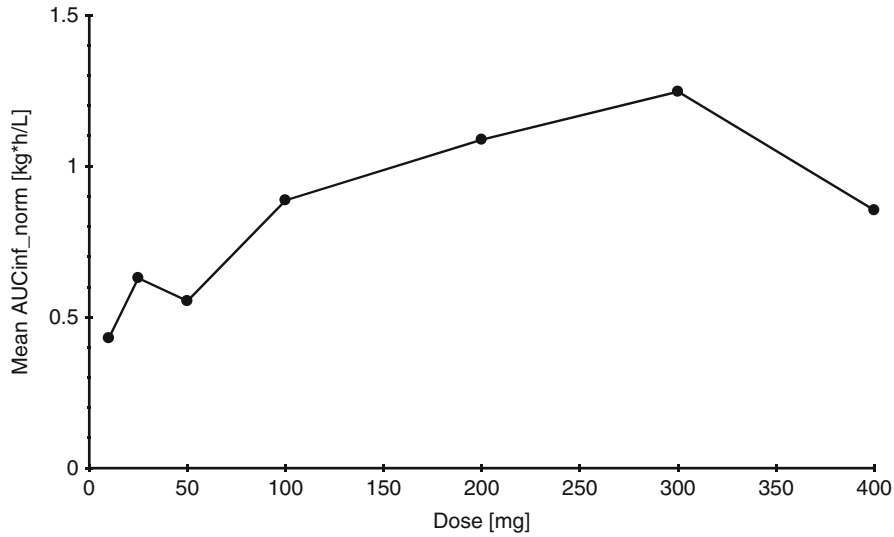

Fig. 45.1 Relationship of "dose per body weight"-normalized AUC_{inf} values for XYZ1234 versus dose

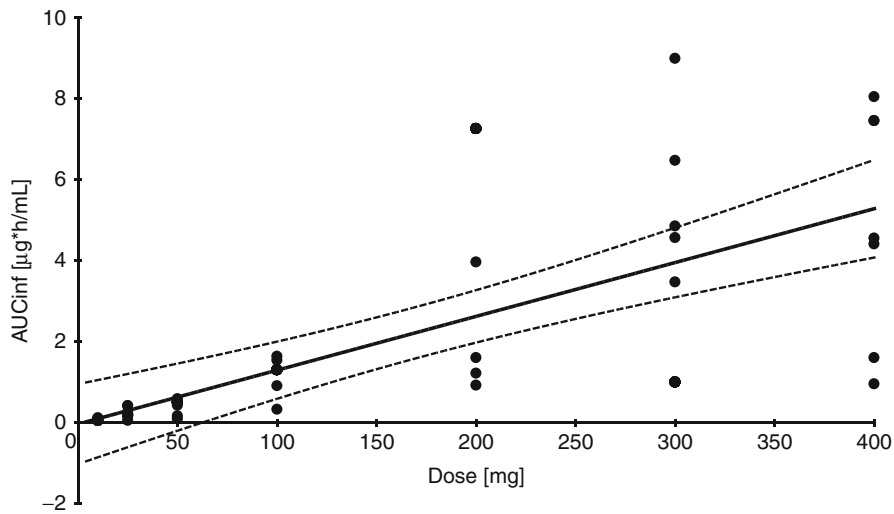

Fig. 45.2 Relationship of observed AUC_{inf} values for XYZ1234 plasma profiles versus dose, with linear regression (*bold line*) and the 95 % confidence range (*dashed line*)

generates a lot of variability. Nevertheless, the example shows that even in this case, predictions to support dose escalation were possible and were even very helpful in that specific study.

45.3 Exploratory Evaluation of Time-Invariant Steady-State Pharmacokinetics

PURPOSE AND RATIONALE

The first tolerability studies early in clinical drug development always provide pharmacokinetic (PK) data over a range of doses, mostly with a single-dose approach. In situations where an explorative proof-of-principle study with escalating multiple doses in patients is needed as the GO/NO GO criterion for further development, the assessment of steady-state conditions in a highly standardized, i.e., a healthy, population has proven to provide meaningful data. Under specific circumstances, information from these studies has a pivotal character for a submission (US FDA Guidance for Industry 2003; ICH E4 1994). The definition of dose linearity/proportionality of drugs in humans after repeated dosing has a crucial impact on the design of large-scale studies in patient settings, including special patient populations (see other contributions in this section).

PROCEDURE
The design of an exploratory assessment of dose linearity/proportionality during the conduct of a multiple-dose study for candidate drug (XYZ123) is presented below. In this case study, a drug was investigated that had shown a terminal half-life of about 24 hours (h) in single-dose trials. In the first clinical studies with different solid formulations, high inter- and intrasubject variability was observed in plasma concentrations and derived pharmacokinetic variables. Data from a study that compared a new formulation to a conventional one and to an oral solution showed that intrasubject variability was markedly reduced. As in that study only one dosage strength had been tested, here different dosage strengths were administered to assess dose linearity/proportionality at steady state.

45.3.1 Protocol Outline

Pharmacokinetics, safety, and tolerability of multiple oral doses of 25-, 50-, and 75- or 100-mg XYZ123 given once daily over 7 days as capsules in healthy men in an open-label study.

45.3.1.1 Primary Objective
To assess pharmacokinetics of multiple oral doses of 25-, 50-, and 75- or 100-mg XYZ123 given once daily over 7 days as capsules.

45.3.1.2 Study Design
Open-label study with three treatment groups, with multiple oral doses of 25 mg (treatment group I) and 50 mg (treatment group II) once daily immediately after intake of a standard breakfast in a parallel-group design. Safety information and bioanalytical data were reviewed to determine the dose for treatment group III (multiple oral doses of 75- or 100-mg XYZ123).

45.3.1.3 Inclusion Criteria
Caucasian men between 30 and 65 years of age, with body mass indices of 18–29 kg/m^2. Volunteers were healthy, nonsmoking, and not receiving regular medication.

45.3.1.4 Treatments
Treatment A: Multiple oral doses of 25-mg XYZ123 as capsules, given once daily immediately after intake of a standard breakfast for 7 days.

Treatment B: Multiple oral doses of 50-mg XYZ123 as capsules, given once daily immediately after intake of a standard breakfast for 7 days.

Treatment C: Multiple oral doses of 75- or 100-mg XYZ123 as capsules, given once daily immediately after intake of a standard breakfast for 7 days.

45.3.1.5 Pharmacokinetic Data
Derived from concentrations of XYZ123 in plasma and urine before and at predefined times after dosing.

EVALUATION
All bioanalytical data, derived PK data, and safety data were listed and descriptive statistics calculated. Individual and median data were plotted. The log-transformed PK parameters AUC and C_{max} were analyzed for dose proportionality. The PK parameters $AUC_{(0–24)}$ and C_{max} were also descriptively analyzed for accumulation ratio.

CRITICAL ASSESSMENT OF THE METHOD
For the definition of the lowest dose, exposure data from animal pharmacological effect models and from single-dose studies in man were used. In addition, information from a food screen and on the elimination half-life were applied for the design of the study: the marked effect of a high-fat high-calorie breakfast on the exposure of XYZ123 resulted in a standard continental breakfast on each dosing day. An elimination half-life of almost 24 h lets one expect that steady state is reached after 5 days of once-daily dosing. To confirm the latter expectation, predose and 12-h postdose plasma concentrations were analyzed on days 2–7. An algorithm for the selection of the highest dose was predefined on the basis of "online" safety and bioanalytical data, i.e., C_{max}.

MODIFICATIONS OF THE METHOD
If the active moiety of a drug (parent or metabolite(s)) exhibits half-lives that are greater than 2 days, the evaluation of steady-state conditions might become problematic in a study with healthy subjects due to long dosing periods. In these situations, the insertion of PK profiling days in patient studies might prove to be helpful. Whereas in single-dose studies a crossover design might be the proper choice to assess intraindividual variability, a parallel-group or sequential design has been preferred in multiple dose settings.

References and Further Readings

ICH E4 (1994) Dose–response information to support drug registration. March 1994
US FDA Guidance for Industry (2003) Exposure-response relationships—study design, data analysis, and regulatory applications. April 2003

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the pharmacokinetic results obtained from the study described above under "Procedure" is presented below.

45.3.1.6 Results

The primary objective of this study was to investigate the pharmacokinetics of XYZ123 after multiple dosing for 7 days at three dose levels. Pharmacokinetic profiles were derived both on day 1 (up to 23.5 h post dose) and on day 7 (up to 119.5 h post dose), with same sampling time points relative to dose, and samples were also taken on days 2–6 to monitor the approach to steady state.

Mean C_{max} and $AUC_{(0–24)}$ (= AUC_{τ}) both on day 1 (C_{max}: 0.46, 1.16, and 3.29 μg/mL; AUC_{τ}: 7.12, 16.37, and 39.85 μg*h/mL) and on day 7 ($C_{max,ss}$: 0.91, 2.31, and 5.95 μg/mL; $AUC_{\tau,ss}$: 14.17, 33.55, and 74.48 μg*h/mL) increased with increasing dose although the increases were marginally higher than strict dose proportionality would predict: the deviation from linearity was, however, minor. The median time to reach maximum plasma concentration was 6 h on both profile days and at all doses. The terminal phase half-life of XYZ123 on day 7 was quite constant with mean values between 19 and 23 h: a slight reduction in half-life (accompanied by a reduced terminal phase volume of distribution) was observed at the higher doses.

The extent of exposure to XYZ123 in this study generally increased in a dose-proportional fashion over the dosing range of 25–100 mg, although there was a slight trend to higher values at the higher doses. Times to reach maximum concentration were constant at all doses and after both single and multiple dosing: terminal phase half-lives were also fairly constant at all doses after multiple dosing. After 7 days of repeated dosing, the observed accumulation of XYZ123 (based on AUC_{τ} and C_{max}) showed a factor of about 2, agreeing well with the theoretical expectation: this suggests linear pharmacokinetic behavior of XYZ123 between single and repeated dosing.

Median $C_{max,ss}$ values of 0.79, 2.3, and 5.0 μg/mL at 6 h post dose were observed: slightly higher increases were apparent than strict dose proportionality would predict.

From the mean trough concentrations of XYZ123 on days 2–7, it is apparent that steady-state conditions were attained at all doses after 7 days of repeated dosing, in line with the observed terminal phase half-life of the compound.

Descriptive statistics for the primary pharmacokinetic parameters of XYZ123 on days 1 and 7 are shown in the text tables below (Table 45.2).

The mean data for $C_{max,init}$ (and to a lesser extent $AUC_{\tau,\ init}$) on day 1 confirm the descriptive observation already made that the increases with increasing dose were slightly higher than strict dose proportionality would predict. Median $T_{max,\ init}$ was very constant (6 h) throughout the dose range studied (Table 45.3).

Table 45.2 Pharmacokinetic parameters of XYZ123 (Day 1)

	MEAN (geometric mean): Day 1		
PK parameter	Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,init}$ (μg/mL)	0.46 (0.45)	1.16 (1.12)	3.29 (3.14)
$T_{max,init}$[a] (h)	6.0[a]	6.0[a]	6.0[a]
$AUC_{\tau,init}$ (μg[a]h/mL)	7.12 (6.97)	16.37 (15.87)	39.85 (38.59)

[a]Median values reported

Table 45.3 Pharmacokinetic parameters of XYZ123 (Day 7)

	MEAN (geometric mean): Day 7		
PK parameter	Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,ss}$ (μg/mL)	0.91 (0.89)	2.31 (2.26)	5.95 (5.71)
$T_{max,ss}$[a] (h)	6.0[a]	6.0[a]	6.0[a]
$C_{av,ss}$ (μg/mL)	0.59 (0.58)	1.40 (1.37)	3.10 (3.02)
$AUC_{\tau,ss}$ (μg[a]h/mL)	14.17 (13.92)	33.55 (32.88)	74.48 (72.34)
$AUC_{(0–t),ss}$ (μg[a]h/mL)	24.88 (24.13)	56.00 (54.40)	112.62 (107.12)
$AUC_{(0–inf),ss}$ (μg[a]h/mL)	27.55 (26.85)	58.60 (56.88)	116.20 (110.55)
$t_{1/2,ss}$ (h)	23.28 (22.86)	20.40 (20.21)	18.76 (18.18)
$V_{z,ss}$ (L)	61.35 (59.22)	45.04 (44.34)	37.92 (36.24)

[a]Median values reported

Table 45.4 XYZ123 PK parameters—dose proportionality

Parameter	Comparison	Estimated ratio	Expected ratio if dose proportional	95 % CI
AUC_{τ} (μg*h/mL)	50:25 mg	2.362	2.000	1.97, 2.84
	100:25 mg	5.197	4.000	4.33, 6.24
	100:50 mg	2.200	2.000	1.83, 2.64
$AUC_{(0–inf)}$ (μg*h/mL)	50:25 mg	2.119	2.000	1.69, 2.66
	100:5 mg	4.118	4.000	3.28, 5.17
	100:50 mg	1.943	2.000	1.55, 2.44
$AUC_{(0–t)}$ (μg*h/mL)	50:25 mg	2.254	2.000	1.79, 2.85
	100:25 mg	4.439	4.000	3.51, 5.61
	100:50 mg	1.969	2.000	1.56, 2.49
C_{max} (μg/mL)	50:25 mg	2.544	2.000	2.07, 3.13
	100:25 mg	6.442	4.000	5.24, 7.92
	100:50 mg	2.532	2.000	2.06, 3.11

Table 45.5 Observed accumulation ratios of geometric mean parameters for XYZ123

	Mean (geometric mean): Day 7: Day 1		
Ratio	Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,ss}/C_{max,init}$	1.98	1.99	1.81
$AUC_{\tau,ss}/AUC_{\tau,init}$	1.99	2.05	1.87

The analysis of the dose adjusted values confirms the faster than linear increase of $C_{max,init}$, $C_{max,ss}$, and $AUC_{(0–24),init}$ with increasing dose.

The same trend for $C_{max,ss}$ and $AUC_{\tau,ss}$ was observed on day 7 as on day 1, i.e., slightly higher than dose-proportional increases were recorded; $AUC_{(0–t),ss}$ and $AUC_{(0–inf),ss}$ on the other hand showed increases very closely related to the increases in dose. As for the single-dose data, $T_{max,ss}$ remained very stable over the dose range investigated, with median values of 6 h. There was an indication for slightly reduced terminal phase half-life times at the higher doses which may explain the reduced differences in $AUC_{(0–t),ss}$ and $AUC_{(0–inf),ss}$. This was also accompanied by a lower volume of distribution associated with the terminal phase at the higher doses (Table 45.4).

Dose proportionality could be shown for the dose-dependent parameters $AUC_{(0–inf)}$ and $AUC_{(0–t)}$ and all the three possibilities of treatment comparisons (B vs. A, C vs. A, C vs. B). The 95 % confidence interval contains all of the expected ratios.

For the parameter $AUC_{(0–24)}$, only the expected ratio of the comparison of C and A is not contained in the confidence interval. So dose proportionality could be shown for the two other comparisons.

For the parameter C_{max}, dose proportionality could not be shown for all of the three comparisons. The three expected comparisons are not contained in the confidence intervals.

A comparison of geometric mean values for C_{max} and AUC_{τ} between day 7 and day 1 indicates ratios of approximately 2 at all dose levels: the ratios are given in the following table (Table 45.5).

These results agree well with the theoretical accumulation anticipated according to the formula:

Accumulation $A_I = 1/1–2^{-\varepsilon}$ where $\varepsilon = \tau/t_{1/2}$ and τ is the dosing interval.

Thus, for a dosing interval of 24 h and a half-life close to 24 h, the predicted accumulation factor is 2, close to the observed value found in this study. This is a good indication for linear kinetic behavior of XYZ123 between single and repeated dosing.

The peak-to-trough ratio (data not shown) increased with dose, reflecting the higher than dose-proportional increase in $C_{max,ss}$.

The course of the median trough plasma concentrations (Fig. 45.3) shows a saturation beyond day 5, which is an indicator for reaching the steady state.

45.4 Special Populations: Exploratory Profiling of the Impact of Gender, High Age, and Food Intake on Drug Disposition

PURPOSE/RATIONALE

In early clinical development, the early steps before entering into larger patient studies are typically an explorative first-in-man study with escalating single doses in healthy volunteers, an explorative proof-of-principle study with escalating multiple doses in healthy volunteers, and then a proof-of-concept with escalating multiple doses in a population as close as possible to the target population for the phase IIb/III development program. Additional supportive studies are required to justify the fastest possible broadening of the population in the proof-of-concept study and thus to accelerate patient recruitment into the larger patient studies. Over and above this industry need, the

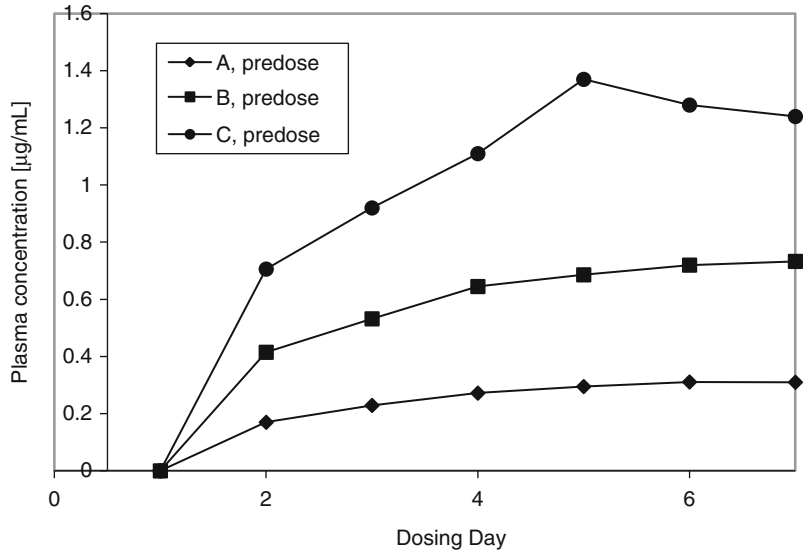

Fig. 45.3 Median XYZ123 trough plasma concentrations Treatment A: multiple oral doses of 25-mg XYZ123 daily (as one capsule) Treatment B: multiple oral doses of 50-mg XYZ123 daily (as two capsules) Treatment C: multiple oral doses of 100-mg XYZ123 daily (as four capsules)

principles of ICH and US Federal Drug Agency (FDA) guidance (US FDA 1978, 1989, 1993; ICH-E7 1994) also require that the pharmacokinetics and safety of a candidate drug are studied in the range of populations likely to receive the drug during the clinical development and later, once the drug is marketed.

The principles of US FDA and European Agency for the Evaluation of Medicines Products (EMEA) guidance also recommend an early profiling of food effects on bioavailability to help guide and select the formulations for further development (US FDA Guidance for Industry 2003; EMEA CPMP/EWP/560/95 1997).

Experience has shown that the exploratory profiling of the impact of gender, high age, and food intake (in the form of a food screen for worst-case food effects) on drug disposition (pharmacokinetics) and safety can be conveniently done in one study with the design described in this chapter. If a notable effect of these parameters is seen, then confirmatory studies need to be performed.

A priori animal data can provide hints with respect to the likelihood of observing some impact of gender, high age, and food intake on candidate drug pharmacokinetics.

The observation of gender-related differences in drug pharmacokinetics in multiple-dose animal studies is one hint that a gender effect might be expected, although gender effects observed in rodent should be viewed with caution. Data from in vitro metabolism assays based on liver factions from men and women could also suggest gender-related differences in metabolism.

Animal studies indicating that the drug candidate is primarily eliminated renally and/or physicochemical characteristics making a renal elimination likely are flags suggesting the need to profile candidate drug pharmacokinetics in elderly—since some degree of renal impairment is often observed this population.

Animal studies reporting an effect of food intake on drug pharmacokinetics, although suggestive, tend to be of low predictivity. This lack of predictivity can be explained by the different physiology and feeding behaviors in animals and man. Thus, if oral dosing is planned, since compliance with dietary restrictions could be an issue in outpatient studies, a screen for notable food effects in man should be included early during clinical development. Food effects are generally greatest when drug product is administered immediately after a meal is ingested. The nutrient and caloric content of the meal can also influence drug availability differently in different drugs, whereby meals that are high in calories and fat content are more likely to result in an effect. The time to dosing and fluid intake can also influence drug disposition.

PROCEDURE

The design of a study providing the suggested exploratory profiling of the impact of gender, high age, and food intake on the pharmacokinetics of candidate drug (Drug XYZ) in a single clinical study is presented below. For the purposes of simplicity, the study

description is limited to the collection, handling, and interpretation of pharmacokinetic data although other parameters were also studied.

In this explorative study, candidate drug is given immediately after ingestion of a high-calorie/high-fat meal (US FDA Guidance for Industry 2003). Since the impact of different food compositions and times to food intake are not tested, this aspect of the study is referred to as a food screen.

45.4.1 Protocol Outline

Explorative pharmacokinetics of Drug XYZ (200 mg, given as tablets), including a food screen, in younger and elderly, obese, but otherwise healthy, adults.

45.4.1.1 Objectives

Primary: The primary objective of the study was to investigate and compare the pharmacokinetics of Drug XYZ, including a food screen, in younger adult and elderly obese, but otherwise healthy, men and women.

Secondary: The secondary objective of the study was to assess the safety of single doses of Drug XYZ in younger and elderly obese, but otherwise healthy, adults.

45.4.1.2 Study Design

A single-center, open-label, randomized, balanced, single-dose, two-treatment (fed vs. fasted), two-period crossover design. In one trial period, subjects were dosed after fasting (= fasted trial period), and in the other trial period, subjects were dosed after consuming a high-fat breakfast (= fed trial period). Subjects were randomized to the sequence of trial periods. The washout period between trial periods was at least 5 days which approximated to >10 Drug XYZ apparent terminal half-lives.

45.4.1.3 Number of Subjects

Twenty-four (24) subjects (12 men/12 women, per gender 6 younger adult subjects, 6 elderly subjects) were recruited.

Due to the variability in the pharmacokinetic parameters seen in earlier studies, no formal sample size justification was given for 12 subjects per population. However, according to the guidance for industry by the US FDA, 12 subjects is the typical minimum sample size for a bioavailability comparison (US FDA 1978); even in a pilot study, "a sufficient number of subjects (e.g., 12) need to complete the study" (US FDA Guidance for Industry 2003). This was fulfilled here for each of the strata: male, female, younger, elderly.

45.4.1.4 Inclusion Criteria

The following inclusion criteria were met: Adults aged between 18 and 45 years (young adults) and aged 65 years or older (elderly); With body mass index (BMI) of 30 and < 40 kg/m^2; Women who are either postmenopausal or surgically sterile or willing to use prescribed barrier contraceptive methods; Who are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; With normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant; Who are non-smoking or light smokers; Elderly subjects with creatinine clearance of >50 mL/min; young adults with creatinine clearance of >80 mL/min.

45.4.1.5 Treatments

A single-dose of Drug XYZ (200 mg) was given orally as tablets. In the fasted trial period, subjects remained fasted until 2 h after dosing. In the fed trial period, subjects were dosed orally after consuming a high-fat breakfast whose composition conformed to US FDA guidance (US FDA Guidance for Industry 2002).

45.4.1.6 Pharmacokinetic Data

Plasma concentrations of Drug XYZ, before and at predetermined times post dose, were measured.

Concentrations of Drug XYZ in urine collected over the profiling period were measured. Volumes of urine collected over each profiling period were recorded.

The volume of urine collected over 24 h and the concentration of creatinine were determined to allow the subjects' creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of the explorative profiling of issues related to age, gender, and the impact of food intake when dosing from study described above was evaluated as follows:

Due to the investigational nature of this study and the small sample size, all variables were only presented descriptively. Where appropriate, individual data were presented together with descriptive statistics.

Descriptive pharmacokinetic parameters (standard parameters including peak concentrations (C_{max}), time of C_{max} (T_{max}), area under the curve (AUC) between time 0 and time t where t = 24 h post dose (AUC_{0-t}), AUC after extrapolation to infinity ($AUC_{0-\infty}$), apparent terminal half-life ($t_{1/2_z}$)) for plasma Drug XYZ were calculated using a noncompartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin® (Pharsight Corp.) protocols. For urine Drug XYZ data, the descriptive pharmacokinetic parameters included fractional/cumulative urinary excretion, and renal clearance of Drug XYZ was calculated using SAS® for Windows™ protocols.

If appropriate, pharmacokinetic parameters were compared descriptively between age groups (with/without stratification), between genders (with/without stratification), and between fasted and fed subjects (with/without stratification and individually). Although not intending to show bioequivalence, the 90 % confidence intervals (CI) for the differences in the log-transformed exposure measurements were calculated.

CRITICAL ASSESSMENT OF THE METHOD

The impact of gender, high age, and food intake on candidate drug pharmacokinetics and safety can be conveniently done in one explorative study with the design described in this chapter. Typically, this study would run shortly after key data are available from the first-in-man study and employs a dose in the upper third of the dose range tested in the first-in-man study. The highest safe dose from the first-in-man study is not usually chosen unless a notable safety margin is given because food intake can cause substantial increases in exposure.

As the clinical development progresses, the dose chosen in this study may prove to be higher or lower than the actually required therapeutic dose. Similarly, the drug formulation available at this early time point may differ from that eventually marketed. These differences may have a notable influence on the magnitude of food effect seen. In the experience of the author, the observed food effects ranged from a 50 % reduction in AUC to a 20-fold increase in AUC when different oral formulations of the same drug were dosed immediately after the same high-fat/high-calorie food.

As mentioned earlier, this type of supportive study provides explorative profiling. If a notable effect of these parameters is seen, then suitable confirmative studies will need to be performed. However, these studies should ideally be performed when the therapeutic dose range and the final drug formulation are known.

Although the impact of gender and high age on the pharmacokinetics of the developmental drug can be studied based on single-dose data as described, the use of steady-state data is required if there is reason to believe that the pharmacokinetics of the drug studied are not accurately predictable from single-dose data.

MODIFICATIONS OF THE METHOD

The type of study described in this chapter provides explorative profiling of issues related to age, gender, and the impact of food intake when dosing. If appropriate, the same principle could be applied to address other variables, for example, to investigate the impact of different types of food composition and/or time of food ingestion using a relatively small population. Typically, this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of gender and high age on the drug disposition during the phase II/III studies.

The most common impact of high age is via a reduced renal and/or hepatic function. In the example given, the study inclusion criteria deliberately allow the inclusion of elderly subjects with mildly impaired renal function, i.e., subject presenting creatinine clearances of >50 mL/min. No measures were taken to ensure that the number of enrolled elderly volunteers with normal and mild renal impairment was balanced. Such measures would have allowed an explorative insight into the impact of mildly impaired renal function at the expense of delaying recruitment since the recruitment of healthy elderly with mild renal impairment is more complicated. If renal elimination is believed to play a notable contribution to developmental drug elimination, then the complication of recruitment may be warranted.

References and Further Readings

EMEA CPMP/EWP/560/95 (1997) Note for guidance on the investigation of drug interactions. December 1997

ICH-E7 (1994) Guideline for industry: studies in support of special populations: geriatrics. August 1994

US FDA (1978) General considerations for the clinical evaluation of drugs, December 1978

US FDA (1989) Guideline for the study of drugs likely to be used in the elderly, November 1989
US FDA (1993) Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs; Notice, July 1993
US FDA Guidance for Industry (2002) Food effect bioavailability and bioequivalence studies. December 2002
US FDA Guidance for Industry (2003) Bioavailability and bioequivalence studies for orally administered drug products — general considerations. (R1) March 2003

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below.

45.4.1.7 Results: Pharmacokinetics

The data from study described above, as summarized in Tables 45.6 and 45.7 and Fig. 45.4 below, can be summarized as follows:

- A trend was seen in all analyzed groups toward a slightly lower Drug XYZ plasma C_{max} under fed conditions than after fasting. Drug XYZ plasma C_{max} was also reached later under fed conditions than after fasting in all analyzed groups.
- From 6 h post dose until at least 48 h post dose, the Drug XYZ plasma concentrations (median values) were slightly higher in the elderly than in young adults and also slightly higher in female than in male subjects, both under fed and under fasting conditions.

Table 45.6 Summary of key Drug XYZ PK parameters: young versus elderly, fasted versus fed

		All PK subjects ($n = 24$)		Young ($n = 12$)		Elderly ($n = 12$)	
Parameter	Statistic	Fasting	Fed	Fasting	Fed	Fasting	Fed
C_{max} (μg/mL)	Geometric mean	1.23	0.96	1.24	0.87	1.22	1.06
	Range	0.73–2.50	0.58–1.96	0.75–1.72	0.58–1.39	0.73–2.50	0.66–1.96
		Geometric mean ratio (90 % CI: upper-lower)		–		–	
		0.7816 (0.6969–0.8767)					
T_{max} (h)	Median	1.0	3.0	1.0	3.0	1.0	3.0
	Range	0.5–2.0	1.0–5.0	0.5–2.0	1.0–4.0	1.0–2.0	1.0–5.0
$AUC_{0-\infty}$ (μg.h/mL)	Geometric mean	9.26	8.99	7.80	7.44	11.0	10.86
	Range	3.52–34.61	3.63–40.06	3.52–14.89	3.63–17.0	6.03–34.61	3.94–40.06
		Geometric mean ratio (90 % CI: upper-lower)		–		–	
		0.9714 (0.8985–1.0502)					
$t_{1/2_z}$ (h)	Geometric mean	12.4	12.0	12.0	11.4	12.7	12.8
	Range	6.8–30.2	6.7–39.4	8.6–18.5	8.5–17.0	6.8–30.2	6.7–39.4

Table 45.7 Summary of key Drug XYZ PK parameters: male versus female

		Male ($n = 12$)		Female ($n = 12$)	
Parameter	Statistic	Fasting	Fed	Fasting	Fed
C_{max} (μg/mL)	Geometric mean	1.31	0.95	1.15	0.97
	Range	1.03–1.60	0.66–1.52	0.73–2.50	0.58–1.96
T_{max} (h)	Median	1.0	3.0	1.0	3.0
	Range	0.5–2.0	1.0–5.0	1.0–2.0	1.0–5.0
$AUC_{0-\infty}$ (μg.h/mL)	Geometric mean	8.12	7.46	10.55	10.84
	Range	3.52–34.61	3.63–32.86	6.03–30.62	5.30–40.06
$t_{1/2_z}$ (h)	Geometric mean	11.5	11.1	13.3	13.1
	Range	6.8–22.3	6.7–25.4	9.7–30.2	8.7–39.4

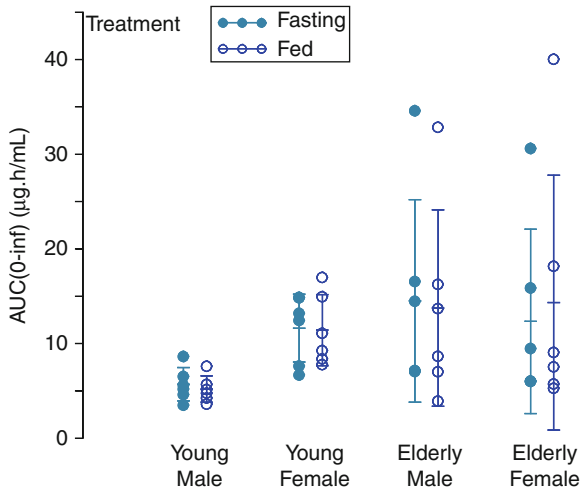

Fig. 45.4 Individual $AUC_{0-\infty}$ values per stratum, with arithmetic mean ±1 SD

- Urinary excretion of unchanged Drug XYZ was low: 48 h after administration, approximately 1 % of the administered dose was excreted unchanged. There was a trend in all analyzed groups toward a slightly lower cumulative Drug XYZ excretion in urine under fed than under fasting conditions. Cumulative excretion seemed slightly higher in the elderly than in the younger adults and in women than in men, both under fed and under fasting conditions.
- Drug XYZ showed delayed absorption due to food intake. The overall geometric mean Drug XYZ plasma C_{max} was 1.23 mg/L under fasting and 0.96 mg/L under fed conditions. Overall median T_{max} was 1 h under fasting and 3 h under fed conditions. Similar differences in C_{max} and T_{max} under fasting and fed conditions were seen in all populations analyzed.
- Overall, no differences were seen in AUC parameters and elimination half-life under fasting and fed conditions.
- A trend was seen toward a smaller $AUC_{0-\infty}$ in the young than in the elderly (geometric mean after fasting, 7.8 and 11.0 mg.h/L, respectively) and in the male than in the female subjects (geometric mean after fasting, 8.12 and 10.55 mg.h/L, respectively).
- There was a trend toward a shorter $t_{1/2_z}$ value in the young than in the elderly (geometric mean after fasting 12.0 and 12.7 h, respectively) and in the male than in the female subjects (geometric mean after fasting 11.5 and 13.3 h, respectively).
- An exploratory analysis was performed using a four-factor ANOVA model, with treatment, period, and sequence as fixed factors and subject within sequence as random factor. The results from the ANOVA were used to calculate the back-transformed 90 % confidence intervals (CI) for the differences between the fed and fasted condition in the log-transformed exposure measurements (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$). For C_{max}, the difference between fasting and fed conditions was found to be statistically significant while this was not the case for the AUC parameters.

45.5 Profiling of Drug Absorption, Distribution, Metabolism, and Elimination in Man: The Hadme Study

PURPOSE/RATIONALE

The aim of this kind of study is to characterize the absorption, distribution, metabolism, and elimination of the investigational product in humans (hADME study) following an administration of the compound in a radiolabeled form. The use of a radiolabel allows identifying metabolites, which were not known beforehand, and characterizing them. In addition, using a radiolabel is—in most cases—the only way to establish a complete balance of the drug and its metabolites, which is required to validate the completeness and predictivity of the results.

A typical hADME study includes an overall balance of excretion of the administered radioactivity (mass balance), protein binding, and metabolic profiles in plasma, urine, and feces and, if possible, the determination of descriptive pharmacokinetic parameters for the radioactivity, the parent compound, and the identified metabolites in plasma and in urine. If feasible and adequate, structures of the observed metabolites will be elucidated.

Knowledge of the absorption, distribution, metabolism, and elimination in man, beyond being a prerequisite for assessing the consequences of dosing in target patient population in phase II studies, will also aid the prediction of potentially relevant drug-drug interactions and of high-risk populations for dosing with the investigational drug.

Presently, there are no official, general guidelines for this kind of study available; the different aspects are covered instead by dedicated guidelines, for example, metabolism, related to drug interaction (US FDA 1997a, 1999a; EU CPMP 1997), or for the related preclinical studies (EU EUDRALEX 3BS11a 1994), or only in a high level form (EU EUDRALEX 3CC3a 1988). The general use of radioactive-labeled drugs in clinical studies is described in a separate FDA regulation (US FDA 2010).

Nevertheless, hADME studies using radioactive-labeled drugs became recently of special interest based on the guidelines on metabolites in safety testing (US FDA 2008; International Conference on Harmonization M3 (R2) 2009). In the FDA guidance document, it is stated: *Human* in vivo *metabolism studies usually have been performed relatively later in drug development, but we strongly recommend* in vivo *metabolic evaluation in humans be performed as early as feasible*. There are slight differences between the ICH and the FDA documents, related to the reference data deciding on the need of metabolite testing, which as consequence might allow the use of alternative (nonradioactive) methods.

This type of hADME studies using radioactive-labeled drug should be run unless phase I studies show that ≥90 % of the dose is excreted unchanged in urine. In this case, a hADME study may not be required. For those drugs, a urine assay should be the prerequisite for phase I trials, and then mass balance may be established in trials where quantitative urine collections are performed. If phase I data indicate that "cold mass balance," i.e., ≥90 % recovery in urine, cannot be obtained, a hADME study has to be scheduled for the development program.

PROCEDURE

The design of a typical hADME study for a candidate drug (XYZ1234) is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are also studied.

45.5.1 Protocol Outline

Absorption, distribution, metabolism, and elimination of XYZ1234 in healthy men following oral administration of ^{14}C-XYZ1234 (100 mg, 4 MBq) as solution.

45.5.1.1 Objective

Primary objective: To investigate the absorption, distribution, biotransformation, and elimination of XYZ1234 in healthy men following oral administration of ^{14}C-XYZ1234. This includes (1) overall balance of excretion of the administered radioactivity (mass balance); (2) metabolic profiles in plasma, urine, and feces; (3) if possible, structures of the observed metabolites; (4) determination of descriptive pharmacokinetic parameters for XYZ1234 and its active metabolite (XYZ5678) in plasma and in urine; and (5) characterization of protein binding in plasma.

45.5.1.2 Study Design

A single-center, open-label, single-dose study design. Subjects did enter the clinical site on the morning 1 day before dosing and remained at the study site at least 7 days after dosing. In case radioactivity measurement showed that recovery in the excreta (urine plus feces) was still below 95 % of the administered dose on day 7 or that the activity in the last plasma sample was still above 1 Bq/mL, then the subjects were requested to stay in the clinic until one of the levels had reached the mentioned limits. The maximum prolongation of the stay in the clinical center was 14 days post dosing. If necessary, after that time, the subjects had to continue daily collection of urine and feces at home until the radioactivity in the excreta samples reached definite threshold criteria (1 Bq both per mL of urine and per homogenate of 100-mg feces, respectively).

45.5.1.3 Sample Size/Population

Six white men aged between 45 and 65 years, with body mass indices (BMI) of 19–29 kg/m^2.

45.5.1.4 Treatments

After overnight fasting, a single oral dose of ^{14}C-XYZ1234 (100 mg, 4 MBq) administered as solution.

45.5.1.5 Pharmacokinetic Data

- Radioactivity administered orally and radioactivity recovered in whole blood, plasma, urine, feces, and expired air
- Concentrations of XYZ1234 and its active metabolite (XYZ5678) in plasma and urine
- Metabolic profiles: number of, and radioactivity attributable to, specific peaks in analyses

(chromatograms) of the radioactivity in plasma, urine, and feces
- Structures of observed metabolites (if possible)
- Protein binding in plasma (in vitro only)

EVALUATION

Where appropriate, individual data were presented together with the descriptive statistics.

If measured radioactivity for plasma, blood, and urine was related to the weight of a sample, the result was converted to the volume of the sample in order to generate the values as concentrations comparable to the "normal" bioanalytical data.

In the case of urine for this conversion, a constant specific weight of 1.02 g/mL is assumed for all subjects. In the case of plasma and whole blood for this conversion, a constant specific weight of 1.03 g/mL (plasma) and of 1.05 g/mL (whole blood), respectively, is assumed for all subjects. In the case of the radioactivity measurements, results are then also listed in terms of measured radioactivity per mL sample and as "concentrations" (i.e., given in μg equivalents per mL sample (μg-eq/mL)), representing the sum of original compound and/or radiolabeled metabolites.

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and pharmacokinetic parameters calculated: number of relevant observations, geometric mean, geometric standard deviation, arithmetic mean, standard deviation, coefficient of variation, median, minimum, and maximum.

CRITICAL ASSESSMENT OF THE METHOD

The required information can be generated in this explorative study with a design described as above. The threshold criteria to stop collecting urine and feces (as described under "study design") guarantee that the combined daily excretion reached a value below 0.1 % of the administered dose. Further collection would not significantly contribute to the overall balance and has to be outweighted to the burden of the subject involved. Using fixed specific weights per matrix for the calculation of concentrations in the respective—originally weighted—samples (urine, plasma, and blood) had proven to be superior to an individual determination of the specific weights: the variability of the results was reduced without a hint for any reduction of the accuracy of the resulting concentration values.

This study could already be conducted after key data are available from the first-in-man study but should use a dose in the upper third of the range of therapeutic doses and a formulation similar to the therapeutic one. The latter cannot always be realized, but just using an oral solution of the radiolabeled drug will, only in rare cases, allow to support all objectives of the study mentioned. These type of hADME studies are increasingly being performed as early as possible in the clinical development (Deroubaix and Coquette 2004), in order to support the assessment of the consequences of dosing in the target patient population, or special populations in order to support the prediction of potentially relevant drug-drug interactions and high-risk populations for dosing with the investigational drug, and to generate the information on human metabolites early enough in order later not to compromise the long-term safety studies in animals and to prevent the need of repeating them. These advantages of conducting the study early have to be balanced against possible uncertainties related to the therapeutic dose and the formulation during early development; timing of the hADME study has therefore to be decided on a project-specific basis.

MODIFICATIONS OF THE METHOD

The example shown comprises protein binding in vitro only (predose samples), since the parent compound in plasma was known to massively exceed all metabolites. If this is not the case, in addition, samples to determine the protein binding ex vivo are required. Depending on the kind of measurement (radioactivity vs. specific), these samples then can indicate a general hint for protein binding across all labeled molecules (weighted by their occurrence in plasma), if only the ex vivo radioactivity is determined, or can provide protein binding data for metabolites, if a specific assay is available.

This type of hADME study always will have an explorative character and typically comprise less than the normal minimum of 12 subjects for a PK study. If the compound is expected to show different and unpredictable ADME characteristics in special populations, then it might be necessary to include these populations in addition to normal, healthy subjects. Or if other conditions might influence ADME in a not predictable manner (e.g., food effects), then it might even be necessary to run the study in a crossover design. The inclusion of females (being

not of childbearing potential) was discussed several times, but it seems to be a rare exception for hADME studies.

Further on, if nonlinearities are observed for the ADME characteristics, instead of a single dose, multiple-dose studies may be needed, where the radiolabeled drug is administered under steady-state conditions.

hADME studies preferentially use compounds labeled with C-14; labeling with H-3 provides, in many cases, less stability and requires a "wet/dry" comparison of the samples during analytics in order to detect free tritiated water. Similar precautions and tests for the in vivo stability of the label are necessary if other nuclides are used as radiolabel.

A promising alternative to the conventional radioanalytical measurement of C-14 in hADME studies is accelerated mass spectrometry (AMS), which can operate as a detector for C-14 with an increase of sensitivity by a factor of 1,000 (Garner 2000). This technique is complex and presently not used routinely. In addition, as an alternative, the last-generation, high-sensitivity liquid scintillation beta counters show an increase of sensitivity by a factor of 10–20 (Deroubaix and Coquette 2004). In those cases where the dosimetric calculations indicate that a traditional dose (2–4 MBq) would result in an unacceptable radiation burden (e.g., due to an intensive and long-lasting binding to melanopherous tissues), then both techniques can certainly provide useful alternatives, since using these radioanalytical tools would allow to administer much lower doses of the radionuclide.

If the drug development has to be done exclusively in compromised patients (some anticancer, anti-AIDS drugs) which may make a comprehensive hADME trial using a radioactive drug difficult to conduct, then other means of establishing mass balance and metabolism information may have to be negotiated.

References and Further Readings

Deroubaix X, Coquette A (2004) The ins and outs of human ADME studies. Business briefing. Pharmatech 1–4

EU CPMP (1997) Note for guidance on the investigation drug interactions. December 1997

EU EUDRALEX 3BS11a (1994) Pharmacokinetics and metabolic studies in the safety evaluation of new medicinal products in animals. April 1994

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Garner RC (2000) Accelerator mass spectrometry in pharmaceutical research and development—an new ultrasensitive analytical method for isotope measurement. Curr Drug Metabol 1: 205–213

International Conference on Harmonization M 3 (R2) (2009) Guidance on nonclinical safety studies for the conduct of human clinical trials, June 2009

US FDA (1997) Drug metabolism/drug interaction studies in the drug development process: studies in vitro. April 1997

US FDA (1999) In vivo drug metabolism/drug interaction studies. November 1999

US FDA (2008) Safety testing of drug metabolites, February 2008

US FDA 21 CFR 361.1 (2010) Radioactive drugs for certain research uses; April, 2010

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below.

45.5.1.6 Results

1. *Balance of excretion*: On average, 34.6 % of the administered radioactivity was excreted in urine and 60.6 % was excreted in feces. No quantifiable radioactivity was found in expired carbon dioxide. The total excretion averaged 95.2 % after 17 days. The urinary excretion of the parent drug averaged 8.85 % of the dose and the urinary excretion of the metabolite XYZ5678 averaged 11.7 %. There was still 14.1 % of the drug that was eliminated in the urine in a form different than XYZ1234 or XYZ5678, as one or several other metabolites.
2. *Metabolic profile*: The metabolites in urine with the largest contribution were the demethyl product (P7) (26 %) and the hydroxylated isomers (P3, P4) (19 % and 6 %). An unidentified metabolite with a molecular weight of 450 g/mol (P5) represented 19 % followed by the unchanged product (P9) (joint contribution of ca. 18 %).

   The contribution of each peak to the total radioactivity in feces was calculated, and the results showed that the metabolites in feces with the largest contribution were the hydroxylated isomers (P3, P4; 38 % and 11 %, respectively) and unknown "450 g/mol" metabolite (P5) (24 %), while the unchanged drug (P9) was found with a contribution of 7 %.

In plasma, XYZ1234 remained mainly unchanged. Only trace levels of the demethyl compound XYZ5678 (P7), glucuronide (P6), and the unidentified "450 g/mol" metabolite (P5) were found.

3. *Structure of metabolites*: The parent Drug XYZ1234 had three main metabolites: XYZ5678, 2-OH XYZ1234, and XYZ1234 glucoside. The XYZ5678 had a metabolite 2-OH XYZ5678.
4. *Pharmacokinetic parameters in plasma and urine*: ^{14}C-XYZ1234 solution was rapidly absorbed from the gastrointestinal tract. The peak radiocarbon concentration in plasma averaged 1.61 μg-eq/mL and occurred between 0.5 and 4 h. $AUCinf_{inf}$ of plasma radiocarbon was, on average, 36.9 μg-eq.h/mL. Elimination of radiocarbon was characterized by a mean half-life of 20.5 h.

   Whole blood concentrations represented approximately 80 % of the plasma concentrations. $C_{max} = 1.33$ μg-eq/mL, $AUCinf_{inf} = 32.7$ μg-eq.h/mL. The mean half-life, 20.2 h, was very close to the plasma half-life. There was radioactivity in the red blood cells, and it declined with an elimination half-life similar to the half-lives in plasma and whole blood radiocarbon concentrations.

   The peak concentration of XYZ1234 averaged 1.54 μg/mL and was reached between 0.5 and 4 h after dosing. $AUCinf_{inf}$ averaged 37.2 μg.h/mL. The elimination half-life (19.8 h) was similar to the half-life observed for radiocarbon.

   The renal clearance of XYZ1234 averaged only 4 mL/min, this being small compared to the nonrenal clearance, which was tenfold higher (42 mL/min). Whereas the renal clearance of the metabolite XYZ5678 averaged 144 mL/min.

   The C_{max} of XYZ1234 and XYZ5678 summed was 99 % of the C_{max} of radiocarbon pharmacokinetic in plasma. The t_{max} for radiocarbon and for XYZ1234 was similar, the t_{max} of XYZ5678 appearing a few hours later. The difference in average urinary excretion between the sum of XYZ5678 and XYZ1234, and the radiocarbon, showed that 14.1 % of the drug was eliminated in the urine in a form different than XYZ1234 or XYZ5678, as one or several other metabolites. This was also confirmed by the metabolic profiles.
5. *Plasma protein binding*: The mean protein binding was 91.3 %; no major interindividual differences occurred.

45.6 Assessment of the Relative and/or Absolute Bioavailability of Drugs

PURPOSE/RATIONALE

The assessment of a drug's bioavailability (BA) is the most important information on its PK. Consequently, numerous guidelines primarily focus on this issue as from the exposure efficacy as well as safety for the patient is deduced (US FDA 2002, 2003a, b, c; ICH E4 1994; EU CPMP 1999, 2001, 2003).

Bioavailability is defined as the rate and extent by which the active moiety becomes available at the site of action. Because neither concentrations nor amounts can generally be determined at the site of action, plasma/serum concentrations are used as a surrogate to determine the rate and extent of bioavailability. Provided that the pharmacokinetics of the drug considered is linear and time invariant, the area under the curve (AUC) is a measure for the fraction of the dose available according to Dost's law of corresponding areas. Absolute bioavailability is deduced from the comparison of an extravascular and an intravascular administration, for example, AUC_{PO}/AUC_{IV}. Relative bioavailability compares the exposure following two different extravascular application forms.

Bioavailability is defined for a formulation, not for a drug.

Bioavailability studies quantify rate and extent of absorption. They compare the efficiency of the disposition of several drug formulations, for example, immediate release versus extended release or capsule versus tablet or tablet A versus tablet B, etc., or they compare the disposition of different routes of administration, for example, oral versus subcutaneous or oral versus intravenous. According to the definition, a comparison to the intravenous bolus injection yields the "absolute" bioavailability.

Bioavailability figures should always be given for the active moiety of a drug.

The criterion of bioequivalence applies if there is a similarity in bioavailability (statistically proven) that is unlikely to result in clinically relevant differences in efficacy and/or safety.

The bioavailability of a drug formulation is best described by C_{max}/T_{max} (rate) and area under the systemic concentration-time curve AUC (extent).

Details on the design of and the interpretation of data from bioavailability studies are given in (US FDA 2002, 2003a, b, c; ICH E4 1994; EU CPMP 1999, 2001, 2003).

PROCEDURE

The design for an absolute bioavailability study for Drug XYZ123 is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although safety parameters were also in the focus.

45.6.1 Protocol Outline

A phase I, open-label, randomized, crossover study to investigate the bioavailability, safety, tolerability, and pharmacodynamics following single oral administration of XYZ123 as capsule and single intravenous administration of XYZ123 in healthy men.

45.6.1.1 Primary Objective

To characterize the bioavailability of XYZ123 drug substance (25 mg) as a capsule formulation following a single oral administration in fasting conditions in healthy male adult volunteers using 10 mg of intravenously administered XYZ123 as reference formulation.

45.6.1.2 Study Design

This was an open-label, single-dose, randomized, 2-period crossover study with a minimum washout period of 7 days. Each treatment group received treatment A (10-mg XYZ123, intravenously administered) and treatment B (25-mg XYZ123 as capsule, orally administered), once each under fasting conditions.

45.6.1.3 Inclusion Criteria

Healthy male subjects, aged 18–45 years (inclusive), with a body mass index between 18 and 27 kg/m^2 (inclusive), normal or clinically irrelevant abnormal findings (in the opinion of the investigator) in the medical history and physical examination, laboratory values, ECG, blood pressure and pulse rate, negative serology (HIV antibody, hepatitis B surface antigen, hepatitis C antibody), and urine screen for drugs of abuse.

45.6.1.4 Treatments

Regimen A (reference treatment):

Intravenous (IV) administration of XYZ123 (10 mg, administered over 30 min)

Regimen B (test treatment):

Oral (PO) administration of XYZ123 (25 mg, as a capsule formulation)

45.6.1.5 Pharmacokinetic Data

Concentrations of unconjugated XYZ123 and cysteine (CYS)-conjugated XYZ123 in plasma were measured predose and at predetermined times up to 48 h post dose.

The primary analysis examined pharmacokinetic parameters calculated from plasma concentrations of CYS-conjugated XYZ123 using noncompartmental techniques. The secondary analysis examined the pharmacokinetic parameters of unconjugated XYZ123.

EVALUATION

The primary analyses consisted of characterizing the bioavailability of oral XYZ123 using intravenous XYZ123 as the reference. Determination of bioavailability was to be based on the plasma concentrations of CYS-conjugated XYZ123. Descriptive statistics and formal statistical analysis were used to summarize and analyze the pharmacokinetic parameters of unconjugated XYZ123 and CYS-conjugated XYZ123 in all evaluable subjects.

The secondary analyses consisted of assessing the safety, tolerability, and pharmacodynamic responses after administration of XYZ123 and XYZ123 in plasma and urine using descriptive statistics.

CRITICAL ASSESSMENT OF THE METHOD

For the oral route of administration, the dose was selected according to the experience from the FIM study, where this dose was safe and well tolerated and was in the dose-proportional range. The dose for the intravenous route of administration was adjusted according to the results from animal bioavailability studies where the absolute bioavailability was in the range of 50 %.

Bioavailability/bioequivalence studies are usually conducted in healthy subjects. Although the inclusion of women is now being encouraged, in this study, only men were enrolled. The study was the second clinical trial in this project.

As the bioequivalence rules are clearly defined, the study population must ensure a high level of

Table 45.8 Summary of the pharmacokinetic parameters in plasma

Treatment	C_{max} (ng/mL)	T_{max}[a] (h)	AUC_{last} (ng.h/mL)	AUC_{0-inf} (ng.h/mL)	$t_{½}$ (h)
XYZ123 (unconjugated)					
10 mg IV	49.5 (30.4–96.7)	0.50 (0.50–0.58)	81.8 (42.7–149.2)	207.4[b] (85.1–602.4)	85[b] (36–165)
25 mg PO	15.1 (8.8–24.3)	1.00 (0.50–4.00)	85.0 (60.5–136.4)	215.9[b] (127.8–337.8)	94[b] (61–192)
Conjugated XYZ123					
10 mg IV	280.8 (198.0–420.6)	0.50 (0.50–0.75)	367.1 (256.5–1344.5)	nd	nd
25 mg PO	98.1 (45.9–209.9)	1.50 (1.00–4.00)	317.1 (139.0–704.7)	nd	nd

nd not determined
[a]For T_{max}, the median (range) is given instead of the geometric mean (range)
[b]$n = 14$

standardization, sometimes limiting the extrapolation to patient settings. Typical enrolment criteria are

- Nonsmoking males between 18 and 45 years
- Normal for weight and BMI
- (Clinically) healthy
- Not using any medication
- Massive dietary and general restrictions
- No hypersensitivities
- No history or presence of any condition that might interfere with the absorption, distribution, metabolism, or elimination of the drug under investigation

MODIFICATIONS OF THE METHOD

In this example, an oral formulation has been compared to an intravenous one, aiming at "absolute" bioavailability. More often, the relative bioavailabilities of different oral formulations are assessed in BA studies. The reference formulation in these studies usually is either a marketed (solid) product or an oral solution.

If the drug under investigation has a toxic potential, BA studies have to be conducted in the patient setting the drug is intended for use.

Deviations from the a.m. high level of standardization might become necessary depending on the properties of the compound.

Almost all clinical study types described in this section deal in any way with bioavailability and/or bioequivalence questions. Specifics are mentioned there.

References and Further Readings

EU CPMP (1999) Note for guidance on modified release oral and transdermal dosage forms: section II (Pharmacokinetic and Clinical Evaluation). July 1999

EU CPMP (2001) Note for guidance on the investigation of Bioavailability and Bioequivalence. July 2001

EU CPMP (2003) Points to consider on the clinical requirements of modified release products to be submitted as a line extension of an existing marketing authorization. June 2003

ICH E4 (1994) Dose–response information to support drug registration. March 1994

US FDA (2002) Guidance for industry: food-effect bioavailability and fed bioequivalence studies, December 2002

US FDA (2003c) Guidance for industry: bioavailability and bioequivalence studies for nasal aerosols and nasal sprays for local action, April 2003

US FDA Guidance for Industry (2003) Bioavailability and bioequivalence studies for orally administered drug products—general considerations. March 2003

US FDA Guidance for Industry (2003) Exposure-Response Relationships—Study Design, Data Analysis, and Regulatory Applications. April 2003

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below. Due to the anticipated mode of action of the drug (blood pressure lowering) in this example, instead of an intravenous bolus injection, an intravenous infusion over 30 min was chosen.

45.6.1.6 Results: Pharmacokinetics

A summary of the pharmacokinetic parameters in plasma is presented in Table 45.8.

After oral treatment with 25-mg XYZ123, plasma concentrations of the parent compound were low as a result of a rapid metabolization into the first metabolite XYZ123. For unconjugated and conjugated XYZ123, C_{max} was reached on average 1–1.5 h after oral treatment, after which a rapid initial elimination phase and a slow terminal elimination phase were observed, with a terminal elimination half-life of 3.5–4 days (unconjugated XYZ123). This long half-life led to a small carryover effect in Period 2 for unconjugated

Table 45.9 Results of bioavailability analysis

Analyte	Parameter	Treatment ratio (PO/IV)	90 % CI
Conjugated XYZ123	AUC_{last}	0.38	0.33, 0.43
Unconjugated XYZ123	AUC_{0-inf}	0.47	0.38, 0.59
Unconjugated XYT123	AUC_{last}	0.45	0.43, 0.47

Note: Data were dose corrected

XYZ123. Due to the relatively high lower limit of quantitation (LLQ) of the assay for conjugated XYZ123, the terminal elimination phase for this analyte could only be observed for one subject. The concentrations of conjugated XYZ123 in plasma were 5–10-fold higher than of unconjugated XYZ123. The AUC_{last} (both analytes) and AUC_{0-inf} (unconjugated XYZ123 only) were similar after treatment with XYZ123 25 mg PO and XYZ123 10 mg IV (Table 45.9).

In summary, the calculated bioavailability on basis of the AUC_{last} of conjugated XYZ123 was 38 %. However, this could be a slight underestimation of the bioavailability since this AUC_{last} could only be determined until 6 h post dose. A calculation of the bioavailability on basis of the AUC_{last} or AUC_{0-inf} of unconjugated XYZ123 yielded a slightly higher bioavailability of 45–47 %.

45.7 Drug–Drug Interaction Studies

PURPOSE/RATIONALE

During preclinical developments, numerous flags can arise which indicate that the drug under study has the potential to interact, i.e., its pharmacokinetics or pharmacodynamics can be altered, by concomitant medications, dietary factors, and/or social habits such as tobacco or alcohol in the target population (US FDA 2006b and references therein). Regulatory guidance suggests that if appropriately performed in vitro studies indicate the lack of such an interaction, then a specific clinical study is not compulsory. However, if the claim "No clinically relevant interaction with Drug X" is desired in the product label, then a confirmatory clinical study is compulsory even if in vitro studies indicated the lack of an interaction (EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010).

When discussing interactions, it is important to differentiate between "detectable" and "clinically relevant" interactions. It is accepted that for compounds with a wide therapeutic margin, pharmacokinetic drug interactions may have little clinical relevance. An interaction is considered clinically relevant when the therapeutic and/or toxicity of a drug is changed to such an extent that a dosage adjustment or medical intervention may be required or when concomitant use of two interacting drugs could occur when both are used as therapeutically recommended (EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010).

The basis of an interaction can be pharmacokinetic, pharmacodynamic, or a combination thereof. Pharmacodynamic interactions may be caused by a wide variety of mechanisms; hence, detailed guidance for pharmacodynamic studies is limited, and the study design must be chosen on a case-by-case basis.

During drug development, both aspects for the involvement of the developmental drug in a potential drug-drug interaction has to be considered: their potential as ***victim***, which means they are the object of a drug-drug interaction, or their potential as ***perpetrator***, which means they are the cause of the drug interaction. The scope of mechanisms causing pharmacokinetic interactions may include alterations in one or more of the absorption, distribution, metabolism, and elimination processes (EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010). The alterations may reflect effects of the developmental drug on the pharmacokinetics of the potential interaction partner and vice versa.

Extensive guidance has been published by regulatory agencies on in vitro and in vivo drug-drug interactions studies and how the results obtained can impact the drug dosing and labeling (US FDA 2006b; EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010; US FDA 1997a). Some of the limitations of this guidance are discussed in a recent review that also provides a summary of current industry practice (Bjornsson et al. 2003). However, there is clearly a need for a further harmonization of study designs and marker substrates employed and in the manner in which the data obtained is interpreted, for example, by the development of classification systems. In addition, while existing guidance mainly covers P450-mediated drug interactions, the importance of other mechanisms such as transported has been recognized and should

also be addressed. Recently, increased interest in drug transporters and research in this area has revealed that drug transporters play an important role in modulating drug absorption, distribution, and elimination. Acting alone or in concert with drug-metabolizing enzymes, they can affect the pharmacokinetics and pharmacodynamics of a drug. This commentary will focus on the potential role that drug transporters may play in drug-drug interactions (see Zhang et al. 2006).

Typically, drug-drug interaction studies include some form of comparison of the bioavailability of some marker substrate, for the example given below of ethinylestradiol, when dosed with or without concomitant dosing with the investigational drug, in this example Drug XYZ. Ethinylestradiol is one active component of oral contraceptives and thus a very common concomitant medication for Drug XYZ whose target population is largely younger women. From earlier in vitro and animal in vivo studies, Drug XYZ was known to be potent inducer of both phase I and phase II metabolizing enzymes, including those enzymes reportedly involved in the clearance of ethinylestradiol. Since a clinically relevant drug-drug interaction between Drug XYZ and oral contraceptives would impact the product label and probably also impact the market value of the drug, the study described below was performed.

PROCEDURE

The design of a typical drug-drug interaction study is presented below. For the purposes of simplicity, the description of this example is limited to the collection, handling, and interpretation of data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions, although other parameters were also studied.

45.7.1 Protocol Outline

Effects of repeated once-daily Drug XYZ doses on the safety, pharmacodynamics, and pharmacokinetics of ethinylestradiol after dosing with monophasic oral contraceptives containing ethinylestradiol in healthy overweight or obese women.

45.7.1.1 Objectives

Primary: The primary objective of the study was to study the effects of repeated once-daily Drug XYZ doses on the pharmacokinetics of ethinylestradiol after dosing with monophasic oral contraceptives containing ethinylestradiol in healthy overweight or obese women.

Secondary: The secondary objective of the study was (1) to assess whether Drug XYZ affects the contraceptive effect of the oral contraceptives containing ethinylestradiol as reflected by changes in serum progesterone and 17-β-estradiol levels and (2) to evaluate the safety, tolerability, and pharmacokinetics of repeated once-daily oral doses of Drug XYZ.

45.7.1.2 Study Design

Single-center, double-blind, randomized crossover study in young healthy overweight or obese women. Subjects crossed over with respect to Drug XYZ or placebo that was given double-blinded. The study ran over two menstrual cycles.

Cycles 1 and 2 involved dosing with either Drug XYZ or matching placebo on days 6–20, dosing with the subjects normal ethinylestradiol containing oral contraceptive on days 8–28, hospitalization on days 19–21, and visits to the study site (lunch times) on days 1 (cycle 1 only), 6, 12, 16, 24, and 28.

Day 1 was the first day of the stop week, i.e., the first day after completing the previous cycle: (menstruation generally starts on day 2 or 3, dosing with the oral contraceptive starts on day 8). Sexually active subjects used double barrier contraception during cycles 1 and 2 and for 28 days after completing cycle 2. Subjects were advised to continue use of these measures for at least 28 days after completing cycle 2. Dropouts were not to be replaced.

45.7.1.3 Number of Subjects

Based on published variability in pharmacokinetic studies of ethinylestradiol in lean subjects, taking confidence intervals of 80–125 %, residual variance ranged between 10 % and 33 %. Based on these residual variance values, calculated samples sizes ranged between 6 and 30 (subjects). For example, based on a residual variance value of 17.5 %, a sample size of 14 was calculated.

The chosen sample size reflects (1) the formal sample size calculation (using a residual variance value of 17.5 %) based on the pharmacokinetics of ethinylestradiol in lean subjects; (2) published sample sizes in other studies of this type of study ranged

between 12 and 34; (3) that this study will include overweight and obese subjects, a population who have been suggested to show a higher variability in their pharmacokinetics and in their menstrual cycles; and finally (4) the plan not to replace dropouts.

Based on the planned analysis of variance on log-transformed data, 90 % confidence intervals for AUC ratio's ethinylestradiol + Drug XYZ and ethinylestradiol alone, 20 subjects complete the study as planned.

45.7.1.4 Inclusion Criteria

The following inclusion criteria were met: women aged between 18 and 35 years; with body mass index (BMI) of $25.0 \leq 35.0$ kg/m^2; who are either postmenopausal or surgically sterile and willing to use prescribed barrier contraceptive methods; who—apart from being overweight or obese—are healthy for the purpose of the study and not receiving regular medication in the month preceding the study (with the exception of oral contraceptives); who are using monophasic contraceptives containing ethinylestradiol as the estrogen compound; who present normal gynecological histories and normal, regular menstrual cycles (within the previous 12 months); without contraindications for treatment with oral contraceptives; who are not pregnant or lactating; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant for the study; who are nonsmoking or light smokers.

45.7.1.5 Treatments

Cycles 1 and 2: Oral administration of (1) the subjects normal oral contraceptive once daily (mornings before breakfast) on days 8–28 of each cycle and (2) Drug XYZ or matching placebo, once daily (mornings before breakfast) on days 6–20 of each cycle (15 doses in total).

45.7.1.6 Pharmacokinetic/ Pharmacodynamic Data

Plasma concentrations of ethinylestradiol (EE) on day 20 in cycles 1 and 2.

Plasma concentrations of Drug XYZ, before and at predetermined times post dose, were measured on days 6, 12, 16, 20, 24, and 28 in cycles 1 and 2.

Serum concentrations of 17-β-estradiol and progesterone on days 6, 12, 16, 20, 24, and 28 in cycles 1 and 2.

EVALUATION

The data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions from study described above was evaluated as follows:

Where appropriate, individual data were presented together with descriptive statistics including mean, standard deviation, standard error of the mean, coefficient of variation (in %), median, minimum, maximum, and the number of relevant observations.

Where applicable, pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0–24}$, $t_{½}$) were calculated using a noncompartmental analysis employing a linear/log trapezoidal method.

Plasma EE: Descriptive statistics and comparison of plasma EE concentrations in cycles 1 and 2. Analysis of variance on log-transformed data, 90 % confidence intervals for AUC ratio of EE + Drug XYZ and EE alone ($AUC_{EE+Drug\ XYZ}/AUC_{EE}$).

Analysis of variance was performed on the log-transformed $AUC_{0–24}$ of ethinylestradiol to estimate intrasubject variability. The intrasubject variability was subsequently used to estimate the 90 % confidence interval of the $AUC_{EE+Drug\ XYZ}/AUC_{EE}$ ratio.

Plasma drug XYZ: Individual plasma concentrations were tabulated together with standard descriptive statistics for each variable.

Serum progesterone: Descriptive statistics and comparison of serum progesterone concentrations in cycles 1 and 2. Descriptive comparison of the proportion of subjects who ovulated while receiving Drug XYZ and contraceptive concomitantly and the proportion of subjects who ovulated while receiving contraceptive alone. Ovulation was assumed if serum progesterone levels exceeded 1.4 ng/mL on day 20 of a menstrual cycle. Individual and mean/median profiles were presented graphically.

Serum 17-β-estradiol: Descriptive statistics and comparison of serum 17-β-estradiol concentrations in cycles 1 and 2. Individual serum concentrations of 17-β-estradiol were tabulated.

CRITICAL ASSESSMENT OF THE METHOD

The study described above could have been powered to study effect of Drug XYZ on pharmacodynamic effects of ethinylestradiol, in this case, to study effects on ovulation.

The study described above did not study interactions with other active components of oral

contraceptives whose pharmacokinetics could also be altered by concomitant dosing with Drug XYZ.

The study described above did not study whether or not the observed effects on the pharmacokinetics of ethinylestradiol were reversible, and if so, the time course thereof.

The lack of prior knowledge of the time course of any Drug XYZ–mediated induction of phase I and II metabolizing enzymes complicated the interpretation of the data obtained from the example study. Based on previous clinical studies, the Drug XYZ dosing regimen was chosen to ensure that enzyme induction was maximal at the time shortly before ovulation. However, it was not known whether or not the observed enzyme induction would fade after prolonged exposure to Drug XYZ, or whether on rechallenge, the same magnitude of enzyme induction would be seen, or if the observed induction was reversible.

This example however followed the recommended basic profiling sequence, i.e., in vitro profiling, confirmation of the in vitro observation in animals, explorative profiling in man. The example study would typically be followed by further profiling of this drug-drug interaction potential with other substrates, representing a range of concomitant medication in the target patient population, for example, using population pharmacokinetic approaches and definitive studies designed to support clear labeling statements.

MODIFICATIONS OF THE METHOD

The following general issues and approaches should be considered (for a more detailed discussion, see references (US FDA 1997a, 2006b; EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010). In the following discussion, the term *substrate* (S, in our example ethinylestradiol) is used to indicate the drug studied to determine if its exposure is changed by another drug, which is termed the *interacting drug* (I, in our example Drug XYZ). Depending on the study objectives, the substrate and the interacting drug may be the developmental drugs or approved products.

45.7.1.7 Study Design

Clinical drug-drug interaction studies are generally designed to compare substrate levels with and without the interacting drug, and thus many of the principles applying to comparative bioavailability studies also apply here. Because a specific study may consider a number of questions and clinical objectives, no one correct study design for studying drug-drug interactions can be defined.

The following considerations may be useful when choosing a study design:

- Interpretation of findings from these studies will be aided by a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects. In certain instances, reliance on endpoints other than pharmacokinetic measures/parameters may be useful.
- The inhibiting/inducing drugs and the substrates should be dosed so that the exposure of both drugs is relevant to their clinical use.
- The time at steady state before collection of endpoint or pharmacokinetic observations depends on whether inhibition or induction is to be studied. Inducers can take several days or longer to exert their effects, while inhibitors generally exert their effects more rapidly. Thus, if induction is to be assessed, a more extended profiling period after attainment of steady state for the substrate and interacting drug may be necessary.
- When attainment of steady state is important, long half-lives of the substrate, interacting drugs, and/or their metabolites should be considered.
- When a substrate and/or an interacting drug are to be studied at steady state, documentation that near steady state has been attained is important.
- Studies can usually be open label (unblinded), unless pharmacodynamic endpoints subject to bias are part of the assessment of the interaction.
- For a rapidly reversible inhibitor, administration of the interacting drug either just before or simultaneously with the substrate on the test day might be the appropriate design to increase sensitivity.

45.7.1.8 Study Population

Clinical drug-drug interaction studies may generally be performed using healthy subjects on the assumption that findings in this population should predict findings in the target patient population. Safety considerations, however, may preclude the use of healthy subjects. In certain circumstances, inclusion of patients from the targeted patient population may offer certain advantages, including the opportunity to study pharmacodynamic endpoints.

45.7.1.9 Choice of Substrate and Interacting Drugs

Substrates for a developmental drug (Developmental drug is the Perpetrator): When testing inhibition, the substrate selected should generally be one whose pharmacokinetics is markedly altered by coadministration of known specific inhibitors of the affected enzyme systems (i.e., a very sensitive substrate should be chosen). If the initial study is positive for inhibition, further studies with other substrates representing a range of substrates based on the likelihood of coadministration may be useful. If the initial study is negative with the most sensitive substrates, it can be presumed that less sensitive substrates will also be unaffected.

Developmental drug as substrate (Developmental drug is the Victim): When testing a developmental drug for the possibility that its metabolism is inhibited or induced (i.e., as a substrate), selection of the interacting drugs should be based on a priori knowledge of the enzyme systems that metabolize the developmental drug. The choice of interacting drug should then be based on known, important inhibitors of the pathway under investigation. If the study results are negative, then absence of a clinically important drug-drug interaction for the metabolic pathway could be claimed.

45.7.1.10 Route of Administration

For a developmental drug used as either an interacting drug or substrate, the route of administration should generally be the one planned for in-product labeling.

45.7.1.11 Dose Selection

For both the substrate and interacting drug, testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and shortest dosing interval of the interacting drug (as inhibitors or inducers) should be used. Doses smaller than those to be used clinically may be needed for substrates on safety grounds and may be more sensitive to the effect of the interacting drug.

45.7.1.12 Sample Size and Statistical Considerations

For both developmental drugs and approved drugs, when used as substrates and/or interacting drugs in drug-drug interaction studies, the desired goal of the analysis is to determine the clinical significance of any increase or decrease in exposure to the substrate in the presence of the interacting drug. Assuming unchanged PK/PD relationships, changes may be evaluated by comparing pharmacokinetic measures of systemic exposure that are most relevant to an understanding of the relationship between dose (exposure) and therapeutic outcome.

Results of drug-drug interaction studies should be reported as 90 % confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with (S + I) and without the interacting drug (S). Confidence intervals provide an estimate of the distribution of the observed systemic exposure measure ratio of S + I versus S alone and convey a probability of the magnitude of the interaction.

When a drug-drug interaction is clearly present, the sponsor should be able to provide specific recommendations regarding the clinical significance of the interaction based on what is known about the dose–response and/or PK/PD relationship for either the investigational agent or the approved drugs used in the study.

The sponsor may wish to make specific claims in the package insert that no drug-drug interaction is expected. In these instances, the sponsor should be able to recommend specific *no effect* boundaries, or clinical equivalence intervals, for a drug-drug interaction. No effect boundaries define the interval within which a change in a systemic exposure measure is considered not clinically meaningful.

References and Further Readings

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CPMP/EWP/560/95/Rev. 1 (2010) Draft guideline on the investigation of drug interactions; April 2010

EMEA CPMP/EWP/560/95 (1997) Note for guidance on the investigation of drug interactions. December 1997

US FDA (1997a) Guidance for industry: drug metabolism/drug interaction studies in the drug development process: studies in vitro, April 1997

US FDA (2006) Draft guidance for industry: drug interaction studies —study design, data analysis, and implications for dosing and labeling. September 2006

Zhang L, Strong JM, Qiu W, Lesko LJ, Huang S-M (2006) Scientific perspectives on drug transporters and their role in drug interactions. Mol Pharm 3(1):62–69

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under *Procedure* is presented below.

45.7.1.13 Results

The data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions from study described above, as given in Table 45.10 below, can be summarized as follows:

In this study, nine different brands of oral contraceptives were used. The dose of ethinylestradiol per pill most commonly taken was 30 μg (by 17 subjects) and ranged from 20 to 50 μg across all subjects, 24 subjects who completed the study as planned.

Pharmacokinetics: When EE was administered in combination with Drug XYZ, arithmetic mean $t_{1/2}$ and T_{max} values were comparable for both treatments. Geometric mean C_{max} and $AUC_{0–24}$ values were approximately 20–30 % lower, respectively, than when EE was administered with placebo. For $AUC_{0–24}$, the lower and upper limits of the 90 % CI were below the predefined 90 % CI of 0.8–1.25. Therefore, a pharmacokinetic interaction between Drug XYZ and EE can be concluded. The mechanism for this statistically significant reduction in systemic exposure to EE is unknown; however, since Drug XYZ is known to induce CYP 1A2 in man, and CYP 1A2 and phase II enzymes in animals, the observed effect could reflect a metabolic interaction between Drug XYZ and EE.

Pharmacodynamics: Serum progesterone and 17-β-estradiol levels are considered reliable indicators for the occurrence of ovulation. Since progesterone and 17-β-estradiol levels were comparable for both treatments, and progesterone serum concentrations did not exceed 1.4 ng/mL (as defined in this study, progesterone concentrations above 1.4 ng/mL on day 20 of a menstrual cycle indicated ovulation) for both treatments, it was concluded that Drug XYZ administration did not affect the contraceptive effect of ethinylestradiol-based oral contraceptives and that no ovulation occurred in any of the subjects.

45.8 Profiling the Effect of Food on Drug Bioavailability

PURPOSE/RATIONALE

Drugs intended for oral administration have to pass through the gastrointestinal tract before they can enter the bloodstream and eventually reach their target site of action. Often already profiled using a food screen in the first-in-man study, the assessment of the influence of food intake on the bioavailability of a drug belongs to the most important steps when describing the drug pharmacokinetics in early clinical development. The outcome of such food screens or formal food interaction studies has a considerable impact on the design of ensuing studies and forms the basis for later labeling recommendations as well as for the package insert (US FDA Guidance for Industry 2002).

Food can alter the bioavailability of drugs either by direct physical or chemical interaction or by the physiological response. Such effects are most prominent when the drug product is administered shortly after a meal. The composition of such a meal should lead to the greatest possible physiological reaction. Consequently, a high-fat, high-calorie breakfast after overnight fasting is recommended. Details on the composition of such a meal and the design of those studies can be found in US FDA Guidance for Industry (2002) and EU CPMP (1999).

The effects of food intake include the physiological effects of food itself, as well as physicochemical interactions between food and the drug under investigation.

Table 45.10 Summary statistics of pharmacokinetic parameters for ethinylestradiol

	EE + placebo				EE + Drug XYZ			
Parameter	*n*	Arithmetic mean	CV (%)	Geometric mean	*n*	Arithmetic mean	CV (%)	Geometric mean
C_{max} (pg/mL)	24	98.3	39	91.1	24	79.4	43	72.5
$AUC_{0–24}$ (pg.h/mL)	24	933	34	879	24	672	32	637
	Treatment ratio and 90 % confidence interval of EE $AUC_{0–24}$ (EE + Drug XYZ over EE + placebo) 0.73 (0.68–0.78)							
$t_{½}$ (h)	24	22	27	21	24	21	28	20
T_{max} (h)	24	1.2	55	–	24	1.2	54	–

For the latter, the categories of the biopharmaceutical classification system (BCS) become important (Amidon et al. 1995). A comprehensive excellent review of the determinants of food effects on clinical pharmacokinetics can be found in Singh (1999).

PROCEDURE

The design of an exploratory food interaction bioavailability study with Drug XYZ123 is presented below. In this given project, the food interaction study was initiated in parallel to a hADME study just after completion of the first-in-man study. In this study, the collection, handling, and interpretation of pharmacokinetic data were in the focus.

45.8.1 Protocol Outline

Study of the effect of food on the pharmacokinetics of film-coated tablets (3 × 200 mg) of XYZ123 in healthy men.

45.8.1.1 Primary Objective

To assess the effect of food on the pharmacokinetics of 600-mg XYZ123 in healthy men.

45.8.1.2 Study Design

An open, randomized, four-period crossover study. There were four sequence groups of five subjects each. Each sequence group received the treatments A, B, C, and D (different time intervals between food intake and medication) in different sequential order (Williams design). Washout periods were at least 4 days.

45.8.1.3 Inclusion Criteria

Healthy men aged between 40 and 65 years. Body weights within −15 % and +10 % of the normal weight according to Broca.

45.8.1.4 Treatments

Treatment A: Single dose of 3 × 200-mg XYZ123 on an empty stomach (overnight fasting) and start of high-fat-food intake 4 h later.

Treatment B: Single dose of 3 × 200-mg XYZ123 together with high-fat food (drug administration 15 min after start of high-fat-food intake).

Treatment C: Single dose of 3 × 200-mg XYZ123 2 h after start of high-fat-food intake.

Treatment D: Single dose of 3 × 200-mg XYZ123 on an empty stomach (overnight fasting) 1 h before start of high-fat-food intake.

45.8.1.5 Pharmacokinetic Data

Concentration of XYZ123 in plasma before and at predefined times after dosing.

EVALUATION

Descriptive statistics of all variables.

Analysis of variance (ANOVA) with treatment, subject (nested within sequence), and period as main factors was performed for C_{max} and AUC_{0-inf}. The 90 % confidence intervals of the point estimates of the ratio of C_{max} and AUC_{0-inf} and of the difference between treatments for t_{max} were determined. Pairwise comparisons to treatment A were made, with treatment A versus treatment B being the primary comparison.

CRITICAL ASSESSMENT OF THE METHOD

The information originating from preceding studies is needed for the proper design of a food interaction study: safety and tolerance data has to be considered as well as the PK results including a food screen. The terminal half-life of the drug or its active metabolite(s) will provide the basis for the washout periods. Single-dose linearity/proportionality will help to define the dose. Safety and tolerance data will justify the dose. The PK comparison in a food screen (FIM study, see below) will influence the sample size.

It depends on the target indication, for example, acute or chronic use, and on the intended dosing regimen, for example, once, twice, or thrice daily, whether a given food effect is acceptable for justification of further development or if it defines the "knockout."

The study design described here is a quite complex approach. A simplification would limit the study conditions to both extremes: a high-fat, high-calorie breakfast starting 0.5 h before the drug administration versus fasting overnight (at least 10 h) + at least 4 h after drug administration.

MODIFICATIONS OF THE METHOD

There is a tendency to include a so-called food screen already in a FIM study. The limitation of such an approach is the nonavailability of information mentioned in the previous chapter. On the other hand,

Table 45.11 Summary of the pharmacokinetic parameters in plasma

	PK parameter (geometric mean + range)				
Treatment	C_{max} (μg/mL)	t_{max}[a] (h)	$t_{½}$ (h)	$AUC_{0–inf}$ (μg.h/mL)	CLtot/F (L/h)
A ($n = 19$)	4.51	0.75	2.78	11.0	51.4
Food 4 h after dosing	(2.00–8.02)	(0.25–2.50)	(2.05–4.98)	(4.96–17.0)	(33.4–115)
B ($n = 19$)	3.60	1.50	2.77	11.4	49.8
Food with dosing	(1.72–8.57)	(0.75–6.00)	(1.78–4.22)	(5.62–19.0)	(29.9–101)
C ($n = 19$)	3.52	1.50	3.10	9.89	57.4
Food 2 h before dosing	(2.02–5.57)	(0.75–2.50)	(1.86–5.98)	(4.12–17.3)	(32.9–138)
D ($n = 19$)	5.41	0.50	3.05	11.8	48.3
Food 1 h after dosing	(2.98–8.65)	(0.50–1.50)	(2.40–4.45)	(6.13–18.2)	(31.2–92.7)

[a]For t_{max}, the median + range is presented

changes in the formulation during the drug development phase, a switch from an immediate-release to an extended-release formulation might necessitate a repetition.

Only if the conditions for a waiver apply, a food interaction study is not needed for a submission package. Under all other circumstances, where drug products are administered orally for systemic exposure, this kind of study is a must. And it must be conducted with the drug product that is intended for the market authorization.

References and Further Readings

Amidon GL, Lennernäs H, Shah VP, Crison JR (1995). A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm Res 12:413–420

EU CPMP (1999) Note for guidance on modified release oral and transdermal dosage forms: section II (Pharmacokinetic and Clinical Evaluation) July 1999

Singh BN (1999) Effects of food on clinical pharmacokinetics. Clin Pharmacokinet 37(3):213–255

US FDA (2002) Guidance for industry: food-effect bioavailability and fed bioequivalence studies, December 2002

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below.

45.8.1.6 Results: Pharmacokinetics

The XYZ123 concentration-time profiles show that food caused a delay in drug absorption and a lower peak concentration. This effect was most pronounced when food and medication were taken at the same time. A summary of the pharmacokinetic parameters in plasma is presented in Table 45.11.

Table 45.12 Summary of the point estimates of the treatment ratios or of the difference between treatments

PK parameter	Treatment	Point estimate of treatment ratio[a]	90 % CI
C_{max}	B/A	0.79	0.69–0.91
	C/A	0.78	0.68–0.89
	D/A	1.20	1.04–1.37
AUC_{inf}	B/A	1.03	0.94–1.12
	C/A	0.89	0.81–0.98
	D/A	1.07	0.97–1.17
t_{max}[a] (h)	B–A	1.13	0.88–1.63
	C–A	0.94	0.63–1.25
	D–A	0.00	−0.13–0.13

[a]Point estimate of the treatment difference (h) is presented for t_{max}

A summary of the point estimates of the treatment ratios or of the difference between treatments for XYZ123 is presented in Table 45.12. The $AUC_{0–inf}$ was similar for all treatments. When compared to treatment A, the $AUC_{0–inf}$ of the other treatments was within the 80–125 % equivalence limits.

The point estimates show that taking food 2 h before, or at the same time as receiving medication, delayed absorption and lowered the maximum plasma concentration. The relative bioavailability, however, was similar for all treatments. The lower limit of the 90 % CI for C_{max} was marginally outside the predefined equivalence range for treatment B and C, but C_{max} was within the equivalence range for treatment D.

The intake of a high-fat meal before or at dosing lowered the maximum plasma concentration (C_{max}) and increased the absorption time (t_{max}).

The relative bioavailability, however, was equivalent when medication was taken with food or under fasting conditions.

45.9 Exploratory Profiling of Enzyme Induction on Drug Disposition

PURPOSE/RATIONALE

Concomitantly administered drugs have the potential to interact, i.e., their pharmacokinetics or pharmacodynamics can be altered (US FDA 2006b and references therein). Regulatory guidance suggests that if appropriately performed in vitro studies indicate the lack of such an interaction, then a specific clinical study is not compulsory. However, if the claim "No clinically relevant interaction with Drug X" is desired in the product label, then a confirmatory clinical study is compulsory even if in vitro studies indicated the lack of an interaction (EMEA CPMP/EWP/560/95 1997; CPMP/EWP/560/95/Rev. 1 2010).

Drug-drug interactions mediated by enzyme induction are less common than those mediated by enzyme inhibition. Drug-drug interactions due to enzyme induction are also less likely to cause safety issues, except toxic if a toxic metabolite is formed by metabolite activation; however, they may affect the activity of the developmental drug itself and of concomitant medications. In this context, it is to mention that induction of CYP 3A4 by a developmental drug is frequently investigated for oral contraceptives, although they are not very sensitive substrates (US FDA 2006b).

Preclinical profiling for enzyme induction is complicated by the observation that, contrary to as seen for enzyme inhibition, the enzyme inductive potential of a drug in man is difficult to assess preclinically, especially in nonhuman systems. There are however numerous flags which can indicate that the drug under study has some activating effect on drug-metabolizing enzymes such as the cytochrome P450 isozymes (CYP) 1A2, 3A4, etc. These flags can include a drop in systemic exposure to developmental drug after multiple dosing, increases in animal liver weights after multiple dosing in toxicology studies, class characteristics, and positive signals in animal and human in vitro enzyme induction screens.

If one or more of these flags are observed, especially positive signals in human in vitro enzyme induction screens, then the in vivo inductive potential of the developmental drug is typically studied in suitable explorative clinical studies. Given the expectation that enzyme induction is more likely to cause a reduced efficacy of developmental drug itself and/or of concomitant medications than to cause safety issues, in the profiling of enzyme induction during early clinical development in man is typically included as secondary objective in other studies. Such explorative clinical studies employ non-indication-specific, but metabolically well-characterized, marker drugs or compounds. If a notable inductive potential is seen in these explorative clinical studies, then more specific studies with drugs with a narrow therapeutic index critical and/or frequent usage in the target population are usually performed.

PROCEDURE

The design of a study providing the suggested exploratory profiling of the effect of Drug XYZ on CYP 1A2- and CYP 3A4-mediated metabolism is presented in the *Protocol Outline* below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of data pertinent to the assessment of potential drug-mediated effects on CYP1A2- and CYP3A4-mediated metabolism, although other parameters were also studied.

45.9.1 Protocol Outline

Safety, tolerability, and the effect on CYP 1A2- and CYP 3A4-mediated metabolism of single and repeated oral doses of 400-mg and 1,200-mg Drug XYZ or placebo in overweight or obese, but otherwise healthy, men.

45.9.1.1 Objectives

Primary: The primary objective of the study was to investigate in overweight or obese, but otherwise healthy, men: (1) the safety and tolerability of single and repeated oral doses of 400-mg and 1,200-mg Drug XYZ and (2) the effect of Drug XYZ on CYP1A2- and CYP3A4-mediated metabolism.

Secondary: The secondary objective of the study was to investigate the pharmacokinetics of Drug XYZ after single and repeated oral doses of 400-mg and 1,200-mg Drug XYZ in overweight or obese, but otherwise healthy, men.

45.9.1.2 Study Design

The study had a single-center, single- and repeated-dose, single-trial period, and double-blind design.

Subjects received single doses of 150-mg caffeine, once before starting treatment with Drug XYZ (day 1) and then again together with Drug XYZ dosing on day 10. In addition, subjects received a single daily dose of 400-mg and 1,200-mg Drug XYZ or matching placebo on day 2 and then again on days 4–10.

On days 1 and 10, CYP 1A2 activity was monitored using the plasma concentrations of caffeine and paraxanthine and CYP 3A4 activity was monitored using the urinary excretion of free cortisol and 6-β-hydroxy-cortisol (Rost and Roots 1994; Fuhr et al. 1996; Streetman et al. 2000; Kovacs et al. 1998; Tran et al. 1999).

The treatment period between trial periods was at least 10 days which approximated to >10 Drug XYZ apparent terminal half-lives.

45.9.1.3 Number of Subjects

The inclusion of eight (8) subjects was considered to be in line with common industry practice for this type of explorative study.

45.9.1.4 Inclusion Criteria

The following inclusion criteria were met: men aged between 18 and 55 years; with body mass index (BMI) of 25–35 kg/m^2; who—apart from being overweight or obese—are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant; who are nonsmokers.

45.9.1.5 Treatments

Single (day 2) and repeated (days 4–10) oral doses of 400-mg and 1,200-mg Drug XYZ or matching placebo after fasting. Single doses of 150-mg caffeine, once on day 1 and then again on day 10.

45.9.1.6 Pharmacokinetic Data

Plasma concentrations of Drug XYZ, caffeine, and paraxanthine, before and at predetermined times post dose, were measured.

Concentrations of Drug XYZ, 6-β-hydroxy-cortisol, and free cortisol in urine collected over the profiling period were measured. Volumes of urine collected over each profiling period were recorded.

The volume of urine collected over 24 h and the concentration of creatinine were determined to allow the subjects' creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of potential drug-mediated effects on CYP 1A2- and CYP 3A4-mediated metabolism from study described above was evaluated as follows: Due to the investigational nature of the study, and the small sample size, all variables were only presented descriptively. Where appropriate, individual data were presented together with descriptive statistics.

Plasma caffeine and paraxanthine: Descriptive pharmacokinetic parameters (standard parameters including peak concentrations (C_{max}), time of C_{max} (T_{max}), area under the curve (AUC) between time 0 and time t where t = 24 h post dose (AUC_{0-t}), AUC after extrapolation to infinity ($AUC_{0-\infty}$), apparent terminal half-life ($t_{1/2_z}$), and total clearance (CL)) for plasma caffeine and paraxanthine on days 1 and 10 were calculated using a noncompartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin® (Pharsight Corp.) protocols. Changes from baseline (day 10–day 1) in caffeine clearance and the area-under-the-curve (AUC) ratio paraxanthine/caffeine were presented individually and with corresponding descriptive statistics. The ratio of paraxanthine AUC/caffeine AUC was calculated.

Urinary 6-β-hydroxy-cortisol and free cortisol: The following pharmacokinetic variables were derived from urine concentration data for 6-β-hydroxy-cortisol and free cortisol on days 1 and 10 using SAS® for Windows™ protocols: amount excreted during each collection interval for 6-β-hydroxy-cortisol and free cortisol, total amount excreted (mg) during 12 h (Ae_{0-12}) and 24 h (Ae_{0-24}) for both compounds, the ratio of Ae_{0-24} of 6-β-hydroxy-cortisol/Ae_{0-24} of free cortisol, the ratio of Ae_{0-12} of 6-β-hydroxy-cortisol/Ae_{0-12} of free cortisol.

CRITICAL ASSESSMENT OF THE METHOD

Ideally, such a study should profile the dose dependence, the time dependence, and the reversibility of the enzyme induction. Also the enzyme substrates used as

markers for the enzyme activity should be drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population.

In the described study, the time dependence and reversibility of the enzyme induction were not studied. Also the enzyme substrate used as a marker for the CYP 1A2 activity was caffeine, which, although frequently encountered in the target population and commonly used as a marker for CYP 1A2 activity, is not a drug with a narrow therapeutic index used by the target population. The enzyme substrate used as a marker for the CYP 3A4 activity, urinary 6-β-hydroxy-cortisol, and free cortisol, although readily amenable to inclusion in studies, is not a drug and is also known to be a relatively insensitive marker for CYP 3A4 induction. Also urinary 6-β-hydroxy-cortisol and free cortisol do not differentiate between intestinal and liver CYP 3A4 activities. But on the other hand, since only endogenous markers are to be determined, this test can be implemented in the early development program without the need to conduct a dedicated study in order to generate a flag for CYP 3A4 induction early.

MODIFICATIONS OF THE METHOD

The design of studies profiling of enzyme induction is typically case specific since the time and dose dependence of enzyme induction differs between the enzyme (s) being induced and the drug causing the induction. There is no clearly defined regulatory guidance on enzyme induction studies beyond the recommendation that study designs should be science based.

It is generally accepted that the dosing regimen should minimally ensure that the anticipated therapeutic steady-state exposure is maintained (or exceeded) for some days since although some inducible enzymes respond rapidly, others require longer exposure before responding. Ideally, such a study should profile the dose dependence, the time dependence, and the reversibility of the enzyme induction. Also the enzyme substrates used as markers for the enzyme activity should be drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population.

The study described in this chapter provides explorative profiling of potential enzyme induction after dosing over 2 weeks at two dose levels and in comparison to placebo. This relatively comprehensive design reflects the combination of (1) the clear expectation that a clinically relevant enzyme induction would be observed based on numerous flags from preclinical studies and experience with other members of this chemical class characteristics for this drug, (2) the chance ability to build in the planned investigation into a tolerability study due to the use of innocuous (caffeine) or endogenous enzyme markers (urinary 6-β-hydroxy-cortisol/free cortisol), and (3) the opinion that a clinically relevant enzyme induction would have severely impacted the market value of a Drug XYZ and thus should be profiled as early as possible.

In practice, the explorative profiling of enzyme induction is even less elaborate than that performed for Drug XYZ because (1) it requires the use of enzyme substrates which are less amenable to inclusion in tolerability studies and (2) risk-benefit considerations do not justify studies a with a range of dose levels.

Instead, specific confirmatory clinical studies employing drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population are typically implemented in the late clinical development as part of the range of drug-drug interaction studies used to support the drug label.

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above is presented below.

45.9.1.7 Results: Pharmacokinetics

The data pertinent to the assessment of potential drug-mediated effects on CYP 1A2- and CYP 3A4-mediated metabolism from study described above, as given in Tables 45.13 and 45.14 below, can be summarized as follows:

- The mean $AUC_{0-\infty}$ for caffeine on day 10 was decreased by approx. 67 % and approx. 80 % when compared to day 1 for subjects treated with 400-mg and 1,200-mg Drug XYZ, respectively, and was more pronounced than for caffeine and paraxanthine mean AUC_{0-t} values, which also decreased substantially.
- Administration with placebo showed no notable change in caffeine or paraxanthine levels.
- On day 10, the mean caffeine clearance [dose/$AUC_{0-\infty}$] was increased by approx. threefold in subjects treated with 400-mg Drug XYZ and fivefold in subjects treated with 1,200 mg.
- All the caffeine/paraxanthine results suggest that Drug XYZ induced CYP 1A2 in humans and that the extent of induction was dependent on the dose administered.
- Given that a natural approx. 20-fold variation in CYP 1A2 activity has been reported in the literature, the observed induction was considered to be clinically irrelevant.
- The ratio of 6-β-hydroxy-cortisol/free cortisol excretion was about 11 for all treatments on both day 1 and 10, suggesting that there was no noticeable effect on CYP 3A4 activity.

Table 45.13 Mean (SD) plasma caffeine and paraxanthine pharmacokinetic variables (ng.h/mL) by treatment

Analyte variable		Treatment group		
Day	*N*	Placebo	400 mg	1,200 mg
Caffeine				
AUC_{0-t}				
Day 1 (baseline)	8	26,626.1 (8,971.9)	16,261.7 (3,908.3)	21,977.9 (4,309.7)
Day 10	8	24,996.9 (5,393.2)	6,464.0 (2,052.7)	6,580.1 (1,493.6)
$AUC_{0-\infty}$				
Day 1 (baseline)	8	43,222.8 (23,538.3)	20,585.0 (8,236.6)	33,769.3 (11,680.6)
Day 10	8	37,447.4 (14,356.4)	6,776.0 (2,326.7)	6,793.7 (1,572.8)
Paraxanthine				
AUC_{0-t}				
Day 1 (baseline)	8	8,258.1 (2,036.8)	7,430.9 (1,166.8)	7,809.7 (2,228.7)
Day 10	8	8,886.6 (2,549.1)	5,624.1 (770.9)	6,054.2 (1,192.0)

45.10 Formulation Interactions

PURPOSE/RATIONALE

The reasons why individuals differ in their responsiveness to drugs in medical products are manifold including age, gender, genetics, disease, and concomitantly administered drugs.

The focus of interaction studies has changed from ad hoc observational studies to rationally designed studies. Depending on the structural and physicochemical characteristics and on animal and human in vitro data, selective in vivo studies are performed. Based on the results of such studies, the risk of clinically relevant interactions may be predicted. As a result, essential information on formulation interactions has become an integral part of the labeling document of a drug product.

Formulation interactions can be of many different kinds, such as formulation interactions with packaging

Table 45.14 Mean (SD) ratio of urinary 6-β-hydroxy-cortisol/free cortisol excreted by treatment

Analyte variable		Treatment group		
Day	*N*	Placebo	400 mg	1,200 mg
Ratio 6-β-hydroxy-cortisol/free cortisol				
Ae_{0-12} Day 1 (baseline)	8	8.75 (2.3)	11.4 (7.9)	9.28 (3.7)
Ae_{0-24} Day 10	8	11.3 (2.0)	11.4 (4.7)	12.7 (6.1)

material (compatibilities), interactions between the active drug and excipient(s), or interactions between formulations (e.g., when mixing the drug product with other (parenteral) drug products). Details about how to deal with changes in components or composition of drug products are described in published regulatory guidances (US FDA 1995, 1997b, 1999b), while no formal guidance exists that in particular covers all types of formulation interactions. It is important to differentiate between detectable interactions and clinically relevant interactions. A clinically relevant interaction, for example, is the concomitant administration of two drugs that interact to such an extent that a dosage adjustment of either drug may be required. Details about clinically relevant (drug-drug) interactions and assessment of equivalence of formulations can be found (US FDA 2001; Steinijans and Hauschke 1997; CPMP/EWP/QWP/1401/98 2002; CPMP/EWP/560/95 1998).

Reasons for change of formulation include, but are not limited to, poor bioavailability of solid oral formulations, limitations in drug load for oral or parenteral formulations, profound food effect, too early/too late onset of action (absorption, distribution), too short/too long duration of action (metabolism, elimination), or high intra- and interindividual variability.

While formulation interactions often are subject to in vitro investigations, the chapter below presents a particular example of a clinical formulation interaction study (Frick et al. 2004): a potential interaction of a drug in medical practice frequently given concomitantly with another drug (i.e., both mixed in a syringe) was subject to a clinical study which is illustrated below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although safety and pharmacodynamic parameters were also assessed.

PROCEDURE

The design of a typical formulation interaction study is presented below.

45.10.1 Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) syringe-mixed versus simultaneously injected 0.1 IU/kg HMR1964 (insulin glulisine) and 0.2 IU/kg NPH insulin in healthy subjects using the euglycemic clamp technique.

45.10.1.1 Objective

To investigate the pharmacokinetics of insulin glulisine following subcutaneous (s.c.) administration of insulin glulisine immediately premixed in a syringe with NPH insulin versus separate simultaneous injections of insulin glulisine and NPH insulin.

45.10.1.2 Design, Treatment, and Sample Size

This was a single-dose, randomized, open-label, two-way, crossover study. 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin separate and simultaneous s.c. injections in the abdominal area or 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin by s.c. injection in the abdominal area, immediately after being premixed in the syringe.

45.10.1.3 Population Treated

Thirty-two (32) healthy male subjects, aged 18–45 years, with body mass index between 18 and 26 kg/m^2, with a minimum body weight of 65 kg were treated.

Pharmacokinetic interaction studies are suggested to be performed in healthy individuals (Schall and Williams 1996). Furthermore, it was anticipated that any interaction, should it occur, would be present in this population.

EVALUATION

45.10.1.4 Statistical Procedures Pharmacokinetics

The interpretation of the pharmacokinetic variables C_{max}, AUCs, and MRT of insulin glulisine was based on 95 % confidence intervals, after ln-transformation of the data. These 95 % confidence intervals were calculated for the respective mean ratios of pair-wise treatment comparisons. In addition, the "test" treatment was compared to the "reference" treatment with respect to the pharmacokinetic variables using an ANOVA with subject, treatment, and period effects, after ln-transformation of the data. The subject sum of squares was partitioned to give a term for sequence (treatment by period interaction) and a term for subject within sequence (a residual term). Due to the

explorative nature of the study, no adjustment of the α-level was made for the multiple testing procedure.

The time to maximum insulin glulisine concentration (T_{max}) was analyzed by nonparametric analyses. Ninety-five percent nonparametric confidence intervals for the respective median difference in treatment ("test reference") were calculated according to (Steinijans and Diletti 1983). Pair-wise treatment comparison was made for the pharmacokinetic variables.

CRITICAL ASSESSMENT OF THE METHOD

45.10.1.5 Introduction

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of Type I and Type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used to optimally control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting insulin to provide basal insulin requirements and control fasting and preprandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. These two insulins are sometimes premixed in the syringe prior to injection. HMR1964 (insulin glulisine) has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously.

45.10.1.6 Study Rationale

The purpose of the study was to compare the time concentration profiles of syringe-premixed versus simultaneously, subcutaneously injected 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin. This is intended to address the use of insulin glulisine in a basal bolus treatment regimen, when it might be injected together with NPH insulin to avoid the need for two separate injections, with the aim of determining whether mixing the two insulin preparations might compromise the rapid-acting properties of insulin glulisine.

45.10.1.7 Dosing Recommendation/ Therapeutic Dose Range

A single dose of 0.1 IU/kg HMR1964 (insulin glulisine) was chosen for this study as this dose is well within the range of recognized average prandial insulin dose in Type I and Type II patients with diabetes mellitus. Based on the primary pharmacokinetics of HMR1964 (insulin glulisine), a single-dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

45.10.1.8 Individual Bioequivalence

In contrast to the standard average bioequivalence approach for which the regulatory requirements (US FDA 2001; CPMP/EWP/QWP/1401/98 2002) have been internationally harmonized, this is not the case for the more recent concept of individual bioequivalence (Steinijans and Hauschke 1997). The main reason for introducing more complex replicate designs and bioequivalence criteria is the highly variable drugs for which the setting of suitable bioequivalence ranges poses a major problem and scaling of the bioequivalence criteria by the intrasubject variability has been suggested. The shortcoming of the present two-treatment, two-period (2×2) crossover design to detect subject-by-formulation interactions provides a second argument in favor of the more complex replicate designs. A unified approach of proposed statistical procedures for the replicate design has been given by Schall and Williams (1996).

References and Further Readings

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under *Procedure* is presented below.

Thirty-two (32) healthy male subjects with demographics as described above completed the study according to the protocol.

The insulin glulisine concentration-time profiles are reflected in the following results:

The total systemic insulin glulisine availability as presented by $AUC_{(0–clamp\ end)}$ was similar for the two modes of administration of insulin glulisine and NPH insulin, mixed in a syringe immediately before injection (treatment B) and the separate simultaneous injections (treatment A).

The maximum insulin glulisine concentration C_{max} was somewhat attenuated, being 27 % less, when insulin glulisine was premixed with NPH insulin as compared to the separate simultaneous administration. Nevertheless, the time to C_{max} did not differ between the two treatments with T_{max} values of 47 and 50 minutes (min).

The MRT of insulin glulisine was somewhat longer when insulin glulisine was premixed with NPH insulin (118 min) compared to their simultaneous administration (92 min), corresponding to an average increase of 27 %.

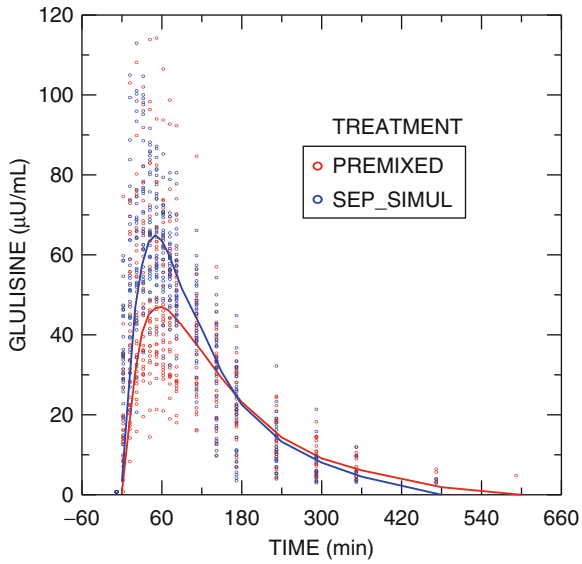

Fig. 45.5 Insulin glulisine concentration-time profiles: individual profiles (*dotted lines*) and the fitted average mean profiles (*solid line*)

In summary, the total insulin glulisine availability [AUC(0-clamp end)] was similar for the two modes of administration, and further, the time to maximum concentration [C_{max}] was not affected when insulin glulisine was mixed with NPH insulin with T_{max} values of 47 and 50 min. C_{max} was somewhat attenuated being 27 % less when insulin glulisine was immediately premixed with NPH insulin as compared to the separate simultaneous administrations of these study medications.

Overall, these data support the possibility of mixing of insulin glulisine with NPH insulin in a syringe immediately prior to administration (Fig. 45.5, Table 45.15).

45.11 Special Population: Subjects with Renal Impairment

PURPOSE/RATIONALE

A pharmacokinetic (PK) study in individuals with impaired renal function is recommended when renal impairment is likely to significantly alter the disposition of a drug/or its active metabolite(s) such that a dose adjustment may be needed. In the main, this is the case primary for drugs that are mainly eliminated (excretion and/or metabolism) by the kidneys and/or if a drug has

Table 45.15 Pharmacokinetic parameters of HMR1964 (insulin glulisine). Separate simultaneous (treatment A) versus immediately premixed (treatment B)

	Geometric mean (arithmetic mean) ($N = 32$)		Point estimate/(95 % CI)[a]
Variable	Simultaneous (A)	Premixed (B)	Premixed (B)/simultaneous (A)
AUC$_{(0\text{–clamp end})}$ (μIU.min.mL^{-1})	9,261.79 (9,401.83)	8,251.20 (8,843.44)	89.1 % (78.1; 101.6 %)
C$_{max}$ (μIU/mL)	69.98 (71.80)	51.32 (55.39)	73.3 % (64.3; 83.6 %)
MRT (min)	92 (96)	118 (121)	127.3 % (120.3; 134.7 %)
T$_{max}$ (min)	47[b]	50[b]	3.9 min (−3.3; 12.4 min)[c]

[a]Point estimates and 95 % confidence intervals for the ratio of treatment means, based on ln-transformed data
[b]Median values
[c]Point estimates and 95 % confidence intervals for the respective median differences from nonparametric data analysis

a narrow therapeutic window. However, severe renal impairment may affect the disposition of a drug through diverse mechanisms; thus, also low renal clearance drugs may significantly be affected by renal impairment.

Most drugs are cleared by elimination of unchanged drug by the kidney and/or by metabolism in the liver. For a drug eliminated primarily via renal excretory mechanisms, impaired renal function may alter its pharmacokinetics (and pharmacodynamics) to an extent that the dosage regimen needs to be changed from that used in patients with normal renal function.

A study also should be considered when a drug or an active metabolite exhibits a combination of high hepatic clearance (relative to hepatic blood flow) and significant plasma protein binding. In this setting, renal impairment could induce a significant increase in the unbound concentrations after parenteral administration due to a decreased plasma protein binding coupled with little or no change in the total clearance (decrease in unbound clearance).

More details about when such studies may be or may not be important, and other aspects as study design and methods, can be found in (EMEA CHMP 2004; US FDA 1998). Although the most obvious type of change arising from renal impairment is a decrease in renal excretion, or possibly renal metabolism of a drug or its metabolites, renal impairment has also been associated with other changes, such as changes in absorption, hepatic metabolism, plasma protein binding, and drug distribution. These changes may be particularly prominent in patients with severely impaired renal function and have been observed even when the renal route is not the primary route of elimination of a drug. Thus, for most drugs that are likely to be administered to patients with renal impairment, pharmacokinetic characterization should be assessed in patients with renal impairment to provide rational dosing recommendations. It may not be feasible to conduct the study in patients with the condition for the intended use of the drug. An acceptable alternative is to use otherwise healthy subjects with different degrees of renal function. It may also be necessary to study the influence of dialysis on the pharmacokinetics of a drug. In this case, it is recommended to study this end-stage-renal-diseased subgroup separately from the renally impaired individuals, as different types of clinical study protocols are suggested to be applied for those undergoing dialysis and those who are not. Renally impaired individuals not undergoing dialysis are subject to the example that is discussed below.

It is also key to distinguish between *explorative* studies conducted during the investigational phase before the confirmatory safety and efficacy trials, aimed to give appropriate dosage recommendations for the patients with reduced renal function and *confirmative* studies (usually conducted in parallel to the confirmatory safety and efficacy trials) when assessing the influence of renal impairment on the pharmacokinetics of a drug, as, for example, study design and timing of study conduct may be different.

In summary, the primary goal of a study in individuals with impaired renal function is to determine whether the pharmacokinetics are altered to such an extent that the dose and/or dosing regimen of a drug should be adjusted from that established in the confirmatory safety and efficacy trials.

The following chapters present a particular study in renally impaired individuals with result illustration and conclusion. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although safety parameters were also studied.

PROCEDURE

The design of a typical renal impairment study is presented below.

45.11.1 Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) after subcutaneous injection of a single dose of 0.15 IU/kg in nondiabetic subjects with different degrees of renal function in an open, parallel-group, single-dose, multicenter study.

45.11.1.1 Objective

To investigate the pharmacokinetics of HMR1964 (insulin glulisine) in nondiabetic subjects with different degrees of renal function.

45.11.1.2 Design, Population, Treatment

Single-dose, open, three-parallel-group, study in nondiabetic subjects with different degrees of renal function (classified according to US FDA 1998). The renal function groups consisted of 8 individuals each and were comparable to each other with respect to age, gender, and weight. The creatinine clearance (CL_{cr}) was the measures of renal function.

Group 1 (normal renal function): $CL_{cr} > 80$ mL/min

Group 3 (moderate renal impairment): CL_{cr} 30–50 mL/min

Group 4 (severe renal impairment): $CL_{cr} < 30$ mL/min, but not requiring hemodialysis.

Individuals were allocated to these renal function groups based on the weighted average of the two pretreatment CL_{cr} values. Individuals received a single dose (0.15 IU/kg body weight) of HMR1964 (insulin glulisine) subcutaneously in the periumbilical abdomen. Blood for determinations of insulin in serum were taken according to the sampling schedule.

45.11.1.3 Inclusion Criteria

Nondiabetic men or women, aged between 18 and 75 years. The renally compromised individuals had to show a reasonably stable renal function in the previous 3 months (e.g., maximum change in CL_{cr} of approximately 15 mL/min, determined by urine and/or serum). Women either postmenopausal, surgically sterilized, or not pregnant and using adequate contraception.

EVALUATION

45.11.2 Statistical Procedures Pharmacokinetics

The relationship between renal impairment and the absorption and disposition of HMR1964 (insulin glulisine) was assessed by regressing pharmacokinetic parameters onto CL_{cr}. Regression parameter estimates ($\pm$ standard error) with confidence intervals and coefficients of correlation (Pearson) with p-values for test of difference from zero were reported. Scatter plots of the concentration-time profiles and pharmacokinetic parameters against creatinine clearance were produced.

Statistical methods were applied to the natural log (ln)-transformed pharmacokinetic parameters, except for T_{max} on which statistical methods on raw data were applied.

CRITICAL ASSESSMENT OF THE METHOD

45.11.2.1 Introduction

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of Type I and Type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used to optimally control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting insulin to provide basal insulin requirements and control fasting and preprandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. HMR1964 (insulin glulisine) has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously (Frick et al. 2003).

Preclinical studies suggest HMR1964 (insulin glulisine) does not differ from the established elimination pathways of endogenous human insulin.

45.11.2.2 Study Rationale

The purpose of this study was to characterize the impact of renal impairment on the absorption and disposition of HMR1964 (insulin glulisine) in nondiabetic subjects with different degrees of renal

function. A control group with normal renal function and similar demographics to the renally impaired subjects was included in this study, as suggested by the CPMP and FDA Guidance for Industry (EMEA CHMP 2004; US FDA 1998). This study was conducted after the investigational phase in parallel to the confirmatory safety and efficacy trials.

45.11.2.3 Population

In order to cover a wide range of renal function, individuals from renal function groups 1, 3, and 4 were enrolled. Individuals with mild impairment (group 2) are often much more difficult to recruit for phase I studies, as mostly not yet diagnosed. Yet those patients can be found in phase II/III studies quite frequently, allowing a population PK approach for mild renal impairment. As described above, individuals with end-stage renal disease are recommended to be studied separately.

For adequate representation of individuals with various degrees of renal impairment, equal numbers of individuals from each renal function group were recruited, and the renally compromised individuals had to show a reasonably stable renal function in the previous 3 months (e.g., maximum change in CL_{cr} of approximately 15 mL/min, determined by urine and/or serum) before study starts. This study followed an alternative approach (EMEA CHMP 2004; US FDA 1998) and used a nonpatient (i.e., otherwise healthy) population with different degrees of renal function with comparable demographic factors such as weight, age, and gender. Individuals were matched for body mass index (BMI) and age between the three renal function groups (BMI matched ±20 % between renal function groups, and with age, between 18 and 75 years, matched ±20 years between renal function groups) and a similar number of men and women were enrolled in each renal function group.

45.11.2.4 Dosing Recommendation/ Therapeutic Dose Range

A single dose of 0.15 IU/kg HMR1964 (insulin glulisine) was chosen for the individuals with renal impairment as this is a well-recognized average prandial insulin dose in Type I and Type II patients with diabetes mellitus. Based on the primary pharmacokinetics of HMR1964 (insulin glulisine), a single-dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

45.11.2.5 Reduced/Staged Study Design

In a reduced design, individuals at the extremes of renal function (normal and severely impaired individuals) are studied. The aim of such a study is to confirm that the pharmacokinetics is not altered to a clinically relevant extent. A prerequisite to this approach is a good reason to believe that renal impairment does not affect the PK to a degree sufficient to warrant dosage adjustment.

45.11.2.6 Full Study Design

In specific situations, it makes sense to study the PK also in groups with mild renal impairment, which as compared to the presented example would cover a GFR range of 50–80 mL/min.

45.11.2.7 Population PK

A population PK evaluation of patients from the safety and efficacy trials can be used to assess the impact of renal function on the disposition of a drug. Special care must be taken that patients with severe renal impairment are adequately represented in the population. The population PK approach assesses the impact of various covariates on the disposition of a drug. Nonlinear mixed effects modeling may be used to model the relationship between various covariates and pharmacokinetic parameters. CL_{cr} as a measure of renal function may be one of the covariates. This type of approach has its advantages as it involves assessment of the effect of renal impairment on the PK in the target population.

45.11.2.8 End-Stage-Renal-Diseased Individuals

Independently of a drug being eliminated by the renal route or not, the dialysis process can significantly alter the PK of a drug. Once significant fractions of a drug are removed by dialysis, the dosing regimen may need to be changed (e.g., supplementary dosing during/after dialysis). A study in individuals with end-stage renal disease also provides information about the value of dialysis as a countermeasure in case of overdose.

45.11.2.9 Crossover Study Design

In case of two study medications, a sound alternative to a parallel design would be the (complete) crossover study design.

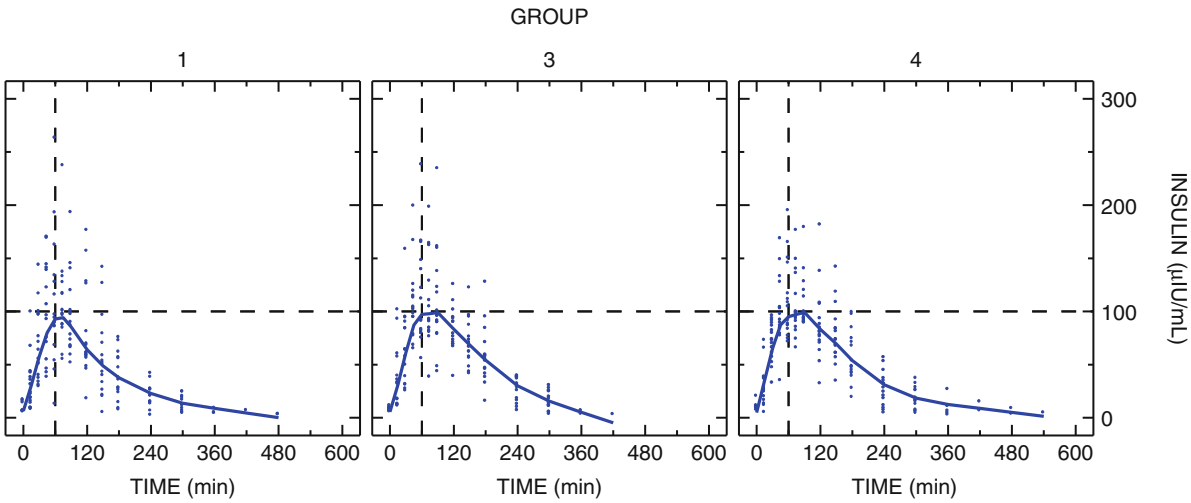

Fig. 45.6 Average insulin glulisine concentration-time profiles of renal function groups 1, 3, 4

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the PK results obtained from the study described above under *Procedure* is presented below.

A population as described above completed the study according to the protocol.

Table 45.16 Pharmacokinetics of 0.15 IU/kg insulin glulisine in subjects with renal impairment

	Geometric mean (CV %)		
	Normal ($n = 8$)	Moderate ($n = 8$)	Severe ($n = 8$)
$AUC_{(0–end)}$ (µIU.min.mL^{-1})	13,215 (31)	18,473 (19)	17,650 (19)
$AUC_{(0–5\ h)}$ (µIU.min.mL^{-1})	13,120 (29)	18,412 (19)	16,912 (16)
$AUC_{(0–2\ h)}$ (µIU.min.mL)	9,005 (24)	11,626 (26)	9,622 (17)
CL_{tot}/F (mL/min)	852 (20)	637 (20)	680 (14)
C_{max} (µIU/mL)	108 (30)	131 (29)	108 (15)
T_{max} (min)[a]	56 (29)	58 (18)	68 (22)

[a]Median values

Note: The apparent relative total clearance was calculated as $CL_{tot}/F = dose/AUC_{(0–5\ h)}$

There was no apparent relationship between PK parameters and degree of renal function, as there were no obvious differences in insulin concentration-time profiles (Fig. 45.6) between the three renal function groups. The PK properties of HMR1964 (insulin glulisine) were similar in subjects with decreased renal function covering a wide range of renal impairment.

In detail, there were no statistically significant correlations (Table 45.16) between renal function as measured by CL_{cr} and the parameters characterizing the rapid-acting properties of HMR1964 (insulin glulisine), $AUC_{(0–2\ h)}$, C_{max}, and T_{max} (Table 45.16). There were weak, though statistically significant,

correlations between renal function and parameters characterizing total exposure ($AUC_{(0–end)}$ and $AUC_{(0–5\ h)}$) and relative total clearance of HMR1964 (insulin glulisine) (CL_{tot}/F).

The predicted changes, which are within the conventional equivalence bounds, are too small to suggest any meaningful clinical consequences.

In conclusion, the rapid-acting insulin, HMR1964 (insulin glulisine), maintains its pharmacokinetic properties in subjects with decreased renal function covering a wide range of renal impairment (Jaros et al. 2004a, b).

As a consequence, no dose adjustments in patients with renal failure are warranted on the basis of intrinsic properties of HMR1964 (insulin glulisine).

45.12 Special Population: Subjects with Hepatic Impairment

PURPOSE/RATIONALE

A pharmacokinetic study in individuals with impaired hepatic function is recommended when hepatic impairment is likely to significantly alter the pharmacokinetics of a drug/or its metabolite(s) such that a dose adjustment may be required.

In liver disease, the likelihood of a pharmacokinetically relevant drug-disease interaction depends on the type of drug (extent of intra-/extrahepatic elimination, hepatic extraction ratio, protein binding) and the nature (flow, enzymatic capacity/reserve) and extent of the liver dysfunction. The likelihood of a pharmacokinetic relevant interaction with the need for dosage adjustment in individuals with impaired hepatic function depends on the changes in systemic exposure.

The majority of drugs is cleared hepatically through a variety of oxidative and conjugative metabolic pathways and/or through biliary excretion of unchanged drug or metabolites. In the main, alterations of these excretory and metabolic activities by hepatic impairment can lead to drug accumulation or, less often, to a failure to form metabolite(s). Liver disease may also alter kidney function, which can lead to accumulation of a drug and its metabolites even when the liver is not primarily responsible for elimination. Moreover, the specific impact of any disease on hepatic function is often poorly described and highly variable, particularly with regard to effects on the pharmacokinetics of a drug.

Hence, a study in individuals with impaired hepatic function may also be recommended if the liver is not the major organ for elimination, or if the drug has a narrow therapeutic window, or in case the metabolism is unknown and other information is missing.

It is also central to distinguish between explorative studies (conducted during the investigational phase before the confirmatory safety and efficacy trials, aimed to give dosage recommendations for patients with reduced hepatic function which otherwise would be treated with the planned clinical dose for patients with normal hepatic function) and confirmative studies (usually conducted in parallel to the confirmatory safety and efficacy trials) when assessing the influence of hepatic impairment on the pharmacokinetics of a drug, as, for example, study design and timing of study conduct may be different.

Measurements such as creatinine clearance have been used successfully to adjust dosing regimens for drugs eliminated primarily by the kidneys. Measures of hepatic function have been sought using endogenous substances affected by the liver, such as bilirubin and albumin, or functional measures such as prothrombin time, or the ability of the liver to eliminate marker substrates such as indocyanine green or galactose (Figg et al. 1995; Tang and Hu 1992). Clinical variables such as ascites or encephalopathy, nutritional status, peripheral edema, and histological evidence of fibrosis have also been used to categorize hepatic impairment (Albers et al. 1989; Pugh et al. 1973; Figg et al. 1995; Tang and Hu 1992; Zakim and Boyer 1996; Dickson et al. 1989; Wiesner et al. 1989; Maddrey et al. 1998; Carithers et al. 1978). Despite extensive efforts, no single measure or group of measures has gained broad clinical use. Yet, the most widely used scheme system to classify the degree of hepatic impairment is the Child-Pugh system (Albers et al. 1989; Pugh et al. 1973; Dickson et al. 1989).

More details about when such studies may or may not be important, and other aspects as study design and methods, can be found in (EMEA CHMP 2005; US 2003d).

In summary, a pharmacokinetic study in individuals with impaired hepatic function is recommended if the extent of hepatic metabolism is unknown, the hepatic metabolism/excretion accounts for >20% of the elimination of parent drug or metabolite(s), and for drugs with a narrow therapeutic window. The primary goal of such a study is to determine if the pharmacokinetic

is altered to an extent that the dose and/or dosing regimen of a drug should be adjusted from that established in the confirmatory safety and efficacy trials.

The following presents a particular clinical study with results and conclusions that was performed in two groups of individuals, hepatic impaired patients and healthy subjects.

For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic (PK) data although clearly safety parameters are also studied.

PROCEDURE

The design of a typical hepatic impairment study is presented below.

45.12.1 Protocol Outline

To investigate the pharmacokinetics of XYZ123 in individuals with hepatic impairment in comparison with those in healthy subjects after multiple oral administration of 800-mg XYZ123 once a day for 7 days in an open, multicenter study.

45.12.1.1 Design, Population, Treatment

Open, nonrandomized, multicenter, repeated-dose study in two groups of individuals. Twelve (12) patients with hepatic impairment with a Child-Pugh score ≥ 5 and ≤ 14 (Table 45.17) and 12 healthy subjects matching for age, weight, and sex. Individuals received a repeated oral administration of 800-mg XYZ123 once a day for 7 days after a standard meal. Samples were collected according to the sampling schedule for determinations of XYZ123 and of its main metabolite XYZ456 in plasma and XYZ123 in urine. The unbound fraction of XYZ123 in plasma was determined by ultra filtration.

45.12.1.2 Inclusion Criteria

Patients with hepatic impairment: Twelve patients with hepatic impairment with a Child-Pugh score ≥ 5 and ≤ 14 (Table 45.17).

Healthy subjects: Twelve healthy subjects with approximately similar range of age and weight, and sex matched, were recruited in the same center as the patient he/she was matching. Age, weight, and sex were matched to those of the patient (individual age within 5 years, weight within 20 % of the matching patient). Women either postmenopausal, surgically sterilized, or not pregnant and using adequate contraception.

Table 45.17 The Child-Pugh classification (Albers et al. 1989; Pugh et al. 1973)

Assessment	Degree of abnormality	Score
Encephalopathy	None	1
	Moderate	2
	Severe	3
Ascites	Absent	1
	Slight	2
	Moderate	3
Bilirubin (mg/dL)	<2	1
	2.1–3	2
	>3	3
Albumin (g/dL)	>3.5	1
	2.8–3.5	2
	<2.8	3
Prothrombin time (seconds > control)	0–3.9	1
	4–6	2
	>6	3
Total score	Group	Severity
5–6	A	Mild
7–9	B	Moderate
10–15	C	Severe

EVALUATION

45.12.1.3 Statistical Procedures: Effect of Hepatic Impairment on PK Parameters

To compare PK parameters of XYZ123 and XYZ456 between the two groups, a one-way analysis of variance was used with group (patients with hepatic impairment and healthy subjects) as main effect in the model, after natural logarithmic transformation of the following pharmacokinetic parameters: C_{max}, AUC(0–24 h) on day 1 and day 7 and $t_{1/2,\lambda z}$ (on day 7 only) for XYZ123 and XYZ456 Ae(0–24 h), CL_R, $t_{1/2,\lambda 1}$ and fu for XYZ123 on day 1 and day 7, and Rac for XYZ123. The ratio of the adjusted means (patients with hepatic impairment/healthy subjects) was obtained by calculating the exponential of the difference of the adjusted means of natural log–transformed parameters. Ninety percent confidence intervals (CI 90 %) of the ratio were constructed using the mean square error (MSE) of the analysis of

variance. For the discrete variable t_{max}, the effect of hepatic impairment was assessed using Kruskall-Wallis nonparametric test.

Plots of pharmacokinetic parameters (C_{max}, AUC of XYZ123 and XZY456, CL_R of XYZ123) versus Child-Pugh score and treatment day were drawn.

CRITICAL ASSESSMENT OF THE METHOD

45.12.1.4 Background to the Example

Drug XYZ123 is rapidly and almost completely absorbed after oral administration. There is a substantial first pass effect, and the oral bioavailability after administration of the tablet is around 60 %. Food does not modify the bioavailability of XYZ123 tablet. In humans, XYZ123 is eliminated mainly by metabolism (70 % of the dose), the metabolites being mainly excreted in feces, and is also excreted unchanged in the urine (13 %) and feces (7 %). Some circulating metabolites have been identified: most of them are present at low concentrations with an AUC representing 2–13 % of that of XYZ123. The metabolites are not likely to contribute to the pharmacological effect. CYP3A4 is partially involved in the metabolism of XYZ123.

45.12.1.5 Study Rationale

The steady-state pharmacokinetics of XYZ123 are not predicted from single dose data, since XYZ123 pharmacokinetics exhibit some moderate time dependency. In healthy subjects a 1.5-fold accumulation was observed which was not predicted from single-dose data. The dosing regimen was based on the therapeutic dose (800 mg twice a day) with a 7-day duration being well within the duration of therapeutic treatment which varies from 5 to 10 days. Given the modest pharmacokinetic changes observed in phase I studies after single-dose administration, the exposure reached after repeated-dose administration should not be much higher than those obtained in healthy subjects and should be in the range of the well-tolerated exposure.

Thus, a multiple-dose study was performed in which all healthy and hepatic impaired individuals received the same dose. It was the aim to include 12 patients with various and well-distributed degrees of hepatic impairment (according to the Child-Pugh score) and 12 pair-matched (based on demographic characteristics) healthy subjects in order to have 10 patients and 10 subjects evaluable. The pharmacokinetics of XYZ123 in plasma (total and unbound) and in urine was assessed after the 1st dose and at steady state after the seventh dose. The pharmacokinetics in plasma of its main metabolite XYZ456 was also assessed.

MODIFICATIONS OF THE METHOD

45.12.1.6 Reduced Study Design

As individuals from the Child-Pugh category with mild impairment could have a normal hepatic function and for the majority of drugs, clinically insignificant differences are more likely to be observed in individuals with moderate and severe impairment, in a reduced design, just individuals with moderate hepatic impairment in combination with a control group are investigated. As a consequence of such a design, the findings in the moderate category would be applied to individuals with a mild Child-Pugh category, and dosing in the severe category would generally be a contraindication in the labeling document.

45.12.1.7 Population PK

A population PK evaluation of patients from the safety and efficacy trials can be used to assess the impact of altered hepatic function (as a covariate) on the PK of a drug. In each of the Child-Pugh category patients should adequately be represented, such that the population PK approach sound evaluates the impact of various covariates on the PK of the drug. Nonlinear mixed effects modeling may be used to model the relationship between various covariates and PK parameters. This type of approach has its advantages as it involves assessment of the effect of hepatic impairment on the PK in the target population. However, this approach may prove difficult in hepatic impairment due to the low prevalence of hepatic disease in the general population.

45.12.1.8 Statistical Procedures PK

Despite the fact that it is difficult to define a relationship between PK parameters and measures of hepatic function, the most appropriate statistical approach is to calculate geometric means and 95 % confidence intervals to compare the healthy and impaired groups (see "Example"). Investigation of the relationships between hepatic functional abnormalities and selected PK

parameters using linear and nonlinear models in order to derive dose recommendations is an appropriate alternative, yet, in spite of many constraints.

45.12.1.9 PK Results and Association with Measures of Hepatic Function

Past experience indicates that it has been difficult to develop a measure or group of measures of hepatic function that predict alterations in drug PK as the grounds for hepatic impairment are manifold (e.g., impaired hepatocellular function, impaired biliary excretion, decreased protein binding, consequences of shunting of blood passing the liver).

In contrast to renal impairment, no obvious marker exists for characterizing hepatic function with respect to predictions of drug elimination capacity. Therefore, dose recommendations may not be as accurate for hepatic impairment as for renal impairment.

Nonetheless, relationships between hepatic functional abnormalities (e.g., hepatic blood flow, serum albumin concentration, prothrombin time, or overall impairment scores such as Child-Pugh) and selected pharmacokinetic parameters (e.g., total body clearance, oral clearance, apparent volume of distribution, unbound clearance, or dose-normalized area under the unbound concentration-time curve) can also be should using linear and nonlinear models. A regression approach for continuous variables describing hepatic impairment and pharmacokinetic parameters is appropriate, with the understanding that some correlations will rely on categorical variables (e.g., Child-Pugh). Typically, modeling results would include parameter estimates of the chosen model and measures of their precision (standard errors or confidence intervals). Prediction error estimates are also desirable to assess appropriateness of the model.

45.12.1.10 Classification of Hepatic Impairment

An alternative to ensure that the individuals have an impaired metabolic capacity is to administer a metabolic probe (e.g., a CYP3A4 probe if the drug under investigation is a CYP3A4 substrate) to evaluate if the pharmacokinetics of the drug is altered.

Exogenous markers that have been used to assess different hepatic drug elimination mechanisms are, for example, antipyrine (Figg et al. 1995) and MEGX (lidocaine metabolite) (Testa et al. 1997), markers for the functional ability of the cytochrome P-450 oxidative pathway, or ICG (indocyanine blue) (Figg et al. 1995), a markers for hepatic blood flow, and galactose (Tang and Hu 1992). Such markers may be used in parallel to the Child-Pugh classification.

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the PK results obtained from the study described above under *Procedure* is presented below.

A population as described above completed the study according to the protocol (Tables 45.18, 45.19).

Table 45.18 Demography

Results—Study subjects and conduct			
Demography, creatinine clearance and Child Pugh score			
Parameter	Statistics	Patients with hepatic impairment	Healthy subjects
Number of subjects	N	13	13
Sex	Male	11	11
	Female	2	2
Age (years)	Mean [Min–Max]	52.2 [38–73]	51.8 [35–74]
Weight (kg)	Mean [Min–Max]	74.4 [54.5–104.7]	75.3 [62.2–101.0]
Height (cm)	Mean [Min–Max]	166.3 [152.4–180.3]	171.9 [154.9–184.0]
Creatinine clearance: (mL/min)	Mean [Min–Max]	112 [71–152]	93 [68–133]
Child Pugh score	Median [Min–Max]	7 [5–11]	NA

NA not applicable

Table 45.19 Child-Pugh score and grade of hepatic impairment

Grade of hepatic impairment	A	B	C
Child-Pugh Score	5–6	7–9	10–15
Number of subjects	4	6	3
Observed Child–Pugh score [min–max]	5–6	7–9	10–11

min minimum, *max* maximum

The demographic characteristics were similar in both groups (Table 45.18). The median Child-Pugh score of patients with hepatic impairment was 7 with a range of 5–11 (Table 45.19).

Steady state was achieved after 2 days of dosing.

Figures 45.7 and 45.8 shows the mean plasma concentration of XYZ123 (Fig. 45.7) and of XYZ456 (Fig. 45.8) after single and repeated doses of XYZ123 (800 mg) once a day. The PK parameters of XYZ123 (Table 45.20) show that:

After single and repeated doses of XYZ123, mean C_{max} and AUC were similar in both groups. Maximum values observed in patients were similar or smaller than those observed in healthy subjects. Between subject variability (CV) was around 30 % for both groups except for patients on day 7 where it was lower with 20 %.

After single and repeated doses of XYZ123, the main elimination half-life ($t_{1/2,\lambda 1}$) and the terminal elimination half-life ($t_{1/2,\lambda z}$) (after repeated doses) were similar in both groups, as well as the between subject variability which was around 20 %.

After single dose of XYZ123, there was a 27 % and 25 % increase in Ae(0–24 h) and renal clearance, respectively, in patients compared to healthy subjects. These increases were not statistically significant probably due to the high between subject variability (around 50 %) observed in patients.

At steady state, the difference between both groups was less marked with 10 and 16 % increases in Ae (0–24 h) and renal clearance when comparing patients to healthy subjects, again with values not statistically different between groups and a between subject variability still around 50 % in patients.

The accumulation ratio (Rac) was similar in both groups with the between subject variability in patients (43 %) higher than in healthy subjects (16 %).

The results showed that whatever the group, there was no statistically significant difference in unbound fraction of XYZ123 whether it was measured at t_{max} or $t_{12\ h}$. Since there was no time effect, the mean unbound fraction (fu) was calculated from fu C_{max} and fu $C_{12\ h}$ on day 1 and on day 7 for both groups.

Figure 45.9 indicates that in patients with hepatic impairment there was no trend toward any relationships between XYZ123 pharmacokinetic parameters and the Child-Pugh score.

The pharmacokinetic parameters of XYZ456 are summarized in Table 45.21 and show that:

After single and repeated doses of XYZ123, mean C_{max} and AUC of XYZ456 were approximately twice as low as in patients compared to healthy subjects. These differences were statistically significant. Between subject variability ranged from 50 % to 60 % in patients and from 20 % to 30 % in healthy subjects.

The terminal elimination half-life was 20 % lower in patients compared to healthy subjects. Probably the elimination half-life was not accurately determined in patients as XYZ456 concentrations were close to the lower limit of quantification. Thus, as the elimination has a biphasic profile, for patients, this elimination half-life probably corresponds to a mix of $t_{1/2,\lambda 1}$ and $t_{1/2,\lambda z}$.

Figure 45.10 indicates that in patients with hepatic impairment there was no trend toward any relationships between XYZ456 pharmacokinetic parameters and the Child-Pugh score.

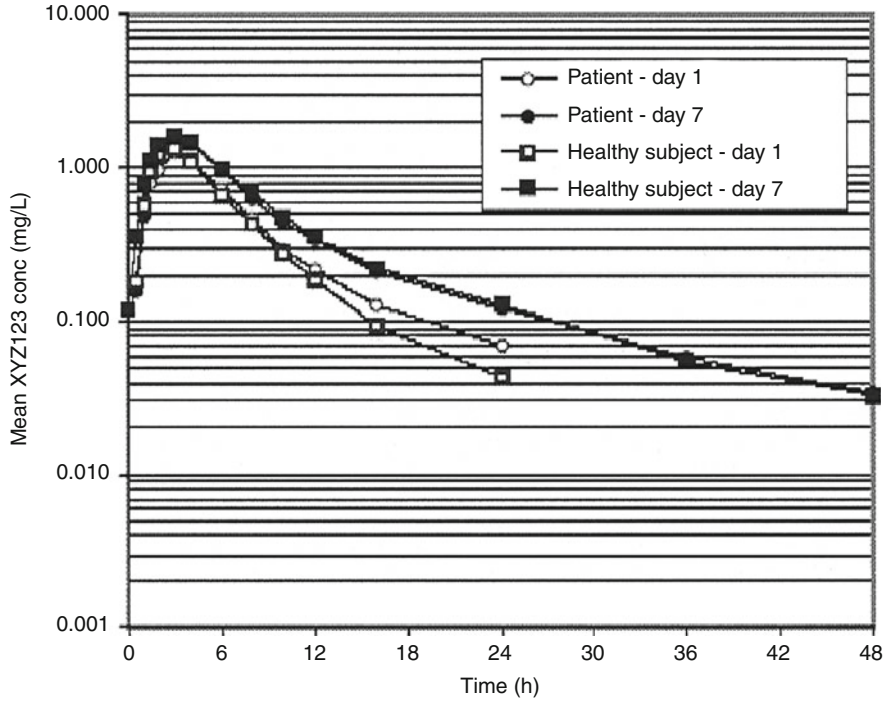


Fig. 45.7 Mean plasma concentration of XYZ123 after single and repeated doses of XYZ123 (800 mg) once a day

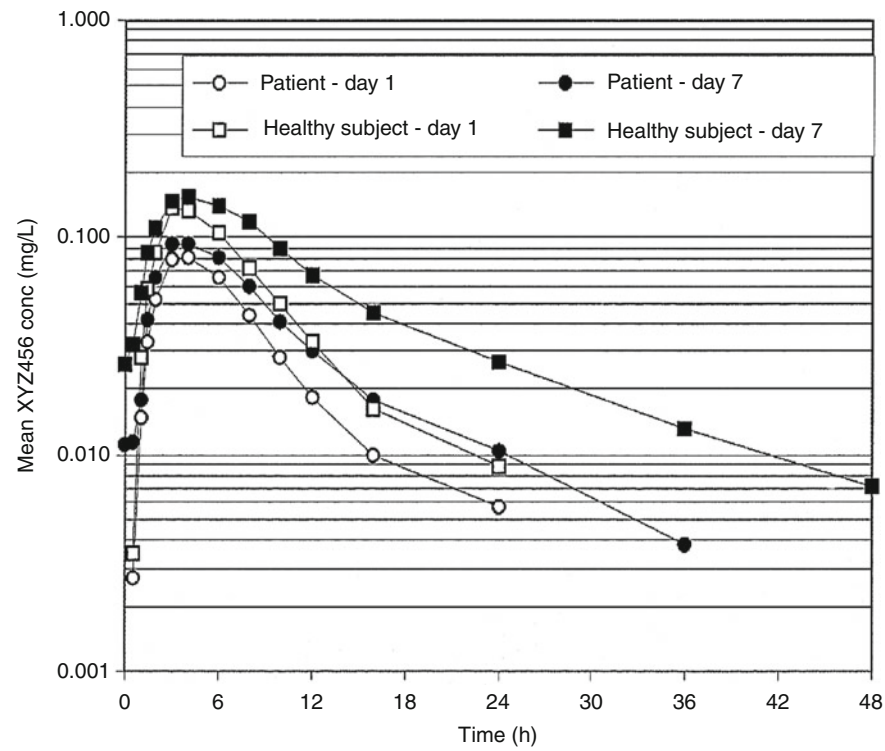


Fig. 45.8 Mean plasma concentration of XYZ456 after single and repeated doses of XYZ123 (800 mg) once a day

Table 45.20 Pharmacokinetic parameters—XYZ123

Parameter	Statistics	Patients with hepatic impairment ($n = 13$)		Healthy subjects ($n = 13$)		Ratio[a] [90 CI (%)] ANOVA[b]	
		Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
C_{max} (mg/L)	**Mean** (CV %) [Min–Max]	**1.54** (33) [0.48–2.52]	**1.80** (23) [1.26–2.49]	**1.73** (32) [0.78–2.66]	**1.92** (30) [0.98–2.99]	**87** [67–113] NS	**96** [79–116] NS
t_{max} (h)	**Median** [Min–Max]	**3.0** [0.5–4.0]	**2.0** [1.5–6.0]	**1.5** [0.5–4.0]	**3.0** [0.5–6.0]	NS	NS
$AUC_{(0–24\ h)}$ (mg.h/L)	**Mean** (CV %) [Min–Max]	**9.11** (36) [4.18–15.60]	**12.43** (20) [8.35–15.93]	**8.79** (32) [5.44–14.96]	**13.26** (27) [8.37–20.17]	**102** [81–128] NS	**95** [81–111] NS
C24 h (mg/L)	**Mean** (CV %) [Min–Max]	**0.069** (82) [0.021–0.243]	**0.122** (41) [0.050–0.220]	**0.044** (43) [0.015–0.076]	**0.127** (45) [0.055–0.254]	–	–
$t_{1/2,\lambda1}$ (h)	**Mean** (CV %) [Min–Max]	**2.94** (23) [1.99–4.41]	**3.89** (21) [2.56–5.33]	**2.80** (21) [1.69–4.00]	**3.84** (22) [2.51–5.51]	**105** [90–122] NS	**101** [88–117] NS
$t_{1/2,\lambda z}$ (h)	**Mean** (CV %) [Min–Max]	–	**11.94** (21) [8.63–16.27]	–	**11.04** (20) [8.80–15.75]	–	**108** [94–123] NS
fu[c] (%)	**Mean** (CV %) [Min–Max]	**23.4** (21) [14.2–32.6]	**24.5** (26) [15.1–34.3]	**20.8** (19) [16.4–28.8]	**21.0** (22) [15.6–32.2]	**112** [97–128] NS	**115** [98–135] NS
$Ae_{(0–24\ h)}$ (% dose)	**Mean** (CV %) [Min–Max]	**16.0** (55) [6.3–30.7]	**22.6** (49) [9.6–50.4]	**11.6** (35) [5.2–20.2]	**19.2** (27) [11.5–31.2]	**127** [93–173] NS	**110** [86–142] NS
CL_R (L/h)	**Mean** (CV %) [Min–Max]	**14.6** (48) [4.8–28.4]	**14.8** (45) [5.9–27.8]	**10.66** (20) [6.82–14.24]	**11.69** (15) [9.20–14.45]	**125** [96–161] NS	**116** [92–147] NS
Rac[d]	**GMean** (CV %) [Min–Max]	**1.43** (43) [0.85–3.25]		**1.53** (16) [1.20–2.06]		**93** [77–113] NS	

[a]Ratio of the adjusted means (patients with hepatic impairment/healthy subjects)
[b]Significance of p-value from ANOVA table; NS: non significant, $^{*}0.01 < p \leq 0.05$, $^{**}0.001 < p \leq 0.01$, $^{***}p \leq 0.001$
[c]fu = (fu C_{max} + fu C12 h)/2
[d]Rac = AUC(0–24 h)–day 7/AUC(0–24 h)–day 1

In summary, the plasma pharmacokinetic profile of XYZ123 is similar to that achieved in healthy subjects with an alteration in one of the XYZ123 elimination pathways in patients with hepatic impairment. This was shown by a marked decrease in XYZ456 metabolite formation, though there was no clear relation between the extent of the decrease and the severity of hepatic impairment. There is a trend for a slight increase in XYZ123 renal clearance with no clear relation between the extent in renal clearance increase and severity of hepatic impairment. Furthermore, it is unlikely that the decrease in XYZ456 formation could modify the overall pharmacological activity as this metabolite is known not to contribute to a clinically relevant extent.

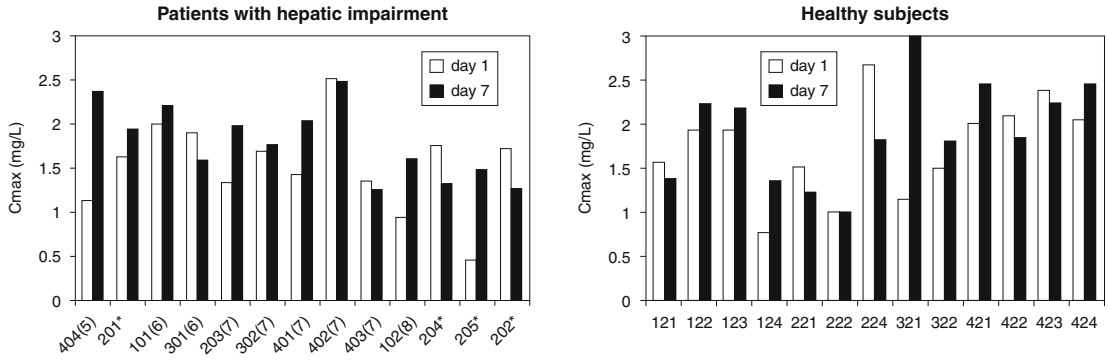
In conclusion, from a pharmacokinetic point of view, the dosage regimen of 800-mg XYZ123 once daily should not be modified in patients with hepatic impairment, provided that the renal function is not severely impaired.

45.13 Special Populations: Profiling the Effect of Obesity on Drug Disposition

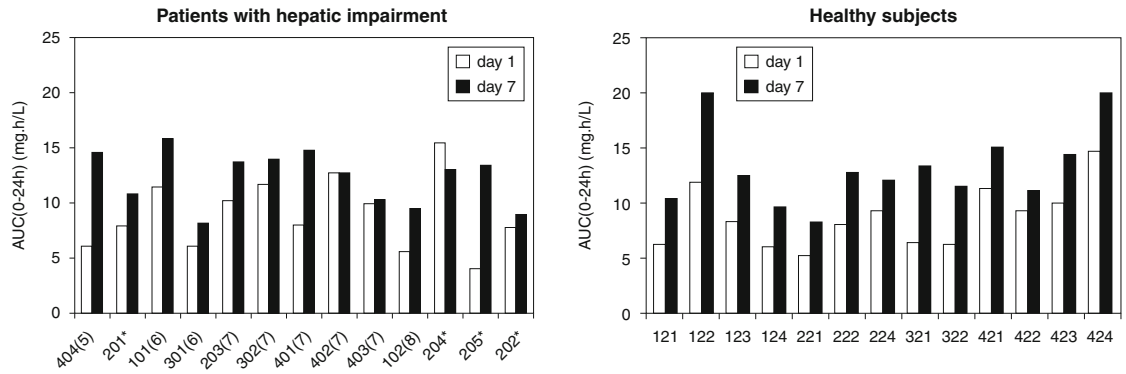
PURPOSE/RATIONALE

Regulatory guidance requires that the pharmacokinetics and tolerability of a candidate drug are studied in the range of populations likely to receive the drug during the clinical development and later, once the drug is marketed (US FDA 1978). Over and above this regulatory need, in some cases, an early switch to using special populations can be advantageous to a clinical development.

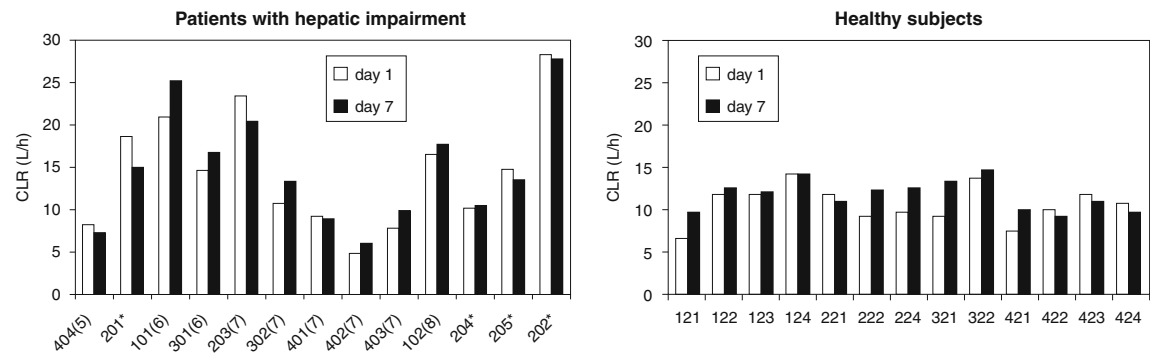
Obesity is associated with physiological changes that can alter the pharmacokinetic parameters of many drugs (Blouin et al. 1987). For example, increases in the apparent volume of distribution and total body clearance have been reported for the antibacterial agents vancomycin and



For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10



For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10



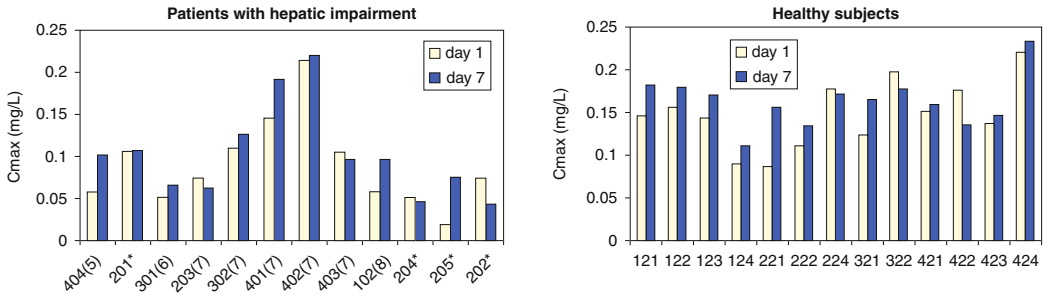
For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10

Fig. 45.9 Individual XYZ123 pharmacokinetic parameters after single and repeated oral doses of XYZ123 (800 mg) once a day

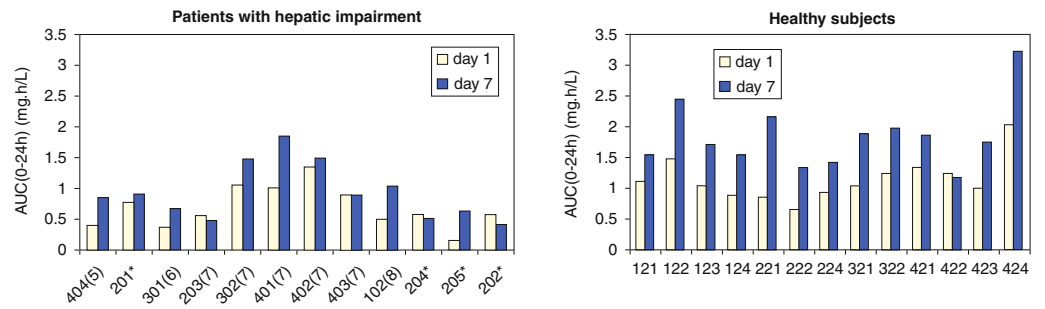
Table 45.21 Pharmacokinetic parameters—XYZ456

Parameter	Statistics	Patients with hepatic impairment ($n = 12$[a])		Healthy subjects ($n = 13$)		Ratio[a] [90 CI (%)] ANOVA[b]	
		Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
$\mathbf{C_{max}}$ (mg/L)	**Mean** (CV %) [Min–Max]	**0.089** (59) [0.018–0.214]	**0.103** (53) [0.043–0.220]	**0.148** (27) [0.087–0.220]	**0.163** (18) [0.111–0.233]	**53** [38–73]**	**57** [44–73]***
$\mathbf{t_{max}}$ (h)	**Median** [Min–Max]	**3.0** [2.0–6.0]	**3.5** [2.0–6.0]	**4.0** [2.0–6.0]	**4.0** [1.5–6.0]	NS	NS
$\mathbf{AUC_{(0–24\ h)}}$ (mg.h/L)	**Mean** (CV %) [Min–Max]	**0.679** (51) [0.138–1.344]	**0.93** (49) [0.41–1.85]	**1.136** (30) [0.661–2.021]	**1.83** (29) [1.16–3.21]	**54** [39–74]**	**47** [36–61]***
$\mathbf{t_{1/2,\lambda z}}$ (h)	**Mean** (CV %) [Min–Max]	–	**9.5** (43) [5.3–18.0]	–	**11.9** (48) [5.3–26.3]	–	**80** [60–108] (NS)

[a]Blood samples not withdrawn for (XYZ456) assay for subject No. 101 (for safety reason linked to hepatic impairment)
[b]Ratio of the adjusted means (patients with hepatic impairment/healthy subjects)
[c]Significance of p-value form ANOVA table. NS: nonsignificant, *$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$



For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10



For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10

Fig. 45.10 Individual XYZ456 pharmacokinetic parameters after single and repeated oral dose of XYZ123 (800 mg) once a day

aminoglycosides (Bearden and Rodvold 2000). For drugs with non-body-weight-adjusted dosing, obese individuals may be notably underdosed and thus show lower efficacy. Since many of these effects are not simply predictable, appropriate profiling of drug pharmacokinetics/pharmacodynamics in obese individuals is essential for drugs targeting indications where the obesity is a prevalent concomitant illness.

European Agency for the Evaluation of Medicines Products (EMEA) and US Federal Drug Administration (FDA) guidance recommend that the pharmacokinetics of candidate weight control drugs are studied in obese (EMEA CPMP/EWP/281/96 (R1) (2007); US FDA 2007).

The good availability of individuals who are obese but are otherwise healthy makes the early profiling of drug pharmacokinetics/pharmacodynamics in healthy obese relatively easy. If no difference is observed between healthy obese and healthy lean individuals, then the recruitment of healthy lean subjects in many studies can be justified. In some cases, for example, when developing drugs for metabolic diseases (e.g., obesity, diabetes), the advantages of an early switch to obese individuals—a population closer to the final patient group than the typical healthy lean subject—early in drug development can justify this limited complication of the recruitment process.

PROCEDURE

The design of a study comparing the steady-state pharmacokinetic/pharmacodynamic profile of Drug XYZ in obese and nonobese healthy adults is presented below. Drug XYZ was developed for both subcutaneous (SC) and intravenous (IV) application routes. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data pertinent to the assessment of the impact of obesity, although other parameters were also studied.

45.13.1 Protocol Outline

A phase I, open-label study of the effect of obesity on the pharmacokinetic/pharmacodynamic profile of subcutaneous and intravenous Drug XYZ.

45.13.1.1 Objectives

Primary: The primary objective of the study was to compare steady-state pharmacokinetic/pharmacodynamic profile of SC Drug XYZ between obese and nonobese healthy adults.

Secondary: The secondary objective of the study was to compare steady-state pharmacokinetic parameters after IV administration and absolute bioavailability of Drug XYZ between obese and nonobese healthy adults.

45.13.1.2 Study Design

The study had an open-label, two-way crossover randomized study. Two groups of obese and nonobese healthy volunteers were administered multiple doses of SC Drug XYZ to reach steady state (treatment A) and single IV dose of Drug XYZ (treatment B). The washout period between trial periods was at least 10 days which approximated to >10 Drug XYZ apparent terminal half-lives.

45.13.1.3 Number of Subjects

Twenty-five (25) obese adults and 25 adults age-, height-, and sex-matched nonobese. Estimations based on previous studies with nonobese showed that the inclusion of 24 subjects per strata (obese, nonobese) would give enough power to allow the detection of a >20 % difference in AUC of Drug XYZ at steady state with alpha = 0.05 and beta = 0.10. According to the guidance for industry by the US FDA, 12 subjects is the typical minimum sample size for a bioavailability comparison; even in a pilot study, "a sufficient number of subjects (e.g., 12) need to complete the study" (US FDA 2003b). This requirement was fulfilled here for each of the substrata obese male, nonobese male, obese female, and nonobese female.

45.13.1.4 Inclusion Criteria

The following inclusion criteria were met: adults aged between 18 and 55 years; obese with body mass index (BMI) of $30.0 \leq 39.9$ kg/m^2 ($\geq$40.0 kg/m^2 on a case-by-case basis) or nonobese volunteers with BMI of $18.5 \leq 24.9$ kg/m^2; men who are willing to use prescribed barrier contraceptive methods; women who are either postmenopausal or surgically sterile and willing to use prescribed barrier contraceptive methods; who—apart from being obese—are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant for the study; who are nonsmoking or light smokers.

45.13.1.5 Treatments

Treatment A: Single (day 1) 1.5 mg/kg Drug XYZ dose given as an IV infusion over 6 hours (h).

Treatment B: Once daily 1.5 mg/kg Drug XYZ SC doses over 4 days.

45.13.1.6 Pharmacokinetic Data

Treatment A: Plasma concentrations of Drug XYZ, before and at predetermined times post dose after IV dosing on day 1. Treatment B: Plasma concentrations of Drug XYZ, before and at predetermined times after SC dosing on days 1 and 4.

A 24-h urine sampling was performed during the IV dosing period of the study that started on day 1 and ended on day 2. The volume of urine collected and the concentration of creatinine were determined to allow the creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of the impact of obesity on the disposition of the developmental drug from study described above was evaluated as follows:

Descriptive pharmacokinetic parameters (total clearance (CL or CL/F), mean residence time (MRT), volume of distribution (Vd or Vd/F), peak concentration (C_{max}), time of peak concentration (T_{max}), area under the curve between time 0 and the time of the last quantifiable concentration (AUC_{0-t}) and/or AUC for the dosing interval ($AUC_{0-\tau}$), apparent terminal half-life ($t_{1/2_z}$) after day 1, and $AUC_{0-\tau}$ after day 4) for plasma Drug XYZ were calculated using a noncompartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin® (Pharsight Corp.) protocols. The absolute bioavailability was calculated by comparison of day 1 data for treatments A and B.

Descriptive statistics (number of observations (n), mean, standard deviation, coefficient of variation in percent (CV %), or median and range) were calculated for each parameter. Statistical tests using SPSS software were as follows:

Homogeneity test of variance (Levene's test) for each dependent variable.

Independent *t*-test on log-transformed parameters: C_{max}, AUC_{0-t}, $AUC_{0-\tau}$, $t_{1/2_z}$ for plasma concentrations of Drug XYZ.

CRITICAL ASSESSMENT OF THE METHOD

The type of study described in this chapter provides profiling of the impact of obesity on the disposition of the developmental drug after dosing via two dosing routes. Although for Drug XYZ this approach was justified given the intention to market the Drug XYZ for both SC and IV applications, typically, however, only the proposed therapeutic dosing route would be tested.

Although the impact of obesity on the disposition of the developmental drug can most comprehensively be studied based on steady-state data as described, the use of single-dose data could also suffice provided there is reason to believe that the pharmacokinetics of the drug studied are accurately predictable from single-dose data.

MODIFICATIONS OF THE METHOD

The use of weight as covariate in PK studies showed to be not optimal for obese patients; therefore, increasingly adjusted weight descriptors are utilized, which assess the fat in the body differentially. These formulas differentiate between males and females and use patient's height or body mass index in addition. The use of a predicted normal weight (PNWT) (Duffull et al. 2004) seems to have advantages in obese patients.

The described supportive study was specifically designed and suitably powered to identify differences in Drug XYZ pharmacokinetics between obese and nonobese. Typically, this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of obesity on the disposition of the developmental drug during the phase II/III studies.

If obesity is a not a prevalent concomitant illness in the targeted indication, then this type of study is unlikely to be performed. Instead, a population pharmacokinetic/pharmacodynamic assessment of the impact of obesity on the disposition of the developmental drug would be performed during the phase II/III studies. A suitably powered clinical study would only be performed if such population pharmacokinetic/pharmacodynamic assessments raised significant questions.

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US FDA (2007) Guidance for industry, draft (R1) developing products for weight management, February 2007

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below.

45.13.1.7 Results: Pharmacokinetics

The data from study described above, as given in Tables 45.22 and 45.23 below, can be summarized as follows:

- After 1.5 mg/kg SC dosing for 4 days, AUC_{0-t} for Drug XYZ was 16 % higher in obese subjects compared to nonobese subjects. This is explained by:
  - A similar, 100 % systemic absorption in both groups
  - A lower weight-adjusted clearance in obese subjects (see below)
- After SC dosing, absorption was slightly delayed in the obese (T_{max} 4 h vs. 3 h) and no difference in C_{max} values was observed after repeated SC dosing. The difference was therefore mainly attributed to the elimination phase. Steady state was reached on day 4 in both obese and nonobese subjects, with almost no unpredicted accumulation.
- After a 6-h IV infusion, Drug XYZ clearance and volume of distribution estimates were over 30 % higher in obese than in controls. On a weight-adjusted basis, these parameters were about 10 % lower in obese. MRT estimates were similar for both groups.
- Exposure (AUC) to Drug XYZ was significantly higher in obese subjects. This observation was explained as follows: while the total clearance (L/h) is much higher in obese subjects (i.e., higher total elimination capacity: 121 mL/min creatinine clearance in obese vs. 83 mL/min in nonobese), the clearance on a weight-adjusted basis was actually lower. Since the dose was weight adjusted, this leads to higher AUC estimates.
- The C_{max} is 15 % higher in obese, which can be explained by the fact that the distribution volume does not increase proportionally with weight either, similar to the argument for clearance.

45.14 Special Population: Pediatric Population

PURPOSE/RATIONALE

The number of medicinal products currently labeled for pediatric use is limited.

There is a general lack of pharmacokinetic (PK) and other clinical information to support the administration of many medicinal products to children which leads to a lack of appropriate dosage recommendations in this population. As a consequence, there is frequent off-

Table 45.22 Mean Drug XYZ pharmacokinetic parameters (CV %) on days 1 and 4 following 1.5 mg/kg SC 24-h dose of Drug XYZ in 24 nonobese and 24 obese healthy subjects

	C_{max} (IU/mL)	T_{max} (h)	AUC_{0-t} (h.IU/mL)	$AUC_{0-\tau}$ (h.IU/mL)	$t_{1/2_z}$ (h)
Day 1					
Nonobese	1.344 (10)	3.50[a] (1.50–4.00)	14.19 (13)	14.87 (14)	4.85 (11)
Obese	1.379 (14)	4.00[a] (3.00–6.00)	15.99 (14)	17.01 (16)	5.09 (18)
Obese/nonobese difference	+3 % (NS)	+0.5 h (NS)	+13 % (p = 0.007)	+14 % (p = 0.006)	+14 % (NS)
Obese PK/nonobese PK ratio difference	1.022	–	1.12	–	–
90 % Confidence interval	0.961–1.086	–	1.05–1.21	–	–
Day 4					
Nonobese	1.488	3.00[a] (2.00–4.00)	16.43	17.52	5.45
Obese	1.563	4.00[a] (3.00–6.00)	19.12	20.78	5.76
Obese/nonobese difference	+5 % (NS)	+1 h (p = 0.005)	+16 % (p = 0.001)	+19 % (p = 0.002)	+6 % (NS)
Obese PK/nonobese PK ratio difference	1.049	–	1.16	–	–
90 % Confidence interval	0.995–1.107	–	1.08–1.25	–	–

[a]Median (range), NS not statistically significant, – not reported

Table 45.23 Mean Drug XYZ pharmacokinetic parameters (CV %) following a 1.5 mg/kg IV dose of Drug XYZ by a 6-h infusion in 21 obese and 21 nonobese healthy subjects

	C_{max} (IU/mL)		AUC_{0-t} (h.IU/mL)	$AUC_{0-\tau}$ (h.IU/mL)	$t_{1/2_z}$ (h)
Nonobese	1.542 (12)		13.32 (14)	13.95 (15)	4.60 (11)
Obese	1.770 (8)		14.90 (13)	15.64 (14)	5.03 (15)
Obese/nonobese difference	+15 % (p = <0.0001)		+12 % (p = 0.013)	+11 % (p = 0.016)	+9 % (p = 0.034)
Obese PK/nonobese PK ratio difference	1.151		1.12		
90 % Confidence interval	1.090–1.216		1.04–1.20		
	CL (L/h/kg)	**CL (L/h)**	**MRT (h)**	**Vd (L/kg)**	**Vd (L)**
Nonobese	0.011 (15)	0.74 (17)	5.99 (12)	0.066 (12)	4.37 (17)
Obese	0.010 (15)	0.99 (23)	5.94 (13)	0.059 11)	5.77 (18)
Obese/nonobese difference	−9 % (p = 0.014)	+34 % (p < 0.0001)	−8 % (NS)	−11 % (p = 0.001)	+32 % (p > 0.0001)

[a]Median (range), NS not statistically significant, – not reported

label use of licensed medicines in children. In 2006, the European Parliament and the Council of the European Union have issued a regulation on medicinal products for pediatric use, implementing a Pediatric Committee (PDCO) to overcome these gaps.

The disposition of a drug may differ considerably between adults and children as in the pediatric population the organs affecting the absorption, distribution, metabolism, and excretion of a drug are under continuous maturation, which at least is expected to lead to additional inter- and intraindividual variances in the PK of a drug in this population. To support the development of a pediatric formulation and especially in order to support the dosing recommendation in different age groups, PK studies should be performed. In case the systemic exposure of a drug is readily related to the pharmacological or therapeutic effect, PK results in the pediatric population can be used for extrapolation of efficacy data from studies performed in adults.

Current guidelines have recently been updated, proposing new pediatric rules and actually encouraging to investigate the safety and efficacy of a medicinal product in children, and also elucidate cases in which the drug blood concentration will become the basis of subsequent determination of the dosage schedule.

As the pediatric subgroup represents a vulnerable population, studies in children are endowed with specific challenges such as additional ethical (e.g., informed consent be obtained from the legal guardian) or clinical-technical (e.g., minimize the amount of blood drawn and the number of venipunctures) issues.

PK studies in the pediatric population are generally conducted in patients with the disease under investigation. This may lead to higher intersubject variability than studies in healthy subjects, but the data better reflect clinical use.

More details about when such studies may or may not be important, and other aspects as study design and methods, can be found in the literature below.

In summary, PK studies in the pediatric population should determine if the dosage regimen in the pediatric population is to be adjusted to achieve approximately the same level of systemic exposure that is safe and effective in adults.

The following chapters present a particular study in a pediatric population (children and adolescents) with result illustration and conclusion. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of PK data although safety and pharmacodynamic parameters were also studied.

PROCEDURE

The design of a typical pediatric study is presented below.

45.14.1 Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) and regular human insulin injected subcutaneously as a single dose in pediatric subjects with type I diabetes in a single-center, double-blind, randomized, two-way crossover study.

45.14.1.1 Objective

To investigate the pharmacokinetics of insulin glulisine and regular human insulin in pediatric type I diabetic subjects.

45.14.1.2 Design, Treatment, and Sample Size

Single-center, single-dose, double-blind, randomized, two-way crossover design; 20 pediatric type I diabetic subjects (10 per age class) of either gender. The two age classes were built by children aged between 5 and 11 years and adolescents aged between 12 and 17 years.

Pediatric patients received a single dose of 0.15 IU/kg body weight of HMR1964 (insulin glulisine) and regular human insulin administered subcutaneously in the periumbilical abdomen 2 minutes (min) before a standardized liquid meal. Volume and frequency of blood sampling for determinations of insulin in serum were minimized and were taken according to the sampling schedule.

45.14.1.3 Population Treated

Twenty type I diabetic pediatric subjects, of both age classes children and adolescents: 10 children (5 male and 5 female), between 7 and 11 years of age, with body mass indices between 16.4 and 22.7 kg/m^2, and 10 adolescents (4 male and 6 female), between 12 and 16 years of age, with body mass indices between 17.7 and 26.3 kg/m^2.

EVALUATION

45.14.2 Statistical Procedures Pharmacokinetics

The relationship between age and pharmacokinetics was assessed by analyses of variance (ANOVA) on AUCs, MRT, and C_{max} with adjustments for treatment, period, sequence, and subject within sequence effects by age class using the natural log–transformed values to compare treatments within age class. Point estimates and 95 % confidence intervals were calculated for the treatment ratios per age class.

ANOVAs with adjustments for age class, period, sequence, and subject within sequence effects were performed by treatment to compare age classes within treatment. Point estimates and 95 % confidence intervals were calculated for the age class ratios per treatment.

T_{max} was analyzed by nonparametric analyses. Ninety-five percent nonparametric confidence intervals for the respective median treatment and age class differences were calculated.

CRITICAL ASSESSMENT OF THE METHOD

45.14.2.1 Introduction

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of type I and type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used to optimally control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting insulin to provide basal insulin requirements and control fasting and preprandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. Insulin glulisine has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously.

Preclinical studies suggest that insulin glulisine does not differ from the established elimination pathways of endogenous human insulin.

45.14.2.2 Study Rationale

Insulin glulisine may be of particular use for children and adolescents who encounter more difficulties in adjusting their daily activities to fixed dosing meal intervals. Given immediately before a meal or thereafter to balance the actual carbohydrate intake may be of significant advantage, provided the rapid and short-acting nature of insulin glulisine also prevails in this patient population.

This study was conducted to investigate the pharmacokinetics of insulin glulisine and regular human insulin subcutaneously administered 2 min before a standard meal in pediatric type 1 diabetic patients from both age classes (10 children and 10 adolescent subjects) in order to verify the sameness of differences seen in adults.

45.14.2.3 Population

Twenty male and female pediatric type 1 diabetic patients from both age classes (10 children and 10 adolescents subjects) were treated.

45.14.2.4 Dosing Recommendation/ Therapeutic Dose Range

A single dose of 0.15 IU/kg insulin glulisine was chosen for the children following a dosing approach estimate on the basis of body weight. Based on the primary pharmacokinetics of insulin glulisine, a single-dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

45.14.2.5 Study Design Aspects

To study the plasma protein binding at least in newborns and infants is recommended, as the protein binding is reduced in the preterm and term infant at birth and in the first weeks of life. Also, the drug elimination capacity is reduced in this subgroup of very young children due to immaturity of both metabolic pathways and renal function.

Initial titrating doses may be estimated on the basis of body weight or surface area from an extrapolation of adult data.

45.14.2.6 Classification of Age Classes

Any classification of the pediatric populations into age categories is to some extent arbitrary.

Suggestions for categorization are presented in the literature below and are as follows:

Neonate: birth to 1 month
Infant: 1 month to 2 years
Child: 2–12 years
Adolescent: 12 years to 16–18 years

The pharmacokinetics of a drug in humans 16–18 years and older is expected to be similar to that of adults.

Depending on the intended use of a drug in the pediatric population, studies should be performed in all pediatric age groups to allow dose adjustment within an individual over time.

45.14.2.7 Population Pharmacokinetics

An alternate approach is the population pharmacokinetic approach or study. This approach relies on infrequent (sparse) sampling of blood from a larger population than would be used in a standard pharmacokinetic study to determine pharmacokinetic measures and/or parameters.

The population pharmacokinetic approach assesses the impact of various covariates on the pharmacokinetics of a drug. Nonlinear mixed effects modeling may be used to investigate the relationship between various covariates and pharmacokinetic parameters. Age or age group may be one of the covariates. This type of approach has its advantages as it involves assessment of the effect of age on the pharmacokinetics in the target population.

45.14.2.8 Crossover Study Design

In case of the comparison of two study medications, a sound alternative could be the (complete) crossover study design (see "Example").

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the PK results obtained from the study described above under *Procedure* is presented below.

Twenty pediatric type I diabetic patients as described above completed the study according to the protocol; 19 subjects were evaluable for PK evaluation of regular human insulin treatment. The demographic data are depicted in Table 45.24.

The concentration-time profile of insulin glulisine differed considerably from regular human insulin (Fig. 45.11a, b, c), with initial fractional AUCs being higher after insulin glulisine than after regular human insulin, while the total AUC (AUC(0–6 h)) was only slightly larger. The C_{max} of insulin glulisine was higher by 71 % and reached earlier with a median T_{max} of 54 min compared to 66 min after regular human insulin. The MRT for insulin glulisine was distinctly shorter with 88 min compared to 137 min for regular human insulin, indicating the shorter residence of insulin glulisine in the systemic circulation (Table 45.25).

Contrasts between the concentration-time profiles of insulin glulisine and regular human insulin obtained in the population as a whole were also present in both age classes, children and adolescents (Table 45.26).

The two age classes, children and adolescents, presented an almost equal PK profile of insulin glulisine, displayed by point estimates close to 100 % with a slight trend toward higher exposure in adolescents (Table 45.27). Nevertheless, T_{max} and MRT were the same in each age class.

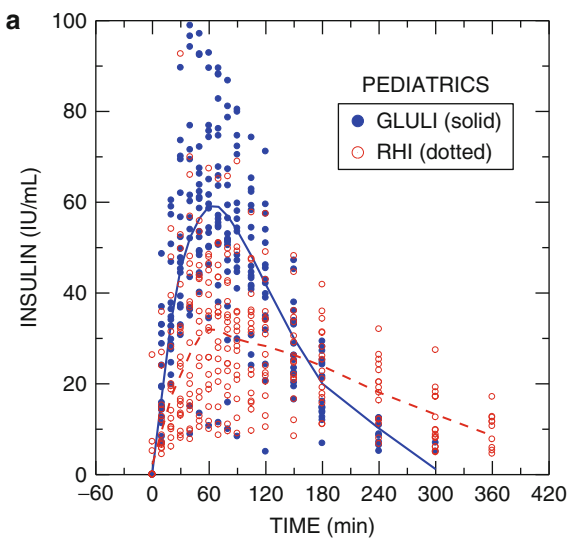
In contrast to the findings with insulin glulisine, the comparison between age classes for regular human insulin revealed on average 60 % higher exposure (AUCs and C_{max}) in adolescents (Table 45.28). Nevertheless, there were no substantial differences in T_{max} and MRT.

In summary, in pediatric type I diabetic subjects, equally in each age class children and adolescents, insulin glulisine was more rapidly absorbed than regular human insulin. The fractional AUCs were larger, and C_{max} was higher with an earlier T_{max} for insulin glulisine. MRT was distinctly shorter, indicating the shorter residence of insulin glulisine in the systemic circulation compared to regular human insulin.

Table 45.24 Demographic variables

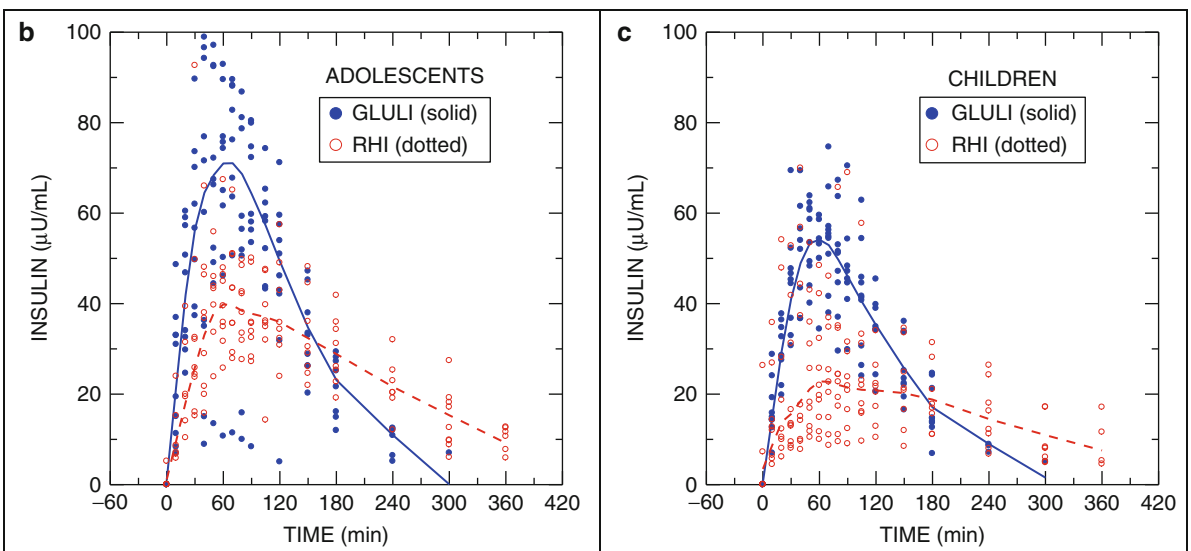
	Arithmetic mean (range)					
	All subjects ($n = 20$)					
Demographic data	All ($n = 20$)		Male ($n = 9$)		Female ($n = 11$)	
Age (years)	12.4 (7–16)		12.6 (8–16)		12.3 (7–16)	
Weight (kg)	52.1 (26.0–82.5)		54.1 (26.0–82.5)		50.5 (27.5–65.0)	
BMI (kg/m^2)	20.9 (16.4–26.3)		20.9 (16.4–26.3)		20.9 (17.6–24.5)	
	Children ($n = 10$)			Adolescents ($n = 10$)		
	All ($n = 10$)	Male ($n = 5$)	Female ($n = 5$)	All ($n = 10$)	Male ($n = 4$)	Female ($n = 6$)
Age (years)	10.1 (7–11)	10.2 (8–11)	10.0 (7–11)	14.7 (12–16)	15.5 (14–16)	14.2 (12–16)
Weight (kg)	40.0 (26.0–50.0)	39.1 (26.0–50.0)	40.8 (27.5–49.0)	64.2 (51.0–82.5)	72.7 (53.0–82.5)	58.6 (51.0–65.0)
BMI (kg/m^2)	19.4 (16.4–22.7)	19.2 (16.4–21.1)	19.7 (17.6–22.7)	22.4 (17.7–26.3)	23.1 (17.7–26.3)	22.0 (20.2–24.5)

Serum insulin concentrations – children and adolescents



Average insulin concentration (μIU/mL) after doses of 0.15 IU/kg insulin glulisine (GLULI) (n=20) and regular human insulin (RHI) (n=19) in pediatric type 1 diabetic subjects.
SYSTAT® Version 9.0 LOWESS function, tension factor 0.3

Serum insulin concentrations – children / adolescents



Average insulin concentration (μIU/mL) after doses of 0.15 IU/kg insulin glulisine (GLULI) (n=10) and regular human insulin (RHI) (n=9) in pediatric type 1 diabetic subjects.
SYSTAT® Version 9.0 LOWESS function, tension factor 0.3

Fig. 45.11 Children and adolescents

The two age classes, children and adolescents, presented an almost equal PK profile after insulin glulisine with a slight trend toward higher exposure in adolescents. In contrast, the comparison between age classes for regular insulin revealed on average 60 % higher exposure in adolescents.

In conclusion, in pediatric type I diabetic subjects, equally in each age class children and adolescents,

insulin glulisine was more rapidly absorbed and had a shorter residence in the systemic circulation compared to regular human insulin. Insulin glulisine displays pharmacokinetic properties in pediatric type 1 diabetic subjects, which classify insulin glulisine as a rapid-acting insulin analogue in this patient population with no specific dose adjustment warranted on the basis of intrinsic properties of HMR1964 (insulin glulisine).

Table 45.25 Pharmacokinetic results: by treatment

	Geometric mean		Point estimate (95 % confidence interval)[a]
Variable	Glulisine ($n = 20$)	RHI ($n = 19$)	Glulisine/RHI ($n = 19$)
$AUC_{(0–1\ h)}$ (μIU.min/mL)	2,287	1,246	176 % (126.9; 243.8 %)
$AUC_{(0–2\ h)}$ (μIU.min/mL)	5,232	2,994	169 % (126.9; 224.3 %)
$AUC_{(0–4\ h)}$ (μIU.min/mL)	7,624	5,703	130 % (99.3; 170.3 %)
$AUC_{(0–6\ h)}$ (μIU.min/mL)	8,361	7,052	116 % (89.5; 149.8 %)
C_{max} (μIU/mL)	58	33	171 % (126.9; 229.4 %)
T_{max} (min)	54[b]	66[b]	−8 min (−24; 7 min)[c]
MRT (min)	88	137	64 % (59.0; 70.4 %)

[a]Point estimates and 95 % confidence intervals for the ratio of treatment means, based on (ln) transformed data
[b]Median
[c]Point estimates and 95 % confidence intervals for the respective median differences from nonparametric data analysis

45.15 Special Populations: Assessment of Ethnic and/or Genetically Determined Differences

PURPOSE/RATIONALE

Special populations become more and more important for clinical trials. It is requested to investigate the influence of organ dysfunctions (kidney, liver), of the underlying and concomitant illness, of gender/age, and also of ethnic differences (ICH-E5 1998). Increasing knowledge about population-determined PD and PK and especially about the genetic background of metabolic capacities raises more and more complex questions during drug development. In this chapter, the assessment of genetically determined (interethnic) differences will be discussed.

During recent years, genotyping of drug-metabolizing enzymes and transporters (DME + T), known to show a polymorph expression, gained more and more importance to predict phenotypes (US FDA 2005). This provides a helpful tool to predefine and select populations on risk and to investigate them specifically in a dedicated phase I study (for specific terminology, see EU CPMP 2002). If one of the known polymorph DME + T's is involved in the metabolism of the drug (or suspected to be), an explorative study—as described in this chapter—might be helpful during early development. Presently, the DME + T's with sufficiently established background knowledge to be considered as "validated" are CYP 2C9, CYP 2C19, CYP 2D6, NAT2, and MDR1, of further

Table 45.26 Pharmacokinetic results: by treatment and age class

	Geometric mean (*arithmetic mean*)					
	Glulisine			RHI		
Variable	All subjects ($n = 20$)	Children ($n = 10$)	Adolescents ($n = 10$)	All subjects ($n = 19$)	Children ($n = 10$)	Adolescents ($n = 9$)
$AUC_{(0–1\ h)}$ (μIU.min/mL)	2,287 (*2,491*)	2,170 (*2,212*)	2,410 (*2,769*)	1,246 (*1,440*)	1,023 (*1,246*)	1,552 (*1,656*)
$AUC_{(0–2\ h)}$ (μIU.min/mL)	5,232 (*5,637*)	4,948 (*5,030*)	5,534 (*6,244*)	2,994 (*3,335*)	2,383 (*2,747*)	3,860 (*3,988*)
$AUC_{(0–4\ h)}$ (μIU.min/mL)	7,624 (*8,190*)	7,193 (*7,314*)	8,081 (*9,067*)	5,703 (*6,231*)	4,530 (*5,068*)	7,367 (*7,523*)
$AUC_{(0–6\ h)}$ (μIU.min/mL)	8,361 (*8,922*)	7,934 (*8,055*)	8,811 (*9,789*)	7,052 (*7,673*)	5,581 (*6,214*)	9,145 (*9,294*)
C_{max} (μIU/mL)	58 (*62*)	55 (*55*)	61 (*69*)	33 (*37*)	25 (*29*)	44 (*46*)
T_{max} (min)	54[a]	55[a]	52[a]	66[a]	59[a]	76[a]
MRT (min)	88 (*90*)	87 (*88*)	90 (*91*)	137 (*139*)	132 (*134*)	144 (*146*)

[a]Median values

Table 45.27 Pharmacokinetic results—children versus adolescents—insulin glulisine. Comparison of pharmacokinetic results for insulin glulisine

Variable	Geometric mean (*arithmetic mean*)		Point estimate (95 % confidence interval)[a]
	Children ($n = 10$)	Adolescents ($n = 10$)	Adolescents/ Children ($n = 10$)
$AUC_{(0–1h)}$ (μIU.min/mL)	2,170 (*2,212*)	2,410 (*2,769*)	111 % (70.4; 175.4 %)
$AUC_{(0–2h)}$ (μIU.min/mL)	4,948 (*5,030*)	5,534 (*6,244*)	112 % (72.0; 173.7 %)
$AUC_{(0–4h)}$ (μIU.min/mL)	7,193 (*7,314*)	8,081 (*9,067*)	112 % (72.1; 175.0 %)
$AUC_{(0–6h)}$ (μIU.min/mL)	7,934 (*8,055*)	8,811 (*9,789*)	111 % (73.0; 169.0 %)
C_{max} (μIU/mL)	55 (*55*)	61 (*69*)	112 % (73.0; 171.8 %)
T_{max} (min)	55[b]	52[b]	−2 min (−9; 11 min)[c]
MRT (min)	87 (*88*)	90 (*91*)	103 % (88.3; 120.9 %)

[a]Point estimates and 95 % confidence intervals for the ratio of treatment means, based on (ln) transformed data
[b]Median
[c]Point estimates and 95 % confidence intervals for the respective median differences from nonparametric data analysis

Table 45.28 Pharmacokinetic results—children versus adolescents—regular human insulin. Comparison of pharmacokinetic results for regular human insulin

Variable	Geometric mean (*arithmetic mean*)		Point estimate (95 % confidence interval)[a]
	Children ($n = 10$)	Adolescents ($n = 9$)	Adolescents/ Children ($n = 9$)
$AUC_{(0–1\ h)}$ (μIU.min/mL)	1,023 (*1,246*)	1,552 (*1,656*)	152 % (89.2; 258.1 %)
$AUC_{(0–2h)}$ (μIU.min/mL)	2,383 (*2,747*)	3,860 (*3,988*)	162 % (105.1; 249.5 %)
$AUC_{(0–4\ h)}$ (μIU.min/mL)	4,530 (*5,068*)	7,367 (*7,523*)	163 % (111.1; 238.0 %)
$AUC_{(0–6\ h)}$ (μIU.min/mL)	5,581 (*6,214*)	9,145 (*9,294*)	164 % (113.8; 235.9 %)
C_{max} (μIU/mL)	25 (*29*)	44 (*46*)	177 % (111.7; 280.5 %)[b]
T_{max} (min)	59[c]	76[c]	9 min (−24; 49 min)
MRT (min)	132 (*134*)	144 (*146*)	110 % (92.4; 130.1 %)

[a]Point estimates and 95 % confidence intervals for the ratio of treatment means, based on (ln) transformed data
[b]Point estimates and 95 % confidence intervals for the respective median differences from nonparametric data analysis
[c]Median

interest became the UGTs. Genetic phenotyping is increasingly recommended for other kind of studies (EU 2010), and thus the investigation described in this chapter provides a tool to assess these influences.

PROCEDURE

The design of an exploratory profiling of the impact of polymorphism of CYP 2C19 on the pharmacokinetics of candidate drug (XYZ1234) in a single clinical study is presented below. Two control groups (both genders) were required, since the poor CYP 2C19 group consisted of males and females (due to the low prevalence in the European population).

For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are also studied.

45.15.1 Protocol Outline

Explorative assessment of the pharmacokinetics of XYZ1234 following a single oral 100-mg dose, given in a special micronized formulation in poor and extensive CYP 2C19 metabolizing healthy male and female subjects.

45.15.1.1 Objective

Comparative assessment of the pharmacokinetics (PK) of XYZ1234 following a single oral 100-mg dose in healthy male and female subjects, which are poor or extensive metabolizers for CYP 2C19 respectively.

45.15.1.2 Study Design

The study was performed open labeled in a single center in three parallel groups.

45.15.1.3 Number of Subjects

Eighteen subjects (6 poor CYP 2C19 metabolizers [2–4 of them males] and 12 extensive CYP 2C19 metabolizers [6 males and 6 females], if possible with matching body mass indices (BMI) and age distribution).

45.15.1.4 Inclusion Criteria

Healthy men or women aged between 18 and 60 years, with BMI of 19.5–29.5 kg/m^2. Screening must have determined the CYP 2C19 metabolizer status beforehand.

45.15.1.5 Treatments

XYZ1234 100 mg per os (orally, PO.) (in a special micronized formulation, under fasting conditions).

45.15.1.6 Pharmacokinetic Data

Concentrations of XYZ1234 in plasma and urine were measured before and at predetermined times up to 72 h (h) post dosing and were used to determine the PK data.

If feasible and adequate, an explorative investigation on potential metabolites in plasma and urine was to be performed.

EVALUATION

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and PK parameters calculated.

This descriptive statistics will be presented by CYP 2C19 metabolizer status and where applicable by gender, including mean (arithmetic and/or geometric), standard deviation (usual and/or dispersion factor), standard error of the mean, coefficient of variation (in %), median, minimum, maximum, and number of observations.

For pharmacokinetics in plasma: Individual concentrations of XYZ1234 will be tabulated together with descriptive statistics and plotted. Median profiles will be presented graphically by CYP 2C19 metabolizer status and gender. Pharmacokinetic parameters (at least C_{max}, t_{max}, $AUC_{(0-t)}$ [$t = 24$ h, and "last > LOQ"], AUC_{inf}, $t_{½z}$, MT, as well as CL/f and Vz/f) will be determined based on plasma concentrations of XYZ1234 using noncompartmental procedures.

For urine data, the individual and mean fractional and cumulative urinary excretion of XYZ1234 will be calculated if feasible and adequate {$Ae_{(t2-t1)}$; $Ae_{(0-72\ h)}$, urinary recovery [% of administered dose], and renal clearance CL_R (fractional and total)}.

CRITICAL ASSESSMENT OF THE METHOD

In the actual development program, this study was needed to exploratively elucidate the influence of the CYP 2C19 phenotype before starting the first multiple-dose study due to the observation of a deviating pharmacokinetics in a poor CYP 2C19 metabolizing subject included in an earlier single-dose study (the first-in-man study). The described study had to clarify if this deviating PK for this subject is based on the status of being a "poor CYP 2C19 metabolizer" or was based on a further individuality of that specific subject (e.g., a different degree of resorption).

Since the prevalence of poor metabolizers for CYP 2C19 in a Caucasian population is only 2–5 %, the recruiting of those subjects became cumbersome, and the consequence for the design of the study was that some study details were more adjusted to the needs of those subjects as in a "normal" phase I study.

The formulation used in this study had proven to be superior to other formulations tested; as dose, 100 mg is selected (instead of the targeted 400 mg) in order to generate an additional safety margin for the case that the metabolizer status consistently influences the extent of exposure.

The impact of selected phenotypes on candidate drug pharmacokinetics and safety can be conveniently investigated in an explorative study with the design described in this chapter. Typically, this study would run shortly after key data are available, indicating the influence of polymorph DME + T. The dose should be adjusted (i.e., reduced, compared to the clinical dose) based on the expected degree of interaction.

But especially since a predicted phenotype based on genotyping data was used to define the study groups, this investigation cannot consider all the extrinsic and intrinsic factors influencing the real activity of enzymes and transporters. During the further clinical development additional (population) analyses, studies might be required to further elucidate the respective influence of the DME + T polymorphism and its consequences.

MODIFICATIONS OF THE METHOD

The design of study described in this chapter is relatively simple but nevertheless provides explorative profiling of issues related to DME + T polymorphism, and the same principle could be applied to address further influencing factors or populations. The more complicated part is to clearly define both relatively small test and control groups based on the DME + T properties or other characteristics.

Typically, this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of genetically determined (interethnic) differences on the disposition of the developmental drug during the phase II/III studies.

References and Further Readings

EU CPMP (2002) Position paper on terminology in pharmacogenetics, November 2002
EU CPMP (2010) Guideline on the investigation of bioequivalence, January 2010
ICH-E5 (1998) Ethnic factors in the acceptability of foreign clinical data (R1). September 1998
US FDA (2005) Guidence for industry: pharmacogenomic data submissions, March 2005

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under *Procedure* is presented below.

45.15.2 Results

Compared to the extensive metabolizers for CYP 2C19, the plasma profiles clearly differ for the poor CYP 2C19 metabolizers: the initial peak is higher and especially the elimination phase takes much longer. There is, in addition, an indication of a difference between the two control groups (females vs. males). Some profiles for the females show peaks similar to those seen for the poor metabolizers, and at least in one case, a similar long elimination phase was to be observed. Nevertheless in average the two control groups of extensive CYP 2C19 metabolizers are highly overlapping and clearly below the profiles for the poor CYP 2C19 metabolizers (see Fig. 45.12).

The peak values (C_{max} as median) are 0.95, 0.35, and 0.61 μg/mL for the poor metabolizers, the male extensive metabolizers, and the female extensive metabolizers, respectively. The differences in the overall exposure (AUC, extrapolated to infinity) are much more prominen, because of the intensive differences in the elimination phase. The median values were 8.83, 0.83, and 2.49 μg*h/mL for the poor metabolizers, the male extensive metabolizers, and the female extensive metabolizers, respectively. This difference is already obvious when comparing the exposure only over the first 24 h: the median values for $AUC_{(0–24\ h)}$ were 4.21, 0.83, and 1.38 μg*h/mL for the poor metabolizers, the male extensive metabolizers, and the female extensive metabolizers, respectively.

Also the half-life of the terminal phase strongly differed. The median values were 43.6, 4.6, and 18.5 h for the poor metabolizers, the male extensive metabolizers, and the female extensive metabolizers, respectively.

The PK parameters showed a significant variability in these small ($n = 6$) groups. In addition, the extrapolated portion of the exposure (AUC:%Extrap) is quite high for the poor metabolizers and also the extensive metabolizing females, indicating that (at least for the poor metabolizers) even the 72-h blood collection period was not sufficient, which in addition contributes to the variability of the PK data.

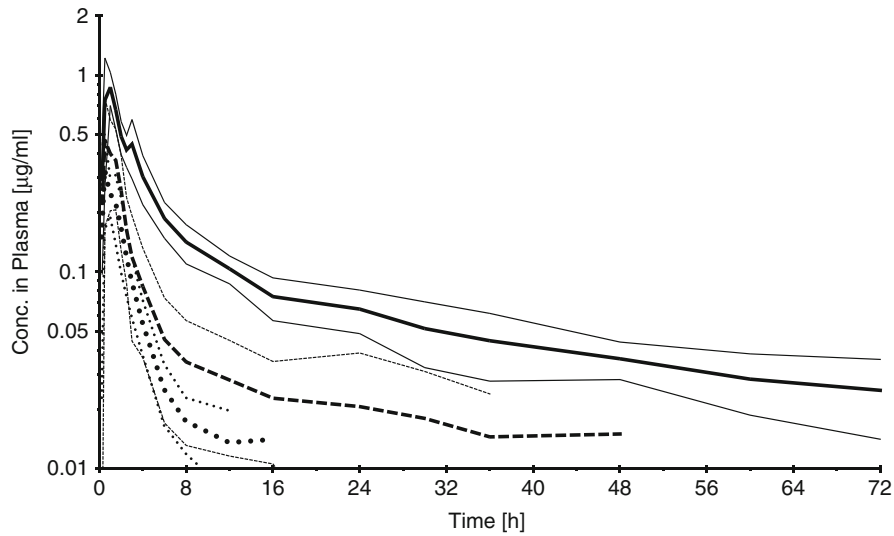


Fig. 45.12 Plasma concentration versus time profiles of XYZ1234 following a single oral 100-mg dose given to poor CYP 2C19 metabolizing male and female subjects (*straight lines*), to extensive CYP 2C19 metabolizing females (*dashed lines*), and to extensive CYP 2C19 metabolizing males (*dotted lines*), respectively. *Mean values (bold) and 90 % confidence interval*

45.16 Profiling of the Gastrointestinal Site of Absorption

PURPOSE/RATIONALE

For several reasons, it may be helpful to investigate the extent of absorption by determining the window for absorption in the gastrointestinal (GI) tract, for example:

- If the drug is intended to reach the colon, a drug loss in the small intestine is not desirable as this portion does not contribute to efficacy, but possibly to intolerance.
- The drug has a (too) short systemic half-life, and the feasibility for the development of an extended-release formulation has to be investigated.

In the present study, example the feasibility of the development of a slow-release formulation was evaluated. Guidelines on the design and conduct of such studies have not yet been published.

PROCEDURE

The design of an exploratory assessment of the absorption window for a high-clearance drug candidate is presented below. The aim of the present study was to compare the regional drug absorption from the proximal small bowel, the distal small bowel, and ascending colon with an immediate-release reference formulation, using pharmacoscintigraphy and remote release via the Enterion™ capsule to evaluate the feasibility of the development of an extended-release formulation.

45.16.1 Protocol Outline

Pharmacoscintigraphic evaluation of the regional drug absorption of XYZ123 in healthy men.

45.16.1.1 Primary Objective

To investigate the pharmacokinetics (PK) of an XYZ123 formulation (200 mg) following topical release in the proximal small bowel, distal small bowel, and colon via the Enterion™ capsule in healthy subjects and to compare to the PK of an XYZ123 immediate-release reference formulation (200 mg).

45.16.1.2 Study Design

Randomized four-way crossover design.

45.16.1.3 Inclusion Criteria

Healthy male subjects aged between 18 and 65 years.

45.16.1.4 Treatments

Regimen A: XYZ123 immediate-release formulation in tablet form (200 mg, reference)

Regimen B: XYZ123 formulation (200 mg) delivered to the proximal small bowel via the Enterion™ capsule

Regimen C: XYZ123 formulation (200 mg) delivered to the distal small bowel via the Enterion™ capsule

Regimen D: XYZ123 formulation (200 mg) delivered to the ascending colon via the Enterion™ capsule

45.16.1.5 Pharmacokinetic Data

Derived from concentrations of the parent drug XYZ123 in whole blood and of the active metabolite ABC4321 in plasma before and at predefined times after dosing (Regimen A) and following activation of the Enterion™ capsule (Regimens B, C, and D).

EVALUATION

PK data: The PK parameters of ABC4321 in plasma were determined by individual PK analyses. The individual and mean concentrations of ABC4321 in plasma were tabulated and plotted. PK variables were listed and summarized by treatment with descriptive statistics. An analysis of variance (ANOVA) including sequence, subject nested within sequence, period, and treatment effects was performed on the ln-transformed parameters (except t_{max}). The mean square error was used to construct the 90 % confidence interval for treatment ratios. The point estimates were calculated as ratio of the antilog of the least square means. Pair-wise comparisons to treatment A were made. Whole blood concentrations of XYZ123 were not used to perform PK analyses.

Scintigraphic data: The activation time post dose and the arrival, residence, and transit times of the capsule in different regions of the GI tract (i.e., dosing time subtracted from the actual clock time for each transit event) were derived from the scintigraphic images and tabulated for each treatment condition and for subjects. Descriptive statistics were performed where appropriate.

CRITICAL ASSESSMENT OF THE METHOD

The pharmacoscintigraphy of a drug vehicle from which the contents can be released via remote signal

was applied to early drug development in recent years with increasing frequency.

Via remote control of its current location, this capsule represents a reliable tool for immediate release at the GI location where the permeation capability of the drug substance should be assessed.

As the capsule can be "loaded" with the drug as powder, suspension, or solution, the physicochemical situations in the different parts of the GI tract can be mimicked.

An in vitro compatibility study ("leakage test") had been performed beforehand: (1) Three capsules were filled with powdered tablet formulation of XYZ123, containing 200 mg of the drug each. (2) Each capsule was transferred to simulated gastric fluid (SGF) and placed in a shaking water bath maintained at 37°C for 2 h. (3) After this 2-h incubation, each capsule was transferred to simulated intestinal fluid (SIF) and placed in a shaking water bath maintained at 37°C for 24 h. (4) Aliquots of SGF were taken at 1 and 2 h and aliquots of SIF were taken at 1 and 24 h and analyzed for XYZ123. (5) The content of the capsules was analyzed for XYZ123.

The leakage of powdered drug formulation (0.01 %) under simulated in vivo conditions was considered acceptable. The recovery of drug from the capsules was within the specification limits for the film-coated tablets.

In the study presented here, absorption from the colon was not reliable, thus making it nonfeasible to develop a formulation with a 24-h (h) release profile reducing the administration frequency to QD.

MODIFICATIONS OF THE METHOD

Other attempts to define the site of absorption were other kinds of vehicles as well as catheters. Three main perfusion methods have been employed in the small intestine: (1) a triple lumen tube including a mixing segment, (2) a multilumen tube with a proximal occluding balloon, and (3) a multilumen tube with two balloons occluding a 10-cm-long intestinal segment. A critical assessment of these perfusion techniques can be found in (Lennernäs 1998). Whereas vehicles inherit the advantage of minimal invasiveness, their transit through the GI tract depends on the individual physiology of the study subject. On the other hand, this is exactly the fate of a solid dosage form.

References and Further Readings

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EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the pharmacokinetic and scintigraphy results obtained from the study described above under *Procedure* is presented below.

45.16.1.6 Results

PK data: PK parameters were calculated for all subjects for Regimens A (immediate release), B (proximal small bowel activation), and C (distal small bowel activation).

However, for Regimen D (ascending colon activation), out of the six subjects available, two subjects had plasma concentrations which were low and $AUC_{(0-\infty)}$ could not be calculated, and a further two subjects had $AUC_{(0-\infty)}$ of >20 % extrapolated from the last observation. Hence, for this parameter, contrasts for Regimen D should be interpreted cautiously. Mean $AUC_{(0-\infty)}$ values for regimens A, B, C, and D were 4.236, 4.175, 2.36, and 0.965 μg/mL*h, respectively. For the contrast Regimen B versus Regimen A, the ratio was 100.3 %: for the contrast Regimen C versus Regimen A, the ratio was 55.9 %; and for the contrast Regimen D versus Regimen A, the ratio was 27.8 %.

Table 45.29 Summary of pharmacokinetic parameters in plasma

Treatment/location		$AUC_{(0-\infty)}$ (μg/mL.h)	$AUC_{(0-t)}$ (μg/mL.h)	AUC ext (%)	C_{max} (μg/mL)	t_{max} (h)	$t_{½}$ (h)
Immediate release (Reference, A)	N	8	8	8	8	8	8
	Mean	4.236	4.168	1.96	2.0226	0.63	2.129
Proximal small bowel (B)	N	8	8	8	8	8	8
	Mean	4.175	4.111	1.68	1.3791	1.31	2.119
Distal small bowel (C)	N	8	8	8	8	8	8
	Mean	2.360	2.298	3.31	0.4563	2.38	2.686
Ascending colon (D)	N	4	5	4	5	5	4
	Mean	0.965	0.722	18.25	0.1020	3.50	7.22

Mean C_{max} values for regimens A, B, C, and D were 2.02, 1.38, 0.46, and 0.10 μg/mL, respectively. For C_{max} for the contrast Regimen B versus Regimen A, the ratio was 64.5 %: for the contrast Regimen C versus Regimen A, the ratio was 20.2 %; and for the contrast Regimen D versus Regimen A, the ratio was 4.0 %.

The t_{max} tended to increase through the regimen order of A (0.63 h) to B (1.31 h) to C (2.38 h) to D (3.50 h).

The $t_{1/2}$ was similar for Regimens A and B (2.1 h) and slightly longer for Regimen C (2.7 h). Half-life was longer for Regimen D (7.2 h) based on the four subjects for which this was calculated (Table 45.29).

Scintigraphic data: Gastric emptying of the Enterion™ capsule occurred on average at 1.15 h post dose for Regimen B, at 1.28 h post dose for Regimen C, and at 2.39 h post dose for Regimen D.

Small intestinal transit times for the Enterion™ capsule were on average 4.19 h for Regimen B, 4.69 h for Regimen C, and 4.11 h for Regimen D.

Colon arrival of the Enterion™ capsule occurred on average at 5.34 h post dose for Regimen B, at 6.25 h post dose for Regimen C, and at 6.49 h post dose for Regimen D.

Capsule recovery occurred on average at 23.86 h post dose for Regimen B, at 34.23 h post dose for Regimen C, and at 30.59 h post dose for Regimen D. This resulted in colonic transit times of 18.52 h for Regimen B, 25.11 h for Regimen C, and 24.53 h for Regimen D.

Enterion™ capsules were successfully activated in eight subjects for Regimen B, eight subjects for Regimen C, and seven subjects for Regimen D.

Gastrointestinal transit data: Two main parameters influence the gastric emptying of pharmaceutical formulations, the physical size of the dosage form and whether it is administered in the fasted or fed state. When the stomach is empty of food, i.e., in the fasted or interdigestive state, indigestible solids, such as large single unit dosage forms, will be emptied by the action of the migrating myoelectric complex (MMC) (Kelly 1980). The MMC is usually divided into four phases of activity (Bieck 1989), which recur approximately every 2 h. Phase III of the MMC is known as the "housekeeper wave" and acts to empty the stomach of swallowed saliva, cellular debris, and indigestible solids. If the formulation is administered just before a "housekeeper wave," then it may be rapidly emptied from the stomach. However, if the preparation is administered just after phase III activity, then it is probable that the Enterion™ capsule would not be emptied until the next "housekeeper wave" occurred some 2 h later.

The Enterion™ capsules demonstrated typical gastric emptying for the majority of subjects for preparations administered in the fasted state. The capsules left the stomach within 3 h of dosing in seven of the eight subjects for Regimen B (proximal small bowel activation), in seven of the eight subjects (eight subjects dosed on nine occasions) for Regimen C (distal small bowel activation), and in five of the seven subjects for Regimen D (ascending colon activation). This suggests that in the majority of individuals, the first cycle of phase III MMC activity was 100 % efficient. Gastric emptying occurred within 5 h of dosing in the majority of the remaining subjects, suggesting that the capsule was removed from the stomach by the second cycle of phase III MMC activity. An extended gastric residence time of 19.08 h was recorded for subject 3 (Regimen C). Extended gastric residence times have been observed previously with a radiotelemetry capsule (RTC), which is comparable in size to an Enterion™

capsule. The RTC did not empty from the fasted stomach within a 12-h study period, even though phase III contractions were noted at 4.5, 6.4, and 8.5 h post dose, therefore indicating a 2 hourly recurring MMC (Kelly 1980). The study demonstrated that the phase III contractions of the MMC were not always efficient at removing large nondisintegrating dosage forms from the stomach.

Mean small intestinal transit times for the Enterion™ capsule of 4.19 ± 1.19, 4.69 ± 1.71, and 4.11 ± 1.45 h for Regimens B, C, and D, respectively, are not unusual. There is always a high degree of intra- and intersubject variability observed in gastrointestinal tract data (Kelly 1980). These intestinal transit times are in general agreement with the 3 h (±1 h, range 1.3–6 h) previously reported for solutions, pellets, and tablets (Davis et al. 1986).

Colon arrival of the Enterion™ capsule occurred on average at 5.34 ± 1.13, 6.25 ± 1.88, and 6.49 ± 2.50 h post dose for Regimens B, C, and D, respectively. For many of the subjects, colon arrival occurred after the ingestion of food at 5 h post dose (lunch). The ingestion of a meal is known to stimulate colonic activity, and this is termed the gastrocolonic response (GCR). The GCR has been shown to play an important role in determining colon arrival of pharmaceutical preparations following an overnight fast or a light meal (Price et al. 1993a, b).

Capsule recovery occurred on average at 23.86 ± 6.39, 34.23 ± 28.85, and 30.59 ± 26.60 post dose for Regimens B, C, and D, respectively. This resulted in average colonic transit times of 18.52 ± 5.65, 25.11 ± 24.00, and 24.53 ± 25.12 h for Regimens B, C, and D, respectively. Colonic transit times of dosage forms ranging from 1 to 60 h have been reported (Hardy et al. 1985, 1987). The results for colonic transit for this study are therefore in good agreement with those previously reported.

The pharmacokinetics of XYZ123 formulations after release in different regions of the gastrointestinal tract reveal similar exposure for the proximal small bowel as compared to the immediate-release formulation, halved exposure for the distal small bowel, and only poor absorption from the ascending colon. Thus, colonic resorption cannot be relied on for the development of an extended-release formulation. Analysis of the scintigraphic data has confirmed release of the formulation at the target locations in the required number of subjects.

45.17 Special Situations for Drug Delivery: Modified-Release Formulations

PURPOSE/RATIONALE

One of the special situations for drug delivery is the assessment of the pharmacokinetic (PK) properties of a modified-release formulation. Modified-release products always gain importance if the PK/PD profile of a drug is not close to optimal for its target indication mostly because the (short) PK or efficacy half-life does not match the intended dosing frequency.

Details on the design, objectives, and interpretation of bioavailability studies on modified-release products can be found in (US 1997c, d, 2003a; EU CPMP 1999, 2003b).

PROCEDURE

The design of an exploratory bioavailability study on modified-release drug products is presented below. For the design of the study, information from a recent bioavailability study with other modified-release products, from a site-of-absorption study and from a Modeling and Simulation experiment was used.

45.17.1 Protocol Outline

Comparison of pharmacokinetics and safety of extended-release formulations of 600-mg XYZ123 with that of an immediate-release formulation—a single-center, open-label, crossover study in healthy men.

45.17.1.1 Primary Objective

To compare the PK characteristics of extended-release (ER) formulations of XYZ123 with the PK of an immediate-release (IR) formulation of XYZ123.

45.17.1.2 Secondary Objective

To assess the influence of food on the PK of ER formulations of XYZ123.

45.17.1.3 Study Design

The study was carried out in a single-center, open-label, single-dose, four period crossover study design with two independent treatment groups.

Single oral doses of 600-mg XYZ123 were given under fasting and under nonfasting conditions.

The order of treatments and of fasting and nonfasting conditions was randomized in a four-way crossover incomplete block design (treatment groups I and II). The washout periods between the administrations of study medication were at least 48 hour (h) each.

45.17.1.4 Inclusion Criteria

Healthy men aged 18–55 years and assessed as healthy based on findings in medical history, physical examination, blood pressure, pulse rate, and electrocardiogram (ECG) during screening.

45.17.1.5 Treatments

Treatment Group I

Treatment A: 600-mg XYZ123 (1 film-coated tablet containing 200 mg + 1 film-coated tablet containing 400 mg given together) as IR formulation under nonfasting (NF) conditions (reference)

Treatment B: 600-mg XYZ123 in ER formulation (LLL matrix tablet) under fasting (F) and NF conditions.

Treatment C: 600-mg XYZ123 in ER formulation (LLL bilayer tablet) under F and NF conditions.

Treatment Group II

Treatment A: 600-mg XYZ123 (1 film-coated tablet containing 200 mg + 1 film-coated tablet containing 400 mg given together) as IR formulation under nonfasting (NF) conditions (reference)

Treatment D: 600-mg XYZ123 in ER formulation (KKK matrix tablet) under F and NF conditions.

Treatment E: 600-mg XYZ123 in ER formulation (KKK bilayer tablet) under F and NF conditions.

45.17.1.6 Pharmacokinetic Data

Concentration of XYZ123 in plasma before and at predefined times after dosing.

EVALUATION

Bioanalytical data: Individual plasma concentrations of XYZ123 were tabulated together with standard descriptive statistics for each treatment. Individual and median profiles were presented graphically.

In vivo *dissolution data*: The individual hypothetical in vivo dissolutions for the four extended-release formulations administered under fasting and nonfasting conditions relating to XYZ123 were estimated by numerical deconvolution using the individual response to the immediate-release formulation given under nonfasting conditions as the weighting (impulse) function using a hidden function of the validated HOEREP-PC software.

Plateau time data: The additional pharmacokinetic characteristics, i.e., plateau times of XYZ123 (time above 200, 500, 800, and 1,000 ng/mL [h]) were calculated in the interval from administration ($t = 0$) to exactly 12 h thereafter from the plasma concentration-time data pairs and subjected to ANOVA. Points of intersection with a specific plateau concentration were obtained by linear interpolation.

PK data: PK parameters were determined based on plasma concentrations of XYZ123 using noncompartmental procedures.

Primary PK Measure: AUC_{0-inf}

Secondary PK Measures: $C_{12\ h}$, $AUC_{0-12\ h}$, AUC_{0-t}, AUC_{ext} [%], C_{max}, t_{max}, MRT, $t_{1/2\lambda z}$, t_{lag}.

The primary measure was subject to an analysis of variance (ANOVA) including sequence, subject nested within sequence (subject (sequence)), period, and treatment effects. According to the treatment groups and fasting conditions, there were five realizations of the variable treatment. The sequence effect was tested using the subject (sequence) mean square from the ANOVA as an error term. All other main effects were tested against the residual error (error mean square) from the ANOVA. The ANOVA was performed on ln-transformed data. The mean square error was used to construct 90 % confidence intervals for treatment ratios. The point estimates were calculated as ratio of the antilogs of the least square means and were expressed as percentages. The ANOVA was performed separately for subjects in treatment group I and subjects in treatment group II. Point estimates and confidence intervals were primarily calculated for the ratio of each ER formulation to the IR reference formulation. Additionally, the ratios fasting/nonfasting for each ER formulation were calculated.

The secondary pharmacokinetic measures were evaluated descriptively.

For t_{max} frequency, distribution tables were given for each group, formulation, and fasting/nonfasting condition.

CRITICAL ASSESSMENT OF THE METHOD

The study described here has a very complex design for its exploratory approach. It combines four different extended-release formulations, each tested under fasting and nonfasting conditions, and compares the

results to the immediate-release drug product as the reference formulation. The bilayer tablets combine an immediate-release component and an extended-release component in one vehicle. In this project, a close cooperation between the galenics department and the clinical pharmacokinetic function was mandatory. The in vitro/in vivo correlation was done by means of the deconvolution which is an appropriate surrogate to describe the in vivo dissolution.

MODIFICATIONS OF THE METHOD

Recommendations exist to conduct in vivo studies first in an animal species (preferably the pig) before going to man. This approach has limited validity because modified release is primarily defined by the absorption properties of a drug. As absorption is influenced by the general composition, the sums of the physicochemical properties, and the length and residence times of each section of the GI tract, no animal species is similar to man in this respect.

Repeated dosing studies are recommended if the drug product is intended for subchronic or chronic use.

References and Further Readings

EU CPMP (1999) Note for guidance on modified release oral and transdermal dosage forms: section II (Pharmacokinetic and Clinical Evaluation), July 1999

EU CPMP (2003a) Points to consider on the clinical requirements of modified release products to be submitted as a line extension of an existing marketing authorization, June 2003

US FDA (1997c) Guidance for industry: extended release oral dosage forms: development, evaluation, and application of in vitro/in vivo correlations, September 1997

US FDA (1997d) Guidance for industry: SUPAC-MR: modified release solid oral dosage forms, September 1997

US FDA (2003a) Guidance for industry exposure-response relationships—study design, data analysis, and regulatory applications, April 2003

EXAMPLE

To illustrate the amount of data that can be obtained using the discussed study type, an overview of the pharmacokinetic and the deconvolution results obtained from the study described above under *Procedure* is presented below.

45.17.1.7 Results: Pharmacokinetics

The primary objective of the present study was to investigate the pharmacokinetic characteristics of four ER formulations of XYZ123. Two LLL formulations (matrix and bilayer tablets, treatments B and C, respectively) and two KKK formulations (matrix and bilayer tablets, treatments D and E) were compared with the pharmacokinetic characteristics of an IR formulation (treatment A) in the nonfasting state. The secondary PK objective was to assess the influence of food on the pharmacokinetics of these ER formulations of XYZ123.

45.17.1.8 Extended-Release Characteristics (Primary Objective)

LLL tablets (treatments B and C) provided lower C_{max} values and lower relative bioavailability compared to the IR formulation (treatment A). A longer half-life was observed for treatment B (matrix tablets), under both fasting and nonfasting conditions, but for treatment C (bilayer tablets), the lower limit of the 90 % confidence was above 100 % only under fasting conditions.

KKK tablets (treatments D and E) provided lower C_{max} values with similar (though not higher) AUC values for treatment E (bilayer tablets) and similar AUC for treatment D—only under nonfasting conditions. Increased half-lives were recorded for treatments D and E, only under fasting conditions.

Higher $C_{12\,h}$ values and longer MRTs, both features important for ER formulations, were obtained for all LLL tablets, compared to treatment A. A similar but less pronounced effect was observed for KKK formulations, notably under fasting conditions. Only treatment C (LLL, bilayer) had significantly greater time values than treatment A for the time above 800 ng/mL.

The effect of the IR component within the bilayer tablets on the absorption profile was most markedly observed in the figures describing the hypothetical in vivo dissolution. The increase in amount absorbed was steeper for treatments C and E, especially under fasting conditions.

45.17.1.9 Effects of Food (Secondary PK Objective)

No effect of food was recorded on $AUC_{0-\infty}$ and MRT values for the bilayer tablets (treatments C and E), with marginal effect on their rate of absorption and on C_{max} values. The absorption rate of matrix tablets, as well as their C_{max} values, was more affected. $C_{12\,h}$ values presented food consumption effects for all formulations, however, to a larger extent for KKK than for LLL tablets.

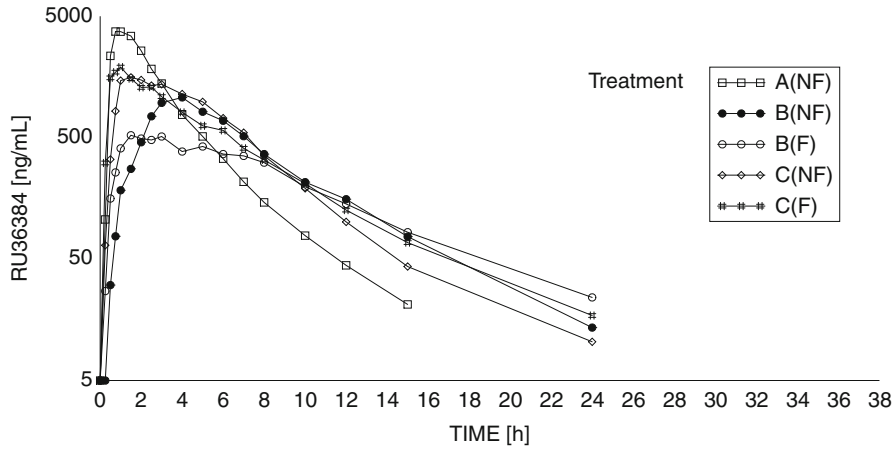

Fig. 45.13 XYZ1234 concentration in plasma versus time following treatments A(NF), B(NF), B(F), C(NF), and C(F) group I. Median plot—log-linear scale

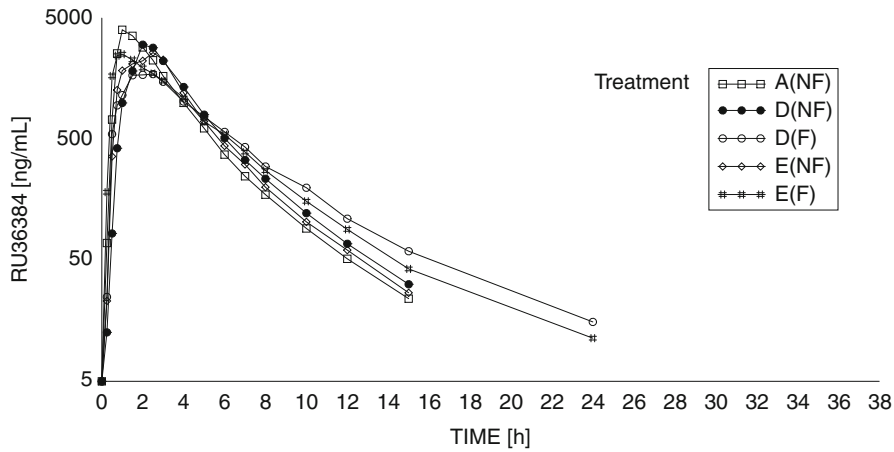

Fig. 45.14 XYZ123 concentration in plasma versus time following treatments A(NF), D(NF), D(F), E(NF), and E(F) Group II. Median plot—log-linear scale

Figures 45.13 and 45.14 show the plots of the median concentration-time profiles of XYZ123 following administration of the several treatments synoptically for the two groups in lin-lin-scaling.

45.17.1.10 Pharmacokinetic Measures and Parameters

The model independent pharmacokinetic characteristics for XYZ123 following single-dose administration of the different treatments were calculated using noncompartmental procedures. The following table gives the arithmetic means, standard deviations, and coefficients of variation as well as the medians and ranges of the primary pharmacokinetic measure $AUC_{0-\infty}$ and of the secondary measures $C_{12\ h}$, C_{max}, and MRT (Table 45.30).

The next table gives further arithmetic mean PK measures and parameters of XYZ123 following oral single-dose administration of XYZ123 (Table 45.31).

45.17.1.11 Statistical/Analytical Issues

The extrapolated part AUC_{ext} of the total areas $AUC_{0-\infty}$ following the four different treatments for XYZ123 did not exceed 4 % in mean and 9 % in maximum.

The primary parameter $AUC_{0-\infty}$ was subjected to an analysis of variance (ANOVA) including sequence, subject nested within sequence (subject (sequence)), period, and treatment (nonfasting/fasting) effects. The sequence effect was tested using the subject (sequence) mean square from the ANOVA as an error term. All other main effects were tested against the residual error (error mean square) from the ANOVA. The ANOVA was performed on ln-transformed data. For ratios, 90 % confidence intervals were constructed. The point estimates and confidence limits were calculated as antilogs and were expressed as percentages. The ANOVA was performed separately for subjects in group I and subjects in group II.

Table 45.30 Arithmetic means and standard deviations (SD) of the primary pharmacokinetic measure $AUC_{0-\infty}$, and of the secondary measures $C_{12\ h}$, C_{max}, and MRT following single-dose administration of the different treatments

Treatment		$AUC_{0-\infty}$ (ng*h/mL)	$C_{12\ h}$ (ng/mL)	C_{max} (ng/mL)	MRT (h)
Group I					
A(NF)	Arithmetic mean	11,076.98	47.66	4,879.50	2.87
	SD	2,357.94	20.93	1,723.06	0.68
B(NF)	Arithmetic mean	7,126.00	149.81	1,343.34	6.65
	SD	2,220.73	61.62	650.08	1.31
B(F)	Arithmetic mean	5,396.57	167.41	660.95	8.27
	SD	1,469.50	95.17	205.40	1.99
C(NF)	Arithmetic mean	9,264.27	110.70	1,955.25	4.80
	SD	2,147.19	53.02	472.25	0.79
C(F)	Arithmetic mean	9,195.10	148.35	2,319.81	5.24
	SD	1,930.40	81.09	598.63	1.13
Group II					
A(NF)	Arithmetic mean	11,079.02	56.68	4,460.81	3.12
	SD	1,988.99	19.80	1,212.04	0.66
D(NF)	Arithmetic mean	10,194.24	68.02	3,226.19	3.90
	SD	1,681.87	18.34	794.93	0.64
D(F)	Arithmetic mean	9,020.77	113.47	1,858.81	5.10
	SD	1,963.15	44.71	355.81	1.36
E(NF)	Arithmetic mean	10,244.97	62.47	3,340.25	3.55
	SD	2,021.91	23.64	902.63	0.48
E(F)	Arithmetic mean	10,634.66	106.72	2,747.50	4.10
	SD	1,506.00	67.24	315.39	0.77

Point estimates and confidence intervals were primarily calculated for the ratio of each ER formulation and the IR reference formulation. Additionally, the ratios fasting/nonfasting for each ER formulation were computed.

No significant effect of either period or sequence was found for the primary parameter $AUC_{0-\infty}$ and for the secondary parameters C_{max} and $t_{1/2\lambda z}$. Treatment effect was highly significant for all three PK parameters, and subject effect was significant for $AUC_{0-\infty}$ and $t_{1/2\lambda z}$ but not for C_{max}.

In addition, the times for which plasma concentrations of XYZ123 were above certain values (200, 500, 800, and 1,000 ng/mL), within the 12-h dosing interval, were calculated for the various formulations and treatment modalities. These results were subjected to ANOVA to assess the statistical significance of the differences. The control treatment A was tested under nonfasting conditions only. In order to get the statistical significance of the differences for the various formulations, without interference of food effects, ANOVA was carried out again comparing treatment A with B, C, D, and E nonfasting. This analysis revealed highly significant treatment effect for both treatment groups I (LLL) and II (KKK) for the time above 200 ng/mL and the time above 500 ng/mL. However, for the time above 800 and 1,000 ng/mL, only group I (LLL) showed statistical significance. Further examination of the results revealed that in fact only treatment C (LLL, bilayer) had greater time values than treatment A (treatment B had lower values).

Table 45.31 Mean pharmacokinetic measures and parameters of XYZ123 following oral single-dose administration of XYZ123

Treatment	$AUC_{0-12\ h}$ (ng*h/L)	AUC_{ext} (%)	t_{max} (h)	$t_{1/2\lambda z}$ (h)	t_{lag} (h)
Treatment group I					
A(NF)	10,881.36	0.83	1.27	2.56	0.02
B(NF)	6,309.06	2.02	3.31	3.66	0.20
B(F)	4,182.51	3.16	2.72	4.86	0.11
C(NF)	8,743.51	0.96	2.34	3.01	0.06
C(F)	8,303.25	1.84	0.98	4.24	0.02
Treatment group II					
A(NF)	10,857.58	0.90	1.52	2.53	0.09
D(NF)	9,908.92	0.95	2.20	2.73	0.11
D(F)	8,282.20	1.44	2.06	4.11	0.08
E(NF)	9,991.45	0.90	2.09	2.76	0.09
E(F)	10,052.60	1.01	1.08	3.25	0.02

The additional pharmacokinetic characteristics, i.e., plateau times of XYZ123 (time above 200, 500, 800, and 1,000 ng/mL [h]) were calculated in the interval from administration ($t = 0$) to exactly 12 h thereafter from the plasma concentration-time data pairs. Points of intersection with a specific plateau concentration were obtained by linear interpolation.

45.17.1.12 Hypothetical in Vivo Dissolution

The individual hypothetical in vivo dissolutions for the four extended-release formulations administered

Table 45.32 Hypothetical dissolution data for XYZ123 obtained by deconvolution using treatment A(NF) as impulse function. Medians

Measures	B(NF)	B(F)	C(NF)	C(F)	D(NF)	D(F)	E(NF)	E(F)
Maximum amount absorbed (mg)	358.97	290.50	485.97	561.49	582.56	460.12	629.25	652.95
Maximum amount absorbed (% of dose)	63.22	51.16	85.58	98.88	102.59	81.03	110.82	114.99
Time to reach maximum amount (h)	15.00	24.00	15.00	15.00	9.00	15.00	2.50	15.00
Time to reach 50 % of maximum amount (h)	2.80	3.34	0.91	0.40	1.04	0.69	0.81	0.37
Time to reach 80 % of maximum amount (h)	5.27	6.95	2.95	1.64	1.79	2.54	1.44	0.48

under fasting and nonfasting conditions relating to XYZ123 were estimated by numerical deconvolution using the individual response to the immediate-release formulation given under nonfasting conditions as the weighting (impulse) function using a hidden function of a validated software.

Deconvolution is used to evaluate in vivo drug release and drug absorption from orally administered drug formulations (i.e., extended release) when data from a known drug input are available. The applied deconvolution method requires data from a formulation with zero order absorption as known input, for example, an oral solution (oral bolus input); the immediate-release formulation used as known input only provides an approximation to the required properties.

The medians and ranges of the hypothetical dissolution data for XYZ123 obtained by deconvolution are listed in the following Table 45.32.

Treatments C and E (the bilayer tablets that contain the IR component) had a steeper amount absorbed profile as compared to the parallel matrix tablets (treatments B and D. This effect was more pronounced under fasting conditions. Only with treatment E (KKK bilayer tablets), the hypothetical in vivo dissolution profiles surpassed the 100 % absorption, both under fasting and nonfasting conditions. For treatment C, this occurred only under fasting conditions, and for treatment D, only under nonfasting conditions.

The mismatch of surpassing 100 % absorption is probably due to method constraints in combination with the immediate-release data, as the deconvolution method requires data from a formulation with zero order absorption for the impulse function, for example, an oral solution (oral bolus input); the immediate-release formulation only provides an approximation to the required properties.

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Population Pharmacokinetics

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PURPOSE AND RATIONALE

Variability in exposure to a drug leads to variability in the clinical response across a patient patient population (Rowland et al. 1985). Estimating the variability of the PK (pharmacokinetics) across a patient population requires data obtained from a large study, typically including more than 100 patients. For ethical and practical reasons, pharmacokinetic properties of a drug are difficult to study in large numbers of patients using the traditional approach.

The PPK (population pharmacokinetic) population approach was suggested by Sheiner et al. (1977) for investigating the typical PK of a drug in a large target population using sparse and unbalanced data obtained without any additional cost during routine care of patients. The PPK approach aims to quantitate the effect of various physiologic factors on drug PK with the overall goal of explaining as much variability as possible.

Using the PPK approach in the development of a new drug has the advantage that the relevant pharmacokinetic parameters for a reasonably large population can be obtained from only a few blood samples per subject. The PPK approach is the method of choice in all situations when only sparse and unbalanced data sparse can be obtained. This situation exists when the PK needs to be studied in elderly, critically ill, and pediatric patients, but also very often in preclinical studies investigating the effects of the drug in animals.

Once such a mathematical model is available, the concentration time courses for various scenarios of administration can be predicted. The dosage can be adjusted to achieve a specific clinical goal like drug exposure within the therapeutic concentration window in the whole population or, if necessary, for special

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subpopulations characterized by their individual physiology. Following the learning and confirming approach (Sheiner 1997), the predicted clinical success for these optimized dose regimens needs to be confirmed in the next clinical study.

The PPK approach estimates the joint distribution of population-specific pharmacokinetic model parameters for a given drug. Fixed effect parameters quantify the relationship, e.g., of clearance to individual physiology like function of liver, kidney, or heart. The volume of distribution is typically related to body size. Random effect parameters quantify the intersubject variability which remains after the fixed effects have been taken into account. Then, the observed concentrations will still be randomly distributed around the concentration time course predicted by the model for an individual subject. This last error term called residual variability needs to be estimated. As fixed and random effects are included, this method is called mixed effects modeling.

The essential features of a population pharmacokinetic study are summarized in a guideline (FDA 1999).

PROCEDURE

The NONMEM (nonlinear mixed effects modeling) software (Beal et al. 1992), mostly used in population pharmacokinetics, was developed at the University of California and is presently distributed by Globomax. For data management, postprocessing, and diagnostic plots, the software S-plus *(Mathsoft)* is frequently used.

Before starting model fitting, all available information obtained in previous studies should be assembled (prior knowledge). The analysis starts with an exploration of the data to generate hypotheses for the model: a statistical summary of demography, plots of the logarithm of the concentration versus time indicate the number of pharmacokinetic compartments involved. With the help of plots of individual time courses in a common coordinate system, subgroups in the population may be identified. Normalizing the curves to unit doses should indicate dose linearity or nonlinearity.

Prior knowledge and various hypotheses are condensed into models. NONMEM determines the parameter vector including fixed and random effects of each model using the maximum likelihood algorithm. NONMEM uses each model to predict the observed data set and selects the best PPK parameter vector minimizing the deviation between model prediction and observed data. Comparing model fits by the criteria discussed in the section "Evaluation" should decide which hypothesis is the most likely. As a general rule, the model should be as simple as possible, and the number of parameters should be at a minimum.

The situation after an IV bolus for a system described by a one compartment model with first order elimination can serve to illustrate the procedure. The observed concentration $c_{i,j}$ of an individual i at the time t_j can be modeled as

$$c_{i,j} = \left(\frac{\text{Dose}}{V_i}\right) e^{-k_i t_j} + \in_{i,j}; \quad \in_{i,j} \sim N(0, \sigma^2) \quad (46.1)$$

with $k_i = \frac{CL_i}{V_i}$, where CL_i and V_i are the individual clearance and the individual volume of subject i. $\epsilon_{i,j}$ is the residual error drawn from a normal distribution with zero mean and variance σ^2 (covariance matrix describing the within subject or residual variability), the intraindividual variability.

The pharmacokinetic parameters themselves are modeled like

$$CL_i = \theta_{CL}\, e^{\eta_{CL_i}}; \qquad \eta_{CL_i} \sim N(0,\ \omega^2), \quad (46.2)$$

where θ_{CL} is a population mean clearance and η_{CL_i} is again a random variable representing the deviation from the population mean of the clearance for the i-th subject. η_{CL_i} is normally distributed with zero mean and variance ω^2 (covariance matrix describing the between subject variability). The unexplained intersubject variability acts as random effect η_{CL_i} on the clearance.

It is important to emphasize that all pharmacokinetic, fixed effect, and random parameters, i.e., θ, ω^2, and σ^2, are fitted in one step as mean values with standard error by NONMEM. A covariance matrix of the random effects can be calculated. For a detailed description of the procedure, see Grasela and Sheiner (1991) and Sheiner and Grasela (1991).

In a subsequent step, the modeler tries to explain part of the unexplained interindividual variability. Fitted individual parameters (or the variable part expressed by η) are plotted against physiological parameters like weight or indicators of renal or metabolic functionality. Identified dependencies should

enter into the model. For example, clearance is very often modeled as depending on the covariate CL_{CR} (creatinine clearance):

$$CL_i = \theta_{CL} \left(\frac{CL_{CR_i}}{4L/h} \right)^{\theta_{CL_{CR}}} e^{\eta_{CL_i}}. \qquad (46.3)$$

In this equation, CL_{CR_i} is the actual creatinine clearance of subject i. The fixed effect parameters are now θ_{CL} corresponding to the clearance of a person with a CL_{CR} of 4 l/h the and $\theta_{CL_{CR}}$ as an exponent describing the increase of CL_i with CL_{CR}.

The relevance of CL_{CR} for clearance is tested using the likelihood ratio test (Beal et al. 1992). The so-called full model 1 (alternative hypothesis) given in Eq. 46.3 is tested against the reduced model with $\theta_{CL_{CR}} = 0$ (null hypothesis) characterized by Eq. 46.2.

The more complex full model is accepted only if the objective function obtained with the full model is more favorable than the objective function obtained with the reduced model (see "Evaluation").

Concentration time courses can be simulated by the model and the demographic parameters for different dose regiments. The final administration of the drug has to be adjusted so that, e.g., 95% of the target population falls into the therapeutic window. If subpopulations differ too much, adjusted administration regiments have to be considered.

EVALUATION

The following criteria determine about the best model:

1. The OF (objective function: negative log of probability, −2 ln(Prob)), calculated by NONMEM, is a measure for the deviation between the model prediction and the observed data. It enters into the likelihood ratio test as follows: if the OF of the full model minus the OF of the reduced model is smaller than −3.84, then the full model can be accepted at a significance level of $p < 0.05$ (Beal et al. 1992).
2. The observed concentrations plotted against the predicted concentrations had to be more randomly distributed around the line of unity.
3. The weighted residuals and the individual residuals plotted against the predicted concentrations had to show the most symmetric distribution around zero.

In order to validate the final model, the data set can be split randomly into two parts. The model is developed with one part, the index data set. With this model and the demographic data of the second part, the validation data set, observations for the validation data set can be predicted. The difference of predicted data and observations is a measure of the accuracy of the model. An alternative is the bootstrap method (Efron 1981).

MODIFICATION OF THE METHOD

Data from individuals drawn from a target population are not completely independent. Concentration time curves (longitudinal data) of a subject are considered to be driven by a functionality depending on individual parameter values. But what is the connection between the same parameters in different persons? Parts of it may be described by a functionality depending on demographic variables. In any case, unexplained intra- and interindividual random effects remain. Mixed effect modeling clearly distinguishes between these two sources of randomness.

Modifications of the method differ in the way they deal with these different levels of random effects, i.e., how they distribute or confound them. It should be noted that the different handling of random effects has also consequences for the fixed effects.

1. In a situation with many data from each individual drawn in an intersubject balanced manner, a two-stage method is very often used: each individual is fitted individually without considering the interindividual dependencies. In a second step, the parameters are resumed as population mean and standard deviation, often considered as interindividual variability. (STS (standard two-stage method), (Steimer et al. 1985)).
2. If only a few data per subject are available, they are sometimes pooled and considered as coming from one hyperanimal. If several observations are available at the same time, they are averaged, and means and standard deviations can be calculated. In a second step, the mean values are fitted to a pharmacokinetic model. (NAD (naive averaging data method) (Steimer et al. 1985)). A different naive technique is the NPD (naive pooled data) method proposed by Sheiner (Sheiner and Beal 1980). Again, all data are pooled but fitted in one step to a pharmacokinetic model. In both cases, intra- and interindividual random effects are confounded. An influence of covariates cannot be determined by this approach.

3. Mixed effect modeling deals with the situation in between. Inter- and intraindividual variabilities are separated and calculated within the same step. Interindividual random effects are calculated for those parameters for which this information can be drawn from the data set. In general, only one residual error is calculated. The method is very well suited for sparse and unbalanced data situations.

Population pharmacokinetics can be extended to pharmacodynamics and PK/PD modeling using a link model like an effect compartment (Sheiner et al. 1979). In huge clinical trials, only a limited number of patients can be included in a pharmacokinetic satellite study. The model is developed in this satellite. Knowing the demographic covariates of the patients in the whole study, concentration time curves and even effect time curves can be predicted.

Alternative software like NPEM uses nonparametric procedures for the statistical part of the models (Jelliffe et al. 1990).

CRITICAL ASSESSMENT OF THE METHOD

The NAD and NPD methods confound several sources of variability and very often give biased estimates of the mean values of the pharmacokinetic parameters (Steimer et al. 1985). But when the population is very homogeneous, the naive approaches already give reasonable results. The widespread STS method requires the estimation of a large number of parameters, reducing the degree of freedom and leading to over parametrization (large SEMs).

Mixed effect modeling is a very flexible one-step method. It can cope with many situations. It is the only method which can deal with sparse data and unbalanced data sparse situations. The method can start in preclinical phases with animal data. In phase I with a homogeneous population and many observations per individual, the structural model, dose linearity, and bioavailability are determined. In phase II and phase III, patients are investigated, and the demographic parameters should spread over a large range in order to determine the variability in the target population. The method is well suited to perform meta-analysis of several studies.

It should be emphasized that models are not the truth and that different models can describe the same data with the same accuracy. Whereas interpolation for doses or covariates is in general possible, extrapolations should be considered with care. Extrapolation with different models, if available, can give a feeling about the range for the observations to be expected.

Simulations should be used for the design of the next experiment (trial). The new observations should be compared to the prediction allowing improvement of the model in an iterative manner (Sheiner 1997).

EXAMPLE

Introduction

Levofloxacin is the l-isomer of the racemate ofloxacin, a quinolone antibacterial agent used worldwide to treat a wide range of infections. The PK profile of levofloxacin was first characterized in healthy volunteers. The following prior knowledge was obtained before the clinical study presented below. Levofloxacin is primarily excreted renally. Increasing doses of levofloxacin showed linear PK over the investigated dose range between 50 and 600 mg. The PPK of levofloxacin used in patients with respiratory tract infections was investigated by Tanigawara et al. (1995).

Objective

Can 500-mg levofloxacin given twice daily achieve the therapeutic goal of plasma levels above 2 mg/l, the MIC (minimum inhibitory concentration) in male and in female patients?

Materials and Methods

The PPK were analyzed in a subpopulation of 44 out of 314 patients with pneumonia being treated with levofloxacin. Patients received two daily doses of 500 mg for 10 to 15 days. Initially, the drug was given intravenously as an infusion for approximately 60 min. The switch from IV to oral treatment was suggested after a minimum of four IV doses. Three to five blood samples were taken from each patient, 199 blood samples in total.

The available concentration time data is typical for a clinical study: there are relatively few observations on each of a large number of patients, and samples are not taken at the same time points (sparse and unbalanced data). Neither the NAD method nor the STS method can be used. A one compartment model with absorption compartment and first order elimination was fitted to the data by mixed effect modeling with

NONMEM. Clearance and volume of distribution were described by

$$CL_i = \theta_{CL}\left(\frac{CL_{CR_i}}{4L/h}\right)e^{\eta_{CL_i}} \quad (46.4)$$

(Eq. 46.3 with $\theta_{CL_{CR}} = 1$)

$$V_i = \theta_V \left(\frac{WT_i}{70\ kg}\right)\ (1 + (2 - SEX_i)\theta_{SEX})\ e^{\eta V_i}. \quad (46.5)$$

The model uses CL_{CR}, WT (body weight [kg]), and SEX (1 = male and 2 = female) as covariates.

Alternatively, the volume model was simplified using LBM (lean body mass in kg) instead of WT and SEX. LBM is related to WT, HT (height in cm), and SEX in the following equation:

$$LBM\begin{pmatrix}\text{male}\\ \text{female}\end{pmatrix} = \begin{pmatrix}1.1\\ 1.07\end{pmatrix} WT - \begin{pmatrix}128\\ 148\end{pmatrix}\left(\frac{WT\ cm}{HT\ kg}\right)^2 kg. \quad (46.6)$$

The model for V (volume of distribution) given as

$$V_i = \theta_V \left(\frac{LBM_i}{50kg}\right)\ e^{\eta V_i} \quad (46.7)$$

needs one parameter, θ_{SEX}, less than the model given in Eq. 46.5. Now, the PPK model uses in total only two covariates, i.e., CLCR and LBM.

RESULTS

PK Differences Between Male and Female Patients? The volume given in Eq. 46.5 as a full model (A) changes with $\theta_{SEX} = 0$ to a reduced model reduced model (B). To perform the likelihood ratio test, both models were fitted with NONMEM, and the OF of the full model (A) was 6.39 points lower than the OF obtained for the reduced model (B). This difference is highly significant, so the full model (A) is preferred when compared to a reduced model (B).

WT and SEX are combined in LBM. To simplify the model, we described the fixed effect on V only with LBM as a single measure of body size. Using Eq. 46.7 in model (C), we repeated the NONMEM fit and compared the OF obtained for model (C) with the previous two fits. Model (A) was still 3.1 points better than model (C). We preferred model (C) because it uses only body size while model (A) uses body size and sex as demographic covariates in the V model. Table 46.1 resumes the values of the PPK parameter vector including θ (vector of parameter, describing the fixed effect model), ω^2, and σ^2 calculated for model (C).

Table 46.1 PK parameters from mixed effect modeling using Eqs. 46.4 and 46.7. The absorption coefficient θ_{KA} was fixed to 1/h

	Parameter	Mean	SEM
Fixed effects θ	θ_{CL} [l/h]	5.3	0.3
	θ_V [l]	76	5
	θ_{KA} [1/h]	1	fix
Random effects ω^2	${\omega_{CL}}^2$	0.12	0.02
	${\omega_V}^2$	0.11	0.04
Residual error σ^2	σ^2	0.03	0.01

Concentration time curves for three individuals with different kidney functions CL_{CR} are shown in Fig. 46.1. The broken lines CL_{CR} represent the time-dependent CL_{CR} as a measure of the kidney function. For the subject shown in the center panel, the CL_{CR} decreases at 3.5 days causing a steep increase in the drug concentration. Dots are observations CONC (observed concentration), and full lines PRED (model predictions for the population with $\eta = 0$) correspond to the model predictions for a typical individual with a specific set of mean covariates CL_{CR}, WT, and SEX (fixed effects). The broken lines IPRE (model predictions for the individual subject with random η_i) are the individual predictions for the subject taking the random effects on volume and clearance into account.

Once the model is in place, simulations can be performed in order to find or to verify the optimal dose regimen.

In Fig. 46.2, mean C_{ss} (concentration at steady state), given in Eq. 46.8, as a function of CL_{CR} is shown.

$$C_{ss,i} = \frac{\text{dose rate}}{CL_i} \quad (46.8)$$

The circles correspond to the observations. The lines are calculated using the fit parameters and Eq. 46.2 for the 2.5%, 50%, and 97.5% quantiles. All

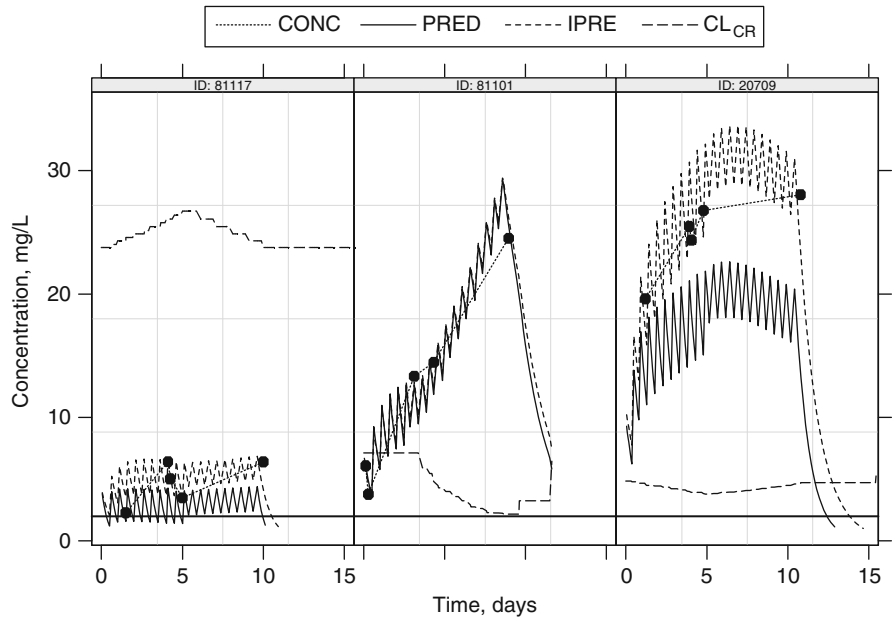

Fig. 46.1 Individual concentration time courses. *CONC* creatinine clearance; *PRED* model predictions for the population with $\eta = 0$; *IPRE* model predictions for the individual subject with random η_i; CL_{CR} creatinine clearance in l/h is calculated as left-hand scale *10/4

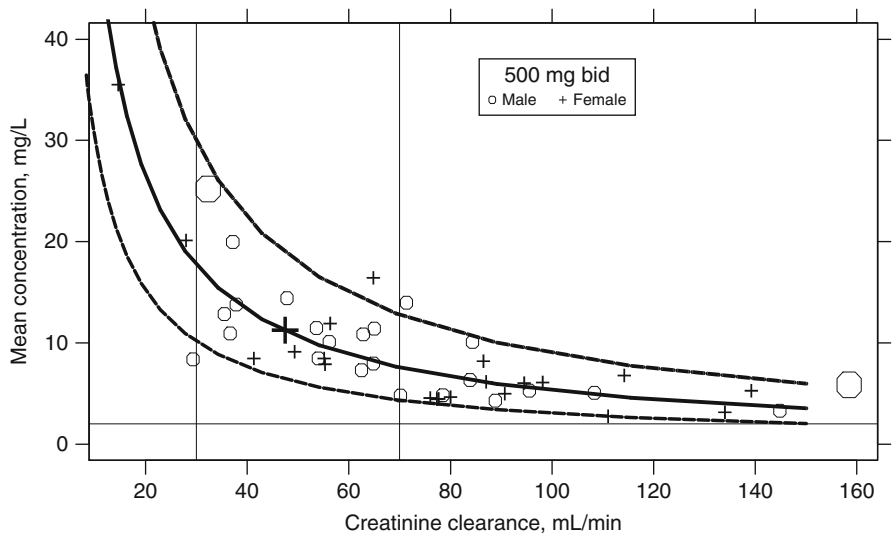

Fig. 46.2 Mean concentrations at steady state after twice daily 500-mg levofloxacin. The circles and crosses correspondent to the individual CL estimates in male and female patients, respectively. Three filled circles correspond to patients which PK is shown in Fig. 46.1. The lines are calculated using the fit parameters given in Table 46.1 and Eq. 46.2 for the 2.5, 50, and 97.5% quantiles. All except two individuals are within these limits. The vertical solid lines mark patients with creatinine clearances of 30 ml/min and 70 ml/min, curves stop at 150 ml/min

except two individuals are within these limits. As reveals from Fig. 46.2, the selected dose regimen of 500 mg twice daily achieves even in more than 95% of male and female patients with normal kidney function C_{ss} concentrations above the MIC of 2 mg/l.

Figure 46.3 shows the joint distribution of V and CL_{tot} (total clearance) for males and females as calculated by the model (C). Volume and clearance are distributed around mean values (center of the ellipse), and they are slightly correlated to

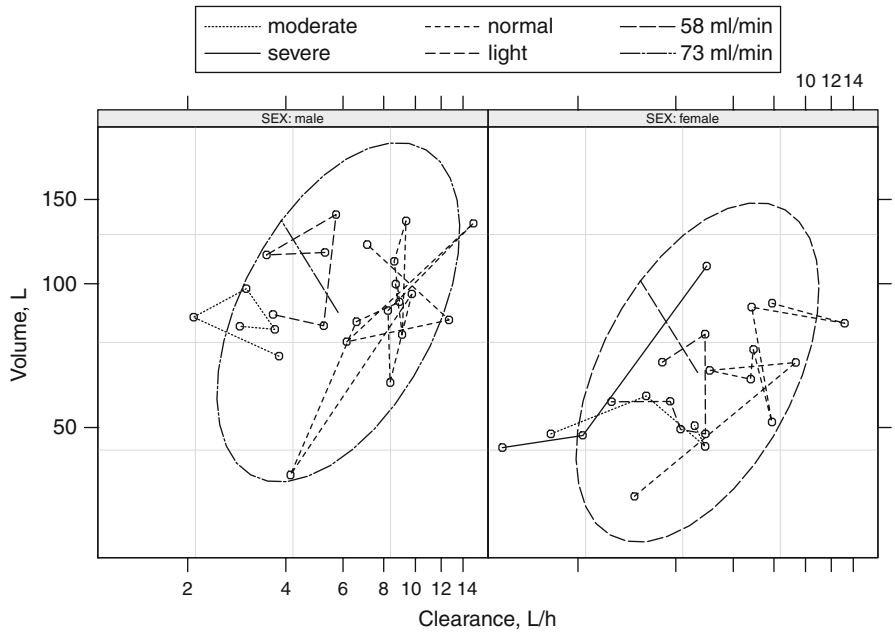

Fig. 46.3 Joint distribution of V and CL_{tot}. Ellipse joint 95% prediction interval in a subpopulation of male patients (CLCR = 72.53 ml/min and LBM = 57.63 kg) and in a subpopulation of female patients (58.32 ml/min and 43.12 kg). The individual V and CL_{tot} estimates calculated by NONMEM are grouped according to the degree of renal failure

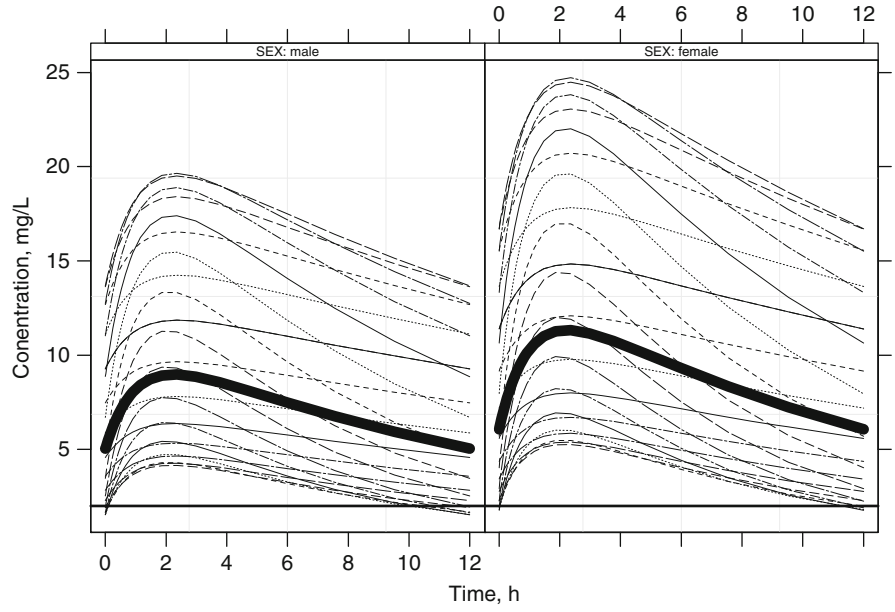

Fig. 46.4 Simulations of the concentration time course under steady state conditions as predicted by the model (C) for a 500-mg twice daily dose regimen. The broad line corresponds to the typical male (CLCR = 72.53 ml/min and LBM = 57.63 kg) and female (58.32 ml/min and 43.12 kg) patients. All other lines are calculated using only CL and V pairs of the 95% contour line of their joint probability of occurrence as shown in Fig. 46.3 as ellipses. The horizontal solid line marks the MIC of 2 mg/l

each other. The 95% contour line of their joint probability of occurrence is shown as ellipses for male and female.

Simulations of the concentration time course under steady state conditions as predicted by the model for a 500-mg twice daily dose regimen are shown in Fig. 46.4. The broad line corresponds to the typical male and female patients. All other lines are calculated using only CL and V pairs of the 95% contour line of their joint probability of occurrence as shown in Fig. 46.3 as ellipses. As reveals from Fig. 46.4, concentration time courses remain within an interval describing the concentrations expected in 95% of male or female patients.

DISCUSSION

The PPK approach uses all the data observed at all sampling times and from all subjects enrolled in the satellite study in a single step to extract the information necessary to optimize a dose regimen.

For the example of levofloxacin given twice daily 500 mg, 95% of male and female patients achieved the therapeutic goal and showed concentrations above 2 mg/l (MIC) for more than 10 hours of the 12-h dose interval. Due to their smaller volume of distribution, peak concentrations are higher, and half-lives are shorter in female patients. The different extents of accumulation as an effect of differences in half-lives become evident when comparing the through levels. The highest concentrations reached are still below the safety limits. Therefore, the same dose regimen for male and female patients was recommended.

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Typical PK/PD Approaches in Preclinical and Clinical Development

47

Willi Weber

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PURPOSE AND RATIONALE

47.1 PKPD Modeling in Drug Development

A development project starts with someone identifying in a patient population an unmet medical need, which may offer a business opportunity. Even when successful treatments are available for the target disease, a medical need may exist when standard treatments fail or unacceptable adverse events (AE) occur after longtime treatment.

An unmet medical need was identified in the population of patients with rheumatoid arthritis (RA). RA belongs to the class of autoimmune diseases. Immunosuppressive drugs like methotrexate (MTX) were successfully introduced as RA standard therapy. However, after long-term treatment with MTX, severe AE like lack of efficacy or liver toxicity were observed.

In that situation, the patient could benefit from a new alternative treatment. As an alternative treatment, leflunomide (Lef) was developed and introduced as an immune-modulating drug into the market in 1998.

In the first part of this chapter, I will describe how medical knowledge about autoimmune diseases is transformed in a general PKPD model for immunosuppressive drugs (Bohl 2006).

In the second part, I will report about the use of the PKPD model during the development of Lef.

47.1.1 Model Described in Words

White blood cells play an important role in the immune defense of pathogens. In case of an infection, white

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blood cells can grow very fast and can double their cell population within hours. Prior to cell division, the DNA content of the cell must be duplicated. A sufficient pool of nucleotides must be built. Pyrimidine is one important precursor for nucleotide formation.

In case of a normal infection, the body rapidly attacks and kills the invading infectious organism. In case of RA autoimmune disease, the immune attack cannot win. The attack is targeted against the body's own joint cartilage, which luckily cannot be destroyed completely. The surviving body's own antigen is continuously activating the immune system.

Immunosuppressive drugs like MTX reduce the immune activity, which otherwise would destroy the body's own joint cartilage. The mechanism of action is well known for MTX. MTX inhibits the pyrimidine neosynthesis. Lack of pyrimidine will reduce the neosynthesis of nucleotides. Lack of nucleotides is slowing down the growth rate of fast-dividing cells like the immune cells. Immune cells, like B- and T-lymphocytes, belong to the white blood cell population.

To build a PKPD model, we need to find first the right questions.

47.1.1.1 What Do We Know About the Disease States in RA?

The autoimmune response to the body's own joint cartilage leads to an increased growth rate of immunocompetent cells. The increased population of these cells attacks the cartilage even more efficiently, leading to accelerated destruction of joint cartilage. To reduce or even stop the autoimmune attack, our drug has to decrease the size of the attacking population of immunocompetent cells. The equilibrium size of the immune cell population during the chronic disease state has to descend to a lower equilibrium size during the drug treatment. The reduced clinical symptoms should increase the frequency of clinical success criteria like ACR20 in RA patients (Arnett et al. 1988). The PKPD model must include the dynamics of cell growth (PD model) and its interaction with the drug exposure (PK model). We assume that the increased size of the immune cell population is destroying the joint cartilage, which is causing severe RA symptoms. The goal of an immunosuppressive therapy is to reduce the size of the immunocompetent cell population and increase the probability of positive clinical outcome criteria.

After describing qualitatively the mechanism of the wanted drug action, we need to get more quantitative insight into the drug effects. We have to translate our words into mathematics. The result of our efforts will give us a PKPD model useful for optimizing immunosuppressive therapy in future clinical studies.

47.1.2 Mathematical Model

Let us start with the dynamics of cell growth. If resources are unlimited in the environment of a small biological cell population, its growth rate

$$\dot{x}(t) = k_f x(t) \tag{47.1}$$

is proportional to the actual size of the population $x(t)$. k_f is the growth rate constant per unit of time. Equation 47.1 describes an exponential growth of the population. The exponential growth will double the population size with a doubling time of $T_2 = log(2)/k_f$. A small number of cells grow independently from each other. Every cell can divide without limitation of resources.

However, with increasing size of the population, the resources in the environment will become more and more limited for the increasing population. The individual cells must share the same limited resources. If a group of cells occupy the resources, others have to wait for the renewal of resources, e.g., the neosynthesis of nucleotides.

Cells cannot divide unlimitedly anymore. The growth rate decreases. Before dividing, the cells must interact with each other. Verhulst introduced a negative quadratic interaction term in the exponential growth in Eq. 47.1. With increasing size, the growth rate is decreasing. The population is approaching an equilibrium state determined by the resources offered by the environment. Verhulst suggested the following equation:

$$\dot{x}(t) = k_f x(t) - k_d x^2(t) \tag{47.2}$$

with k_d as the fractional cell death per unit of time.

During the progress of RA, the immune cell population is growing due to the antigen stimulus. The population starts at an initial condition x_0 and approaches with time to its equilibrium population size K, which is given as

$$\dot{x}(t) = 0 \rightarrow \lim_{t\to\infty} x(t) = K = \frac{k_f}{k_d} \tag{47.3}$$

determined by the strength of the antigen stimulus.

Any maintenance treatment cares most on the equilibrium state conditions. In the light of a maintenance therapy, the equilibrium population size K is especially important to understand the Verhulst equation. Factoring out the expression $k_f\,x(t)$ and introducing the equilibrium population size K, we obtain a version of the Verhulst equation:

$$\dot{x}(t) = k_f x(t)\left\{1 - \frac{x(t)}{K}\right\} \tag{47.4}$$

which is the logistic differential equation (DG). The analytical solution of the Verhulst equation is given as:

$$x(t) = \frac{K}{1 + \left\{\frac{K}{x_0} - 1\right\} e^{-k_f t}}. \tag{47.5}$$

Both Eqs. 47.1 and 47.4 contain an exponential growth term $k_f x(t)$. While the doubling time T_D remains constant for exponential growth, the doubling time for the Verhulst equation prolongs more and more with growing population until an equilibrium state K of the population is reached. Growth and dying processes are now balanced out.

An equilibrium state will remain constant as far as the environmental condition remains unchanged. Changes of the environment will disturb the equilibrium. With increasing resources, the population will grow again until the additional resources are consumed. The goal of our immunosuppressive therapy is the opposite. We decrease the resources. After limiting cell division, the population starts to shrink until the smaller population can again balance the death rate loss.

The relation between $x(t)$ and K in Eq. 47.4 defines the sign of the growth rate. In case of a patient with autoimmune disease, we can differentiate three different states of the disease.

The disease state can

1. Progress, $x_0 < K \leftrightarrow \dot{x}(t) > 0$. Disease stimuli cause sigmoid growth with a positive cell turnover rate until the increased equilibrium size of the cell population K can respond with the requested immune activity.
2. Be stable, $x_0 = K \leftrightarrow \dot{x}(t) = 0$. The cell population remains constantly at the equilibrium size K with constantly increased immune activity. The cell turnover is balanced out.
3. Improve, $x_0 > \widetilde{K} \leftrightarrow \dot{x}(t) < 0$. After the start of an immunosuppressive treatment, a sudden lack of precursors slows down the cell growth rate. The now overcrowded cell population x_0 decreases with a negative turnover rate until the lower equilibrium size $\widetilde{K} < K$ with sufficient precursor production is reached.

47.1.3 Dynamics of Drug Effects on the Disease State

We assume that the probability of observing disease symptoms is directly related to the size of the immune cell population. Now we can start to think about the treatment effects in terms of the cell growth PKPD model described above. We assume the patients reached a stable equilibrium autoimmune disease state K before treatment starts. Unfortunately the size K of the immune cell population is increased by the autoimmune disease, causing severe damage of the joint cartilage and causing severe pain. The treatment idea is that decreasing the size of the immune cell population to a lower level $\widetilde{K}$ will attenuate the immune activity and immune attack of body's own tissue becomes less aggressive. The joint destruction slows down or stops. The body may even regenerate its damaged cartilage. Clinical symptoms in the patient should improve with time.

We start the treatment period from the baseline disease state K with severe symptoms before treatment. The drug will decrease the neosynthesis of required precursors for the cell division. The growth rate k_f will be reduced with increasing drug concentration $C(t)$. The drug effect on k_f is introduced by using the concentration-dependent inhibitor function $I(C(t))$ (Lambertus et al. 2005), which transfers the time dependency to $k_f^\star$ and to K^* as follows:

$$k_f^\star(t) = k_f \times I(C(t)) \tag{47.6}$$

$$K^\star(t) = \frac{k_f^\star(t)}{k_d} = K \times I(C(t)) \tag{47.7}$$

The link between PK and PD is described by the system of two linear DGs

$$PK \Rightarrow \dot{C}(t) = k_e C(t) + \frac{\dot{D}}{V}\sigma\left(t - T_{inf}\right) \tag{47.8}$$

$$PD \Rightarrow \dot{x}(t) = k_f^{\star}(C(t))x(t)\left\{1 - \frac{x(t)}{K^{\star}(C(t))}\right\} \tag{47.9}$$

using the ratio between the clearance CL and the volume of distribution V as pharmacokinetic elimination rate constant $k_e = \frac{CL}{V}$. The mean residence time (MRT) is calculated as the inverse of the elimination rate constant. We will use a constant infusion rate $\dot{D}$ as a step input function $\sigma(t–T_{inf})$ for the drug. During the infusion $\sigma(t \leq T_{inf}) = 1$ obtain the unity value, outside the zero value.

The solution of the Verhulst Eq. 47.5 holds only for a time-independent growth rate constant k_f. In case of a time-dependent change of $\widetilde{k_f}(t)$ as result of a time-dependent drug concentration, the solution of the system of two differential Eqs. 47.14 and 47.15 is much more complex than the solution given in Eq. 47.5. The dynamics of the growth rate inhibited by $\widetilde{k_f}(t)$ must be numerically calculated. However, if we reached a pharmacokinetic steady state with a constant steady state concentration C_{ss}, $\widetilde{k_f}(C_{ss})$ is a time-independent constant again dependent on the steady state concentration. The transition of the cell population from x_0=K to $\widetilde{K}(C_{ss})$ is described by Eq. 47.5 using $\widetilde{k_f}(C_{ss})$ instead of k_f.

The extent of the enzyme inhibition depends on the ratio between the drug concentration and the drug potency IC_{50}. The sigmoid transition between no effect and full effect is well described using the logistic probability distribution. Enzyme inhibition may be limited to a maximum extent of I_{max}. The value range $0 < P_{logis} < 1$ of the logistic distribution describes an increasing inhibition with increasing drug concentration as follows:

$$I(C(t)) = 1 - I_{max}P_{logis}(C(t)) \tag{47.10}$$

$$P_{logis} = \frac{e^{\eta}}{1 + e^{\eta}}, \quad \text{with} \quad \eta = logit(P) = S \times log\left(\frac{C(t)}{IC_{50}}\right) \tag{47.11}$$

The reverse of slope S is the standard deviation of the logistic probability distribution P_{logis}.

The time dependency of the concentration-dependent inhibition of $\widetilde{k_f}(t)$ interconnects the PK and the PD of our PKPD system. Before introducing the inhibited growth rate constant into the PD system given in Eq. 47.4, we will make life easier by introducing dimensionless transformations of our variables. The dynamics of the DG system is easier overseen with the use of the following dimensionless variables:

$$\widetilde{t} = kt = \frac{t}{MRT}, \quad \widetilde{C} = \frac{C}{IC50}, \quad \widetilde{\dot{D}} = \frac{\dot{D}}{\dot{D}_{50}}, \quad \dot{D}_{50} = CL \times IC_{50} \tag{47.12}$$

$$\widetilde{x} = \frac{x}{x(t=0)}, \quad \kappa = \frac{k_f}{k_e}, \quad \widetilde{K} = \frac{K^{\star}}{K} = I(\widetilde{C}) \tag{47.13}$$

After transforming the system DGs 47.4 using dimensionless variables 47.12 and 47.13, we obtain the following equations:

$$\frac{d\widetilde{C}}{d\widetilde{t}} = -\widetilde{C} + \widetilde{\dot{D}} \tag{47.14}$$

$$\frac{d\widetilde{x}}{d\widetilde{t}} = \kappa\widetilde{x}\left\{I(\widetilde{C}) - \widetilde{x}\right\} \tag{47.15}$$

The new equilibrium disease state relative to the disease state before treatment is only dependent on $\widetilde{C}$, the ratio between drug concentration and drug potency. The dynamics of the transition depends on the ratio κ between the PD and the PK rate constants. Figure 47.3 illustrates the dynamics of the described PKPD model.

After the start of drug treatment, the dynamics of PK and PD progress simultaneously to their equilibrium states. To estimate the time needed until the onset of efficacy is obtained, we need to solve differential equation system given in Eqs. 47.14 and 47.15. Because the inhibition is changing with increasing concentration (PK 47.14), the turnover rate constant of the PD system 47.15 is time dependent as well. In such complex situation, using numerical methods is best applicable to solve the DG.

47.1.4 PD Dynamics When PK is at Steady State

Daily administration of the maintenance dose can often be approximated by a constant drug infusion rate $\dot{D}$. Without use of a loading dose, the steady state concentration $\widetilde{C}_{ss} \approx \dot{D}$ is reached after approximately $\widetilde{t} = 3.7$ corresponding to 3.7 MRT (mean residence time, $MRT = k_e^{-1}$) time units. With a constant concentration, the inhibition function I will cause a constant decrease of k_f as well. With $k_f^\star$ no longer time dependent, we can again use the analytical solution of Verhulst Eq. 47.5. Using $\widetilde{x}(t)$ and the inhibited constants K^* and $k_f^\star$, Eq. 47.5 describes the change of the disease state relative to the disease state before treatment.

The PK time scale kt is no longer important because $\kappa \times t^* = k_f t$. We can return to the PD typical time $k_f t$. The patient is assumed in equilibrium disease state $\widetilde{x}_0 = 1$ before the treatment. The treatment period starts the transition from $\widetilde{K} = 1$ to the new equilibrium disease state $\widetilde{K} = I(\widetilde{C}_{ss}) < 1$.

We can establish the PK steady state from the start of treatment using an appropriate loading dose. If we are able to extract the drug after end of infusion almost immediately, the inhibition of the turnover rate constant would stop immediately. The undisturbed turnover rate constant k_f would determine the return to the pretreatment equilibrium state K. With $\widetilde{x}_0 = \widetilde{K}$, the transition back to the pretreatment disease state will again follow the Verhulst solution given in Eq. 47.5.

47.1.5 Equilibrium Disease State During PKPD Steady State

The PD system will follow the PK system in approaching its equilibrium state. The equilibrium disease state of the full PKPD system is only dependent on the inhibitor function I Eq. 47.10:

$$\lim_{t \to \infty} \widetilde{x}(t) = I(\widetilde{C}_{ss}) \tag{47.16}$$

In the following chapter, we will show the usefulness of the described PKPD model during the development of leflunomide for patients with rheumatoid arthritis (RA).

EVALUATION OF THE METHOD

47.2 PKPD of Leflunomide

Like methotrexate MTX, Lef (Rozman 2002) is used as immunosuppressive therapy in rheumatoid arthritis. Both are inhibiting the pyrimidine neosynthesis. The mechanism of action, although similar, revealed core enzymes, which are different between both drugs. Teriflunomide, the active metabolite of Lef, inhibits the dihydroorotate dehydrogenase DHODH enzyme in the metabolic pathway of pyrimidine neosynthesis.

47.2.1 Drug Potency for Enzyme Inhibition

To know the target enzyme is very important to estimate drug potency IC50, which determines the target concentration range of drug action. In preclinical experiments, the DHODH inhibition of various Lef concentrations was investigated. Experiments were performed using human DHODH enzyme and human cell cultures. Both Lef and its active metabolite teriflunomide are more than 99% bound to plasma proteins. Protein binding was found similar between healthy subjects and patients with RA. The potency IC50 was approximately 10 mg/L total concentration. The results for the slope $S \approx 5$ remained imprecise. The influence of S on the relationship between extent of inhibition and dose rate is shown in Fig. 47.1. The sigmoid relationship between the relative enzyme inhibition I and the dimensionless dose rate $\dot{D}^\star$ for S values between 1 and 6 is shown in the left panel. Inhibition up to 90 % would need for S values between $2 \le S \le 5$ a dimensionless dose rate $1.5 \le \dot{D}^\star \le 3$. For $S < 2$, the sigmoid curves are rather similar.

47.2.2 Probability of Success or Failure

We assume the change $I(\widetilde{C}_{ss})$ in the disease state $K \to K^*$ is proportional to the change of the probability of observing clinical symptoms like pain, number of swollen joints, and inflammation indicators. The change of clinical symptoms is summarized as a binary variable like ACR20 (Arnett et al. 1988), with P(Y = y) as the probability of observing

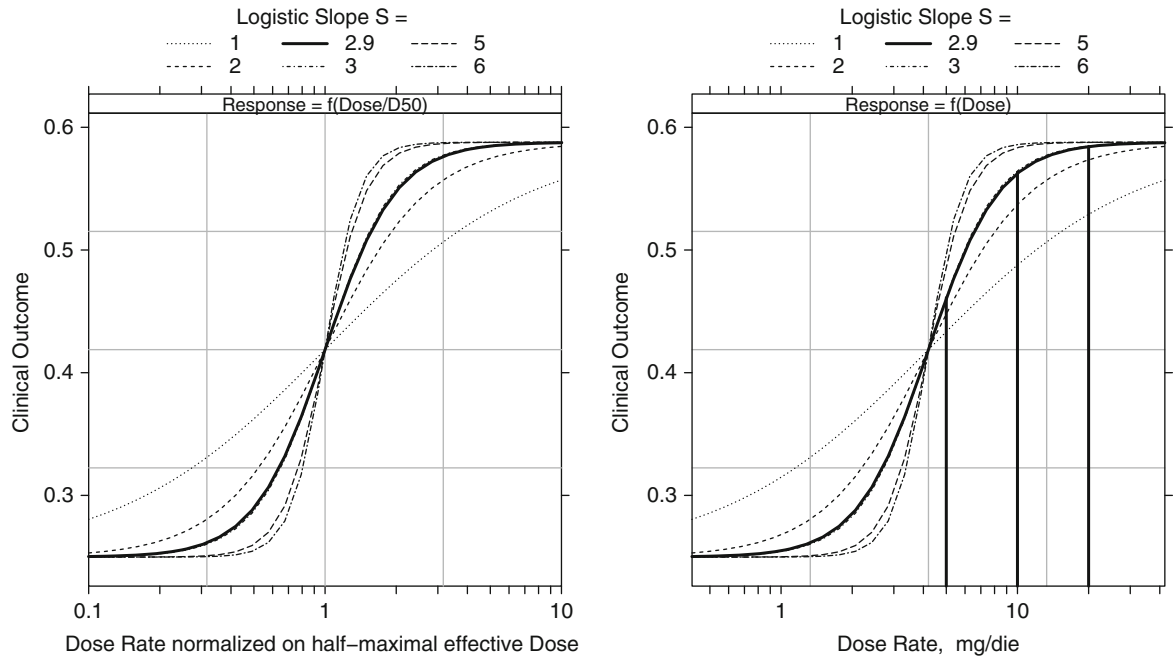

Fig. 47.1 Relationship between the D50-related dose rate D^* and drug response. The dose rate for a target response rate is influenced by the steepness of the logistic curve S = 1:6

a success (y = 1) or a failure (y = 0) as treatment effect. Clinical outcomes are compared to the pretreatment disease equilibrium states.

The success probability is given as the sum of the drug and placebo effects (see (Collett 2003), page 117). We used the following extended Aboth's formula:

$$P_i = P_0 + (P_{max} - P_0) \times P_{logis}\left(\widetilde{\dot{D}}_i\right) \qquad (47.17)$$

$$P(Y = y) = P_i^y \times (1 - P_i)^{1-y}; y = \{0, 1\} \qquad (47.18)$$

P_{max} and P_0 are the maximum values for the probability of success observed in the treatment group and placebo group, respectively.

Appropriate dose regimen should at least reach the range of IC50 concentrations. The pooled phase I data were analyzed using the population approach. The dose rate achieving 50% of the maximum inhibition is given as:

$$\dot{D}_{50} = CL \times IC_{50} \qquad (47.19)$$

Using the population mean estimate of the clearance CL = 0.6 L/day and the IC50 = 10 mg/L, we obtained a target dose rate of $\dot{D}_{50} \approx 6\,\text{mg/day}$ (Weber and Harnisch 1997a). Simulations of dose regimens of 5, 10, and 25 mg/day revealed reasonable exposure to cover active concentrations in the range of IC50 (see Fig. 47.1, right panel).

47.2.3 Clinical Outcome at PKPD Equilibrium

A clinical dose finding study was performed comparing the effect of 5-, 10-, and 25-mg daily Lef doses against placebo. Approximately 100 patients with severe RA were treated in each subgroup for 6 months (Balant and Aarons 1997; Mladenovic et al. 1995; Weber and Harnisch 1997b).

The treatment effect was estimated at monthly visits. The ACR20 criterion was chosen as primary clinical outcome variable. ACR20 summarizes 6 variables to a single binary response of success or failure. ACR20 was estimated at every visit. Only the last visit data was used to prove clinical efficacy of Lef (see Table 47.1).

The maximum success rate was $P_{max} = 0.58$. There was a remarkable placebo response of $P_0 = 0.25$. The

Table 47.1 Data used to calculate the relationship between dose/concentration and effect at steady state after 6 months of treatment

Dose mg/day	0	5	10	25
No. of patients treated	102	95	100	101
No. of successful outcome	25	32	56	59
Fraction of success %	24.5	33.7	56.0	58.4

difference (P_{max}-P_0) in the probability of success should be dependent on drug exposure.

The PK data obtained simultaneously was analyzed using the population approach. The clinical outcome data was analyzed by a logistic regression analysis (Groß 2010) using Eq. 47.17. The binary outcome data was used as dependent variable and the steady state concentration C_{ss} as predictor variable.

We assumed PK steady state for all patients after 6-month treatment. We estimated the individual steady state concentration $C_{ss,i} = \dot{D}_i / CL_i$ using the Bayesian estimates calculated from the observed individual concentration data and the population PK results. The following mean parameter estimates and the coefficient of variation CV diagonal of the covariance matrix:

$$\theta_{PK} = \{CL = 0.06/day,\ \ V = 10\,L,\ \ k_a \approx 1\,h\} \tag{47.20}$$

$$CV_{PK} = \{CL = 0.6,\ \ V = 0.2,\ \ k_a = 0\} \tag{47.21}$$

$$\theta_{PD} = \{P0 = .25,\ \ P_{max} = 0.58,\ \ IC_{50} = 10\,mg/L,\ \ S = 5\} \tag{47.22}$$

$$CV_{PD} = \{P0 = 0,\ \ P_{max} = 0,\ \ IC_{50} = 0.2,\ \ S = 0\} \tag{47.23}$$

were estimated.

The relationship between the probability of success and failure, respectively, and the steady state concentration $C_{ss,i}$ is shown in Fig. 47.2. Summarizing the complete data, the full relationship is shown in the right panel. The data obtained for each treatment group is shown separately in the left panel. Although the success fraction obtained in the 10- and 25-mg dose group was not significantly different, the PKPD analysis revealed a relevant fraction of patients missing the full potential of the drug after 10-mg maintenance dose. The 25-mg dose group got the full benefit of leflunomide. However, an increased frequency of increased liver enzymes was observed. On the basis of this PKPD analysis, we decided to investigate 20 mg daily in the large clinical phase III trial (Horn and Oed 2003; Poor and Strand 2004).

47.2.4 Onset of Efficacy

The cell population model given in the differential equation system for the PK Eq. 47.14 and the PD Eq. 47.15 part and the estimated population PKPD parameters given in Eqs. 47.20, 47.21, 47.22, and 47.23 were used to simulate various dose regimens from 1 to 40 mg/day with and without loading doses. The PK and PD time courses for typical patients are shown in Fig. 47.3. The upper panels show the courses without the lower panels with loading doses.

Comparing the maintenance dose results with and without loading doses revealed an earlier onset of efficacy for lower-dose regimens. We accepted the time needed to reduce the relative disease state by ≈50 % as onset of efficacy. The results of simulations are given in Tables 47.2 and 47.3 without loading dose and with loading dose, respectively. For 40- and 20-mg daily onset of efficacy needed independently of a loading dose given or not given approximately 1.9 MRT units. However, for lower-maintenance dose regimens, a loading dose reduced the time to onset of efficacy slightly. Administering 10 mg daily, an additional loading dose shortens the time interval from 2.86 to 2.4 MRT units.

Increasing the maintenance dose from 10 to 20 mg daily alone reduced the time to onset of efficacy from 2.9 to 1.9 MRT units. The MRT of Lef was ≈25 days. Onset of 80% of efficacy using a maintenance dose of 10 and 20 mg daily is expected after 7 and 10 weeks, respectively. A single MRT unit of 25 days is clinically relevant for RA patients suffering severe pain. Because the coefficient of variation was 60% for the between-subject variability of the clearance, the MRT, as ratio between volume of distribution and clearance, is highly variable too. In extreme cases, the MRT was longer than 2 months. In such patient, starting the Lef treatment with 10 mg daily instead of the recommended 20-mg maintenance dose would delay the onset of efficacy for 2 months.

The effects of various loading doses alone were shown in Figure 47.4. A fast and extended disturbance

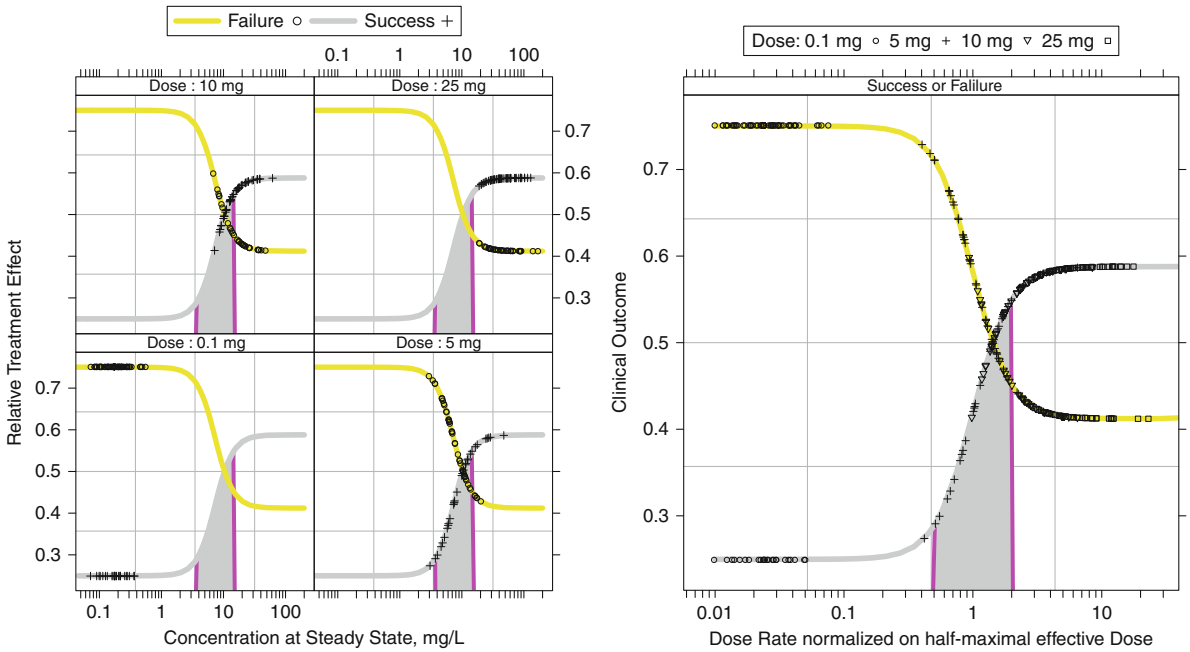

Fig. 47.2 Relationship between clinical outcome and drug exposure after 6 months of treatment. *Left panel*: complete data split per treatment group. Target steady state concentration ranges between 4 and 14 mg/L linked to 30 and 55% success, respectively. *Right panel*: complete data. Target standardized dose $\frac{\dot{D}}{CL \times IC_{50}}$ ranges between 0.5 and 2. To show the placebo data, a very low ineffective dose was used. Success data is following the increasing and failure data following the decreasing sigmoid curves

of the equilibrium disease state is followed by a slow return to the baseline. Drug concentration was almost diminished during the return to baseline. The return to baseline is determined predominantly by the dynamics of the PD system alone.

Our PKPD analysis estimated a turnover time for our PD system of approximately 17 days (Danhof and Steimer 1998; Weber and Harnisch 1998). The PK turnover time or MRT was approximately 25 days. We decided to investigate a dose rate of 20 mg daily in phase III. To obtain the onset of efficacy as early as possible, we started treatment with a loading dose. For a typical patient, an onset of efficacy after 6–8 weeks and an almost maximum probability of success (55%) were observed (Poor and Strand 2004).

47.3 Summary

The typical PKPD approach is describing the relationship between an administered dose and the observed clinical effects. Ideas about new innovative treatments are first described qualitatively in words. The PKPD approach should use all ideas and scientific knowledge as elements of the PKPD model. Using mathematical models helps thinking more quantitatively. PKPD modeling is always a learning process.

Autoimmune diseases are chronic diseases because the body's own antigen cannot be destroyed completely. The antigen continuously stimulates the immune system. A chronic increased population of immune cells is causing hyperactivity against the body's own structures. In case of RA, the joint cartilage is damaged. Inflammation and pain are the typical clinical symptoms.

Knowing about the circumstances of the disease, we can translate our knowledge into mathematics. PKPD modeling is the use of mathematics in medicine. The increased population of immune cells is the basic problem of autoimmune diseases. The right answer was found with immunosuppressive drugs reducing the critical population moderately.

Preclinical PKPD experiments with Lef revealed an antiproliferative effect on cells. Immune cells are

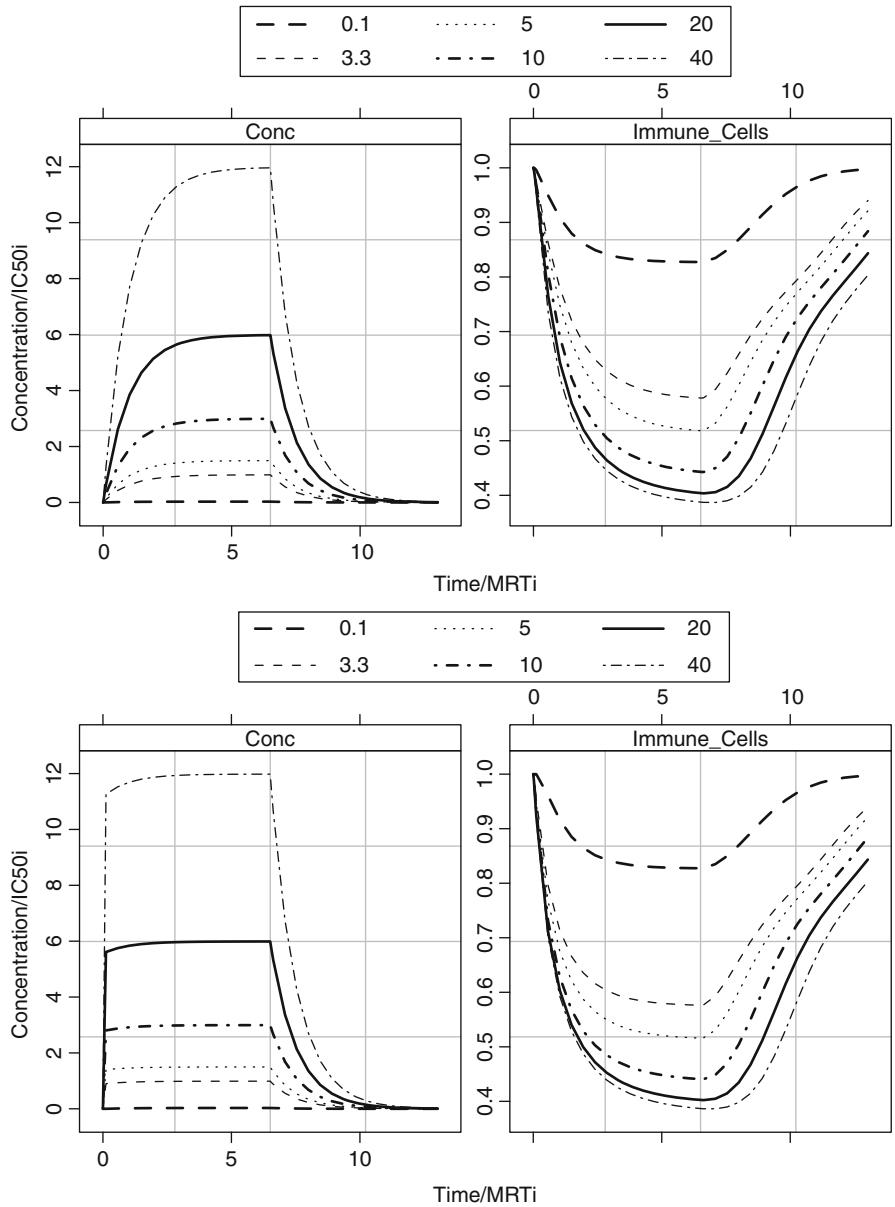

Fig. 47.3 Relationship between the PK and drug response and the dimensionless time normalized on individual mean residence time MRTi are shown in the *left* and *right panels*, respectively. Simulations with and without loading doses are shown in the *upper* and *lower panels*, respectively. Different maintenance doses of Lef are indicated by the line types given in the key panel. Inactive maintenance dose of 0.1 mg/day shows the placebo response. *Left panel*: drug concentration relative to its potency IC50. *Right panel*: Immune cell population relative to its pretreatment state

Table 47.2 Onset time to 0.8 P_{max}: no loading dose (Fig. 47.3)

	Dose, mg/day	Time/MRT	Day	1-I(t)
1	10	2.86	72	0.51
2	20	1.94	49	0.53
3	40	1.94	49	0.52

Table 47.3 Onset time to 0.8 P_{max} after loading dose (Figs. 47.3 and 47.4)

	Load, mg	Dose, mg/day	Time/MRT	Day	1-I(t)
1	84	10	2.40	61	0.50
2	168	20	1.94	49	0.51
3	337	40	1.94	49	0.51

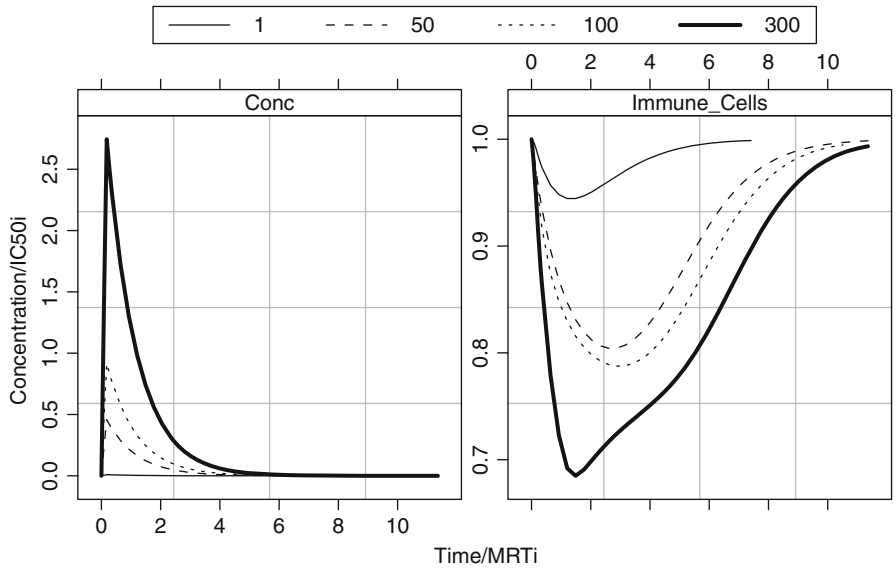

Fig. 47.4 PKPD effects of the loading dose alone. Different loading doses of Lef are indicated by the line types given in the key panel. Inactive loading dose of 1 mg shows the placebo response. Time normalized on individual mean residence time MRTi. *Left panel*: drug concentration relative to its potency IC50. *Right panel*: Immune cell population relative to its pretreatment state

fast-dividing cells which are sensitive to antiproliferative effects.

The growth rate of such population is well described using the Verhulst equation. The PD effect of Lef inhibits the formation of precursors required for cell division. The size of the immune cell population shrinks, and the autoimmune attack against the body's own tissues is attenuated.

In our PKPD model for Lef, we assume that the probability of observing a clinical success is a linear function of the extent of the decrease of the immune cell population. The disease improves and the clinical outcome criteria will change more frequently to success.

The available PKPD model with the mean population parameter and its variance were used to predict the dose regimen for the successfully finished phase III study. The PKPD results obtained from the phase III data were important for the labeling and the question and answer process during the submission. Lef is on the market since 1998.

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Part 3

Safety Toxicology

Safety Toxicology: Introduction

48

Dieter Mayer

In 2006, the first Edition of *Drug Discovery and Evaluation. Safety and Pharmacokinetic Assays* was published by Springer. As methodologies of safety assays developed rapidly it appeared necessary to work on a second edition. The work on the second edition was initiated by our friend and honorable teacher Prof. Dr. Gerhard Vogel, Frankfurt, who passed away last year. Therefore, I would like to devote this section to him respectfully.

Major additions to the methods available at the end of 2005 happened in the area of in vitro toxicology, such as assays in isolated cells (e.g., renal cells). Phospholipidosis, hepatic cholestasis, and mitochondrial dysfunction were considered as important endpoints and new assays were developed.

In the area of genotoxicity, no major changes were noted with one small exception: the in vivo Comet Assay, which is used increasingly. Relevant protocols were developed for a large variety of tissues which can be used in the Comet assay.

With regard to reproductive effects, methodologies were enriched by OECD guidelines for combined studies with reproductive and classical toxicity endpoints. However, these new guidelines are mainly adopted in the area of industrial toxicology (REACH). In summary, the reproductive toxicology methods seemed to be rather stable.

No major methodologies were added in the field of in silico methods. However, efforts were conducted in order to further evaluate the predictive value of in silico methods such as MulticCASE and DEREK.

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International Guidelines

49

Gerd Bode

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49.1 General Considerations

The *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use* (ICH) is a project that brings together the regulatory authorities of *Europe*, *Japan*, and the *United States* and experts from the *pharmaceutical industry* and academia in the three regions to discuss scientific and technical aspects of pharmaceutical product registration of small molecules and biotechnology-derived compounds.

The International Conferences on Harmonization continue their endeavor to harmonize the regulatory recommendations for the development of pharmaceutical compounds by meetings of experts from the three supporting regions twice per annum. No big conferences are being organized any longer, because they are considered to be too expensive. The global cooperation continues by limited numbers of experts and Topic Leaders who publicize the progress of their documents in the internet www.ich.org. New guidelines have come into existence and older ones are being updated and improved. This holds true for all disciplines like Quality, nonclinical Safety, and Efficacy. This chapter will refer to the existing guidelines and report about the newer developments relevant for nonclinical Safety. ICH has produced a comprehensive set of safety Guidelines to uncover potential risks like carcinogenicity, genotoxicity, and reprotoxicity. ICH created a breakthrough with functional nonclinical testing strategy for assessing the QT interval prolongation liability: the single most important cause of drug withdrawals in recent years. With multidisciplinary guidelines ICH implemented crosscutting topics which do not fit uniquely into one of the Quality, Safety, and Efficacy categories.

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These multidisciplinary guidances include, e.g., the ICH medical terminology (MedDRA), the Common Technical Document (CTD), and the development of Electronic Standards for the Transfer of Regulatory Information (ESTRI).

Toxicology and safety pharmacology studies support the detection of undesirable adverse effects of pharmaceutical or chemical compounds and identify hazards; they describe the type and degree of toxicity and asses the risks. They support the management of the risks in humans, when participating in clinical trials or later as patients after market authorization, and analyze the mechanisms behind the alterations; they extrapolate preclinical hazards to humans and finally help to communicate these risks to populations concerned with exposure of that particular substance.

Toxicology therefore contributes to the safe use of pharmaceutical compounds in humans and facilitates the acceptability of submitted documents by regulatory agencies and later as therapeutic principles by patients.

For the safety of pharmaceutical compounds, a great number of in vitro and/or in vivo experiments are being conducted today. Test strategies for toxicological investigations have been refined and the extrapolation of preclinical results to humans improved. The prevention of toxic events in man became the main purpose of preclinical experimentation.

Considerable differences were observed in the creation of guidance and legal conditions. Accordingly, in different regions differently designed studies were conducted, but the repetition of similar studies in different regions did not support the safety but only increased the number of animals being used.

Accordingly, public resistance and concerns among researchers increased the desire to harmonize test procedures and recommendation worldwide. With the creation of the International Conferences of Harmonization (ICH) in 1989 these initiatives were channeled into a global continuing process.

The ICH focuses on guidances for Quality, Preclinical Safety (= Toxicology), and Efficacy (clinical effective substances) of pharmaceutical compounds. This section illustrates all ICH Guidelines important for the safe use of drugs worldwide.

49.2 Background to the ICH Conference

The International Conferences was jointly supported and organized by the Commission of the European Communities (CEC), the US Food and Drug Administration (FDA), the Japanese Ministry of Health and Welfare (MHW), together with the pharmaceutical industry, as represented by the International Federation of Pharmaceutical Manufacturers Associations (IFPMA), the European Federation of Pharmaceutical Industry Associations (EFPIA), the US Pharmaceutical Manufacturers Association (PMA), and the Japanese Pharmaceutical Manufacturers Association (PMA).

Many important initiatives have been undertaken between regulatory authorities and industry associations, particularly on a bilateral basis, to promote harmonization of regulatory requirements between the three regions Japan, USA, and the European Community. ICH owes much to these initiatives of experts during international symposia.

The Main Objectives of the ICH:

1. To provide a forum for a constructive dialogue between regulatory authorities and the pharmaceutical industry on the real and perceived differences in the technical requirements for product registration in the CEC, USA, and Japan.
2. To identify areas where modifications in technical requirements or greater mutual acceptance of research and development procedures could lead to a more economical use of human, animal, and material resources, without compromising safety.
3. To make recommendations on practical ways to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for registration.

49.3 Preparatory Activities for ICH Conferences

A Steering Committee was appointed with members from EU, FDA, MHW, EFPIA, JPMA, PMA, and IFPMA, with observers from WHO and from the regulatory authorities of Canada and Switzerland (for EFTA).

The Steering Committee set up three joint industry/regulatory Expert Working Groups to deal with the technical aspects of the three main subject

areas—Quality, Safety, and Efficacy, these were discussed in three parallel workshops during the conferences. Each of the Expert Working Groups has members, representing EU, FDA, MHW, EFPIA, PMA, and JPMA. With advice from these technical Working Groups, the Steering Committee was responsible for the selection and prioritization of the topics discussed at the Workshops at the ICH conferences.

The International Conference on Harmonization differs from many other harmonization initiatives in that it has a recognized status and is backed by a commitment on the part of both industry and regulators. Commitment to these objectives, set out in the Terms of Reference, was reaffirmed by the Steering Committee in a statement issued following the meeting held in Tokyo, October 1990.

49.4 Success of the ICHs

There is considerable success of the international discussions and constructive solutions documented in the guidelines. There are more than 36 guidelines focusing on issues concerning the quality of compounds. There are more than 10 guidelines dealing with safety issues to be solved during development or later for marketing authorization and there are more than 20 guidelines dealing with problems of efficacy during clinical trials or after marketing authorization. Implemented guidelines are summarized in the following (Tables 49.1–4).

49.5 Description of the ICH Guidelines on Preclinical Safety

49.5.1 Carcinogenicity

In the following a survey is given in regard to the guidelines on preclinical safety.

There are a number of endpoints which cannot be tested in humans, these are mainly: genotoxicity, teratogenicity, and cancerogenicity. Especially, the cancerogenic risk can usually not be tested in humans, it is ethically forbidden and such a risk for patients is unacceptable.

The testing for carcinogenic potential today employs short-, mid-, and long-term studies in rodents, which have a relatively high power of predictivity for the carcinogenic risk in humans.

49.5.1.1 General Regulatory Background

Treatment with compounds associated with carcinogenic potential is unacceptable for banal indications; for severe indications like life-threatening cancer diseases the treatment with carcinogenic compounds often does not increase the overall risk of the underlying disease, especially when end-stage patients with limited life expectancy are being treated.

Carcinogenicity studies are the longest (lifelong or of 2 year duration) and most expensive (approximately 1.5–4 million dollars or Euros) preclinical studies. They should therefore be well designed and conducted in such a way that they clearly indicate any risk involved. These conditions explain why a great number of international and regional guidelines exist and give best advice for the researchers.

49.5.1.2 Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals (S1A)

This guideline was adopted in 1997 and implemented in all regions.

The objectives of carcinogenicity studies are:

- To identify a tumorigenic potential in animals
- To assess the relevance of these identified risks for humans. Any cause for concern derived from laboratory investigations, animal toxicology studies, and data in humans, may lead to a need for carcinogenicity studies

Carcinogenicity studies should be performed for any pharmaceutical whose expected clinical use is continuous for at least 6 months. Certain classes of compounds may not be used continuously over a minimum of 6 months but may be expected to be used repeatedly in an intermittent manner. For pharmaceuticals used frequently in an intermittent manner during the treatment of chronic or recurrent conditions, carcinogenicity studies are generally needed. Examples of such conditions include allergic rhinitis, depression, and anxiety.

Pharmaceuticals administered infrequently or for short duration of exposure (e.g., anesthetics and radiolabel imaging agents) do not need carcinogenicity studies unless there is cause for concern.

Table 49.1 Summary of all guidelines which have been agreed upon in the area of Quality: Finalized Guidelines (*Step 4/5*)

Q1A(R2)	Stability Testing of New Drug Substances and Products (Second Revision)	Feb. 2003
Q1B	Stability Testing: Photostability Testing of New Drug Substances and Products	Nov. 1996
Q1C	Stability Testing for New Dosage Forms	Nov. 1996
Q1D	Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products	Feb. 2002
Q1E	Evaluation for Stability Data	Feb. 2003
Q1F*	Stability Data Package for Registration Applications in Climatic Zones III and IV *(Guideline withdrawn in June 2006)*	Feb. 2003
Q2(R1)	Validation of Analytical Procedures: Text and Methodology *(The Addendum dated November 1996 has been incorporated into the core guideline in November 2005)*	Oct. 1994
Q3A(R2)	Impurities in New Drug Substances	Oct. 2006
Q3B(R2)	Impurities in New Drug Products	Jun. 2006
Q3C(R5)	Impurities: Guideline for Residual Solvents *(including the two Revised PDE for THF and NMP dated September 2002 and October 2002 incorporated in core Guideline in November 2005 and revised PDE for Cumene incorporated in core Guideline in February 2011)*	Feb. 2011
Q4B	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions	Nov. 2007
Q4B Annex 1(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Residue on Ignition/Sulphated Ash General Chapter	Sep. 2010
Q4B Annex 2(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Extractable Volume of Parenteral Preparations General Chapter	Sep. 2010
Q4B Annex 3(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Particulate Contamination: Sub-Visible Particles General Chapter	Sep. 2010
Q4B Annex 4A(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests, General Chapter	Sep. 2010
Q4B Annex 4B(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Tests for Specified Micro-Organisms General Chapter	Sep. 2010
Q4B Annex 4C(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use General Chapter	Sep. 2010
Q4B Annex 5(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Disintegration Test General Chapter	Sep. 2010
Q4B Annex 7(R2)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Dissolution Test General Chapter	Nov. 2010
Q4B Annex 8(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Sterility Chapter General Chapter	Sep. 2010
Q4B Annex 9(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Tablet Friability General Chapter	Sep. 2010
Q4B Annex 10(R1)	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Polyacrylamide Gel Electrophoresis General Chapter	Sep. 2010
Q4B Annex 11	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Capillary Electrophoresis General Chapter	June 2010
Q4B Annex 12	Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Analytical Sieving General Chapter	June 2010
Q5A(R1)	Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin	Sep. 1999
Q5B	Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products	Nov. 1995
Q5C	Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products	Nov. 1995
Q5D	Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products	Jul. 1997

(*continued*)

Table 49.1 (continued)

Q5E	Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process	Nov. 2004
Q6A	Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances	Oct. 1999
Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products	Mar. 1999
Q7	Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients	Nov. 2000
Q8(R1)	Pharmaceutical Development	Nov. 2008
Q9	Quality Risk Management	Nov. 2005
Q10	Pharmaceutical Quality System	June 2008

Table 49.2 A summary of all guidelines which have been issued for the nonclinical safety

S1A	Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals	Nov. 1995
S1B	Testing for Carcinogenicity of Pharmaceuticals	July 1997
S1C(R2)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals	March 2008
S2(R1)	Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for human Use	Nov 2011
S3A	Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies	Oct. 1994
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	Oct. 1994
S4	Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing)	Sept. 1998
S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility *(the addendum dated November 1995 has been incorporated into the core guideline in November 2005)*	June 1993
S6(R1)	Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals	June 2011
S7A	Safety Pharmacology Studies for Human Pharmaceuticals	Nov 2000
S7B	The Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals	May 2005
S8	Immunotoxicity Studies for Human Pharmaceuticals	Sept. 2005
S9	Nonclinical Evaluation for Anticancer Pharmaceuticals	Oct. 2009

Carcinogenicity studies may be recommended if there is cause for concern, e.g., due to:

- Previous demonstration of carcinogenic potential in the product class that is considered relevant to humans.
- Structure-activity relationship suggesting carcinogenic risk.
- Evidence of preneoplastic lesions in repeated dose toxicity studies.
- Long-term tissue retention of parent compound or metabolite(s) resulting in local tissue reactions or other pathophysiological responses.
- Unequivocally genotoxic compounds need not to be subjected to long-term carcinogenicity studies. However, if such a drug is intended to be administered chronically to humans a chronic toxicity study (up to 1 year) may be necessary to detect early tumorigenic effects. In practice, this option has not been used since 1997. Main concern of industry is the lack of historical comparison data. The tumor evaluation was always based on 2 year data.
- In instances, where the life expectancy in the indicated population is short (i.e., less than 2–3 years) no long-term carcinogenicity studies may be required. For example, oncolytic agents intended for treatment of advanced systemic disease do not generally need carcinogenicity studies. It is assumed that the limited life

Table 49.3 A summary of all guidelines which have been issued for the clinical efficacy

E1	The Extent of Population Exposure to Assess Clinical Safety for Drugs Intended for Long-Term Treatment of Non-Life-Threatening Conditions	Oct. 1994
E2A	Clinical Safety Data Management: Definitions and Standards for Expedited Reporting	Oct. 1994
E2B(R2)	*Clinical Safety Data Management: Data Elements for Transmission of Individual Case Safety Reports* (This guideline is re-opened for revision under *Step 2*. See E2B(R3))	Feb. 2001
E2C(R1)	Clinical Safety Data Management: Periodic Safety Update Reports for Marketed Drugs *(The Addendum dated February 2003 has been incorporated into the core guideline in November 2005)*	Nov. 1996
E2D	Post-Approval Safety Data Management: Definitions and Standards for Expedited Reporting	Nov. 2003
E2E	Pharmacovigilance Planning	Nov. 2004
E2F	Development Safety Update Report	Aug. 2010
E3	Structure and Content of Clinical Study Reports	Nov. 1995
E4	Dose-Response Information to Support Drug Registration	March 1994
E5(R1)	Ethnic Factors in the Acceptability of Foreign Clinical Data	March 1998
E6(R1)	Good Clinical Practice: Consolidated Guideline	May 1996
E7	Studies in Support of Special Populations: Geriatrics	June 1993
E7	Q & A Studies in Support of Special Populations: Geriatrics Questions & Answers	July 2010
E8	General Considerations for Clinical Trials	July 1997
E9	Statistical Principles for Clinical Trials	Feb. 1998
E10	Choice of Control Group and Related Issues in Clinical Trials	July 2000
E11	Clinical Investigation of Medicinal Products in the Pediatric Population	July 2000
E14	The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs	May 2005
E15	Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories	Nov. 2007
E16	Biomarkers Related to Drug or Biotechnology Product Development: Context, Structure and Format of Qualification Submissions	Aug. 2010

Table 49.4 A summary of all guidelines which have been agreed upon on multidisciplinary subjects

M2 ICSR (R2)	Electronic Transmission of Individual Case Safety Reports Message Specification (ICH ICSRDTD Version 2.1) companion document to E2B(R3)	Feb. 2001
M3(R2)	Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals	June 2009
M3(R2) Q&As (R2)	Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals Questions & Answers	March 2012
M4(R3)	Organisation of the Common Technical Document for the Registration of Pharmaceuticals for Human Use *(Edited with Numbering and Section Header Changes, September 2002)* Including the Annex : the Granularity Document (Revised November 2003)	Nov 2000
M4Q(R1)	The Common Technical Document for the Registration of Pharmaceuticals for Human Use : **Quality** *(Edited with Numbering and Section Header Changes, September 2002)*	Nov 2000
M4S(R2)	The Common Technical Document for the Registration of Pharmaceuticals for Human Use : **Safety** *(Edited with Numbering and Section Header Changes, September 2002)*	Nov 2000
M4E(R1)	The Common Technical Document for the Registration of Pharmaceuticals for Human Use : **Efficacy** *(Edited with Numbering and Section Header Changes, September 2002)*	Nov 2000

expectancy is too short for the new development of a neoplasia.

- In cases, where the therapeutic agent for cancer is generally successful and life is significantly prolonged, there may be requirements to provide knowledge about the tumorigenic risk.
- When such pharmaceuticals are intended for adjuvant therapy in tumor free patients or for prolonged

Table 49.5 S1B: Basic principle for testing the carcinogenic potential

One long-term rodent carcinogenicity study plus
One short- or medium-term study that
Supplements the long-term carcinogenicity study and
Provides additional information not readily available from the long-term assay

use in noncancer indications, carcinogenicity studies are usually needed.

- Pharmaceuticals showing poor systemic exposure from topical routes in humans may not need studies by the oral route to assess the carcinogenic potential to internal organs.
- Carcinogenicity studies are not generally needed for endogenous substances, when given essentially as replacement therapy (i.e., physiological levels), particularly, where there is previous clinical experience with similar products (e.g., animal insulins, pituitary hormones).

49.5.1.3 Testing for Carcinogenicity of Pharmaceuticals (S1B)

S1B informs about the testing strategies, this guideline was adopted and implemented in 1997.

Historically, the regulatory requirements for the assessment of the carcinogenic potential were based on the conduct of long-term carcinogenicity studies in two rodent species, usually the rat and the mouse. It was the mission of ICH to examine whether this practice could be reduced without compromising human safety. The discussion in the Expert Working Group soon revealed that the rule of testing in two species had to continue, the American consumer societies demanded continuation with the same standard of safety, based on two species testing.

As the new experimental approach to testing for carcinogenic potential, a basic scheme was set up to comprise one long-term rodent carcinogenicity study, plus one other study of the type that supplements the long-term carcinogenicity study and provides additional information that is not readily available from the long-term assay (Table 49.5).

The species selected should be appropriate, based on considerations on pharmacology, repeated-dose toxicology data, metabolism (see also Guidelines S1C and S3A), toxicokinetics (see also Guidelines S1C, S3A, and S3B), and route of administration (e.g., less common routes such as dermal and inhalation). In the absence of clear evidence favoring one species, it is recommended that the rat be selected for the long-term study.

Additional tests may either be short- or medium-term in vivo rodent test systems, usually using the mouse. These models of carcinogenesis may use transgenic or neonatal rodents. The guideline also includes models of initiation promotion in rodents; but these models are today considered to be useful models for hepatic carcinogenesis or adequate mechanistic studies, but not as assays appropriate as general screen for drug-induced carcinogenesis.

A long-term carcinogenicity study in a second rodent species (e.g., mice) is still considered acceptable. This strategy is often used, because of the long-time experience with this model for decades and the rich historical data base.

The guideline describes in the "Notes" important information about the new models. Note 1 informs about the SHE assay; Note 2 on conditions to limit testing with one species only: if the findings of a short- or long-term carcinogenicity study and of genotoxicity tests and other data indicate that a pharmaceutical clearly poses a carcinogenic hazard to humans, a second carcinogenicity study would not usually be useful; Note 3 provides details about the short- or midterm models. Table 49.6 is a representative list of some approaches that may meet these criteria.

Evidence of tumorigenic effects of the drug in rodent models should be evaluated in light of the tumor incidence and latency, the pharmacokinetics of the drug in the rodent models as compared to humans, and data from any ancillary or mechanistic studies that are informative with respect to the relevance of the observed effects to humans.

The results from any tests cited above should be considered as part of the overall "weight of evidence" taking into account the scientific status of the test systems.

These new models have been accepted by Regulatory Agencies like FDA, EMA, and Japan. Most frequently, TgHras2 and p53 knock out models are being used, both models are considered suitable for detecting genotoxic potential, TgHras2 in addition also non-genotoxic mechanisms. XPA model is susceptible to faults, e.g., UV light, and is not recommended by regulators any longer (Table 49.7).

Table 49.6 New Approaches for testing the carcinogenic potential

Approach	Including
Several transgenic mouse assays	σp53+ / − deficient model
	σ Tg.AC model
	σ TgHras2 model
	σ XPA deficient model, etc.
The neonatal rodent tumorigenicity model	

Table 49.7 Transgenic mouse models

Model	Including
Activated oncogenes	TgHras2 model (Japan)
	Tg.AC skin model (USA)
	Also gavage
Inactivated tumor suppressor gene	p53 knock out (=p53+ / −) model (USA)
Inactivated DNA repair gene	XPA− / − (NL)

Table 49.8 General design of long-term cancerogenicity studies

Aspect	Parameters
Species	Rats, mice, rarely hamster
Duration	24 month rats, 24 months mice
Route	As in clinical conditions, in feed, water, by gavage, inhalation
Dose levels	Mostly 3, mostly with a factor (e.g., 1:3:9)
Animal numbers	50/group/sex
Results available	Prior to NDA
Cost	1.0–1.5 million Euros

The FDA considerations for assay selection are:

- P53+/−: If clearly or equivocally genotoxic
- TgAC: For dermally applied products
- Neonatal: If clearly or equivocally genotoxic
- TgRasH2: For genotoxic or non-genotoxic products

The EU evaluation as discussed in the Safety Working Party EMEA reaches the following conclusions:

- p53 and Tg.RasH2 are equally sensitive to genotoxic compounds (some false positives and false negatives).
- TgRasH2 is more sensitive to peroxisome proliferators.
- p53 and TgRasH2 are acceptable in a regulatory context.

49.5.1.4 Dose Selection for Carcinogenicity Studies of Pharmaceuticals (S1C)

The S1C guideline was adopted and implemented in the different regions in 1997.

Traditionally, carcinogenicity studies for chemical agents have relied upon the maximally tolerated dose (MTD) as the standard method for high dose selection.

The MTD is generally chosen based on data derived from toxicity studies of 3 months' duration. Testing options for dose-range-finding studies are as follows:

- Usually:
  - 3 months for long-term studies
  - 1 month for neonatal or transgenic mice
- Range of different dose levels, often 5
- Focus on toxicity endpoints, determination of MTD
- Profiling of AUC
  - E.g., 1 and 4 months for rats
  - E.g., 1 and 4 weeks for alternatives (transgenic = wild type).

Table 49.8 illustrates the general design of long-term tumorigenicity assays.

Ideally, the doses selected for rodent bioassays for non-genotoxic pharmaceuticals should provide an exposure to the agent, adequate margin of safety, no significant chronic physiological dysfunction, and compatible with good survival.

The guideline calls for a flexible approach to dose selection.

The guideline proposes five different approaches:

1. Toxicity-based endpoints (MTD = Maximum Tolerated Dose or Minimum Toxic Dose)
2. Pharmacokinetic endpoints (25 times the human AUC)
3. Saturation of absorption
4. Pharmacodynamic endpoints
5. Maximum feasible dose

Ad 1) The ICH Expert Working Group on Safety has agreed to continue the use of the MTD as an acceptable toxicity-based endpoint for high dose selection for carcinogenicity studies.

The MTD is defined as the top dose or maximum tolerated dose that produces a minimum toxic effect

Table 49.9 History of genotoxicity guidelines (ICH/S2A and S2B) replaced by ICH S2(R)

ICH No.	Title	CPMP Doc. No.	Step
S2A	Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	CPMP/ICH/141/95	Step 5
S2B	Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals	CPMP/ICH/174/95	Step 5
S2(R1)	Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use	Nov 2011	Step 5

over the course of the carcinogenicity study. Factors to consider are alterations in physiological function, which would alter the animal's normal lifespan or interfere with interpretation of the study. Such factors include: no more than 10% decrease in body weight gain relative to controls, target organ toxicity, or significant alterations in clinical pathological parameters.

Ad 2) A systemic exposure representing a 25 times multiple of the human AUC (at the maximum recommended daily dose) may be an appropriate endpoint for dose selection for carcinogenicity studies for non-genotoxic pharmaceuticals, as a pragmatic solution.

Ad 3) High dose selection based on saturation of absorption measured by systemic availability of drug-related substances is acceptable. The mid and low doses selected for the carcinogenicity study should take into account saturation of metabolic and elimination pathways.

Ad 4) Pharmacodynamic endpoints for high dose selection will be highly compound specific. The high dose selected should produce a pharmacodynamic response in dosed animals of such magnitude as would preclude further dose escalation. However, the dose should not produce disturbances of physiology or homeostasis. Examples include hypotension, inhibition of blood clotting, or insulin-like effects.

Ad 5) The maximum feasible dose by dietary administration was considered 5% of diet. By many scientists this amount of drug is considered to be too high. Therefore, a new and more reasonable solution was formulated in the Guideline ICH/S1C (R), which follows.

49.5.1.5 Addendum to Dose Selection for Carcinogenicity Studies of Pharmaceuticals (Integrated into S1C)

Addition of a limit dose and related notes of pharmaceuticals. This addendum S1C (R) was adopted in 1998. This Addendum is today integrated in to the Guideline S1C. In determining the high dose for carcinogenicity studies, it may not be necessary to exceed a dose of 1,500 mg/kg/day. This limit dose applies in cases and where the maximum recommended human dose does not exceed 500 mg/day.

49.5.1.6 Genotoxicity Guidelines

A permanent alteration of genes or chromosomes can cause heritable effects leading to malformations and dysfunctions or affecting the individual by inducing tumors. Initiation of tumors can begin with a mutation of a single DNA base of an oncogene or a tumor suppressor gene leading to changes of normal activity and control. Initiation of neoplasia may also occur when there is displacement or translocation of a section of a chromosome which contains an oncogene leading to loss of normal control in its new location after breakage of chromosomes. Table 49.9 lists the former guidelines S2A and B, which were replaced in November 2011 by the new ICH guidelines S2(R1).

Testing of new drugs for their genotoxic potential is an important contribution to the safety of humans. The appropriate approaches for assessing the genotoxic potential of pharmaceutical compounds are recommended today in the ICH guidelines: ICH/S2(R) "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use."

Valid information on gene mutations, structure chromosome aberrations (clastogenicity), and numerical chromosome aberrations (aneugenicity) is required. No single test is capable of detecting all relevant genotoxic agents, therefore, a battery of in vitro and in vivo tests is considered appropriate.

According to ICH/S2(R) there is a need to measure point mutation, chromosome damage, and

Table 49.10 Toxicokinetics/Pharmacokinetics (ICH/S3A and S3B)

ICH No.	Title	CPMP Doc. No.	Step
S3A	Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies	CPMP/ICH/384/95	Step 5
S3B	PharmacoKinetics: Guidance for Repeated Dose Tissue Distribution Studies	CPMP/ICH/385/95	Step 5
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and non Rodent Toxicity Testing)	CPMP/ICH/300/95	Step 5

chromosome number changes. It is recommended to include a metabolic activation system with in vitro tests, usually rat liver S9 and to use predictive computerized SAR programs, e.g., DEREK and MCASE before testing begins.

Today there are two battery options:

Option I:

1. A test for gene mutations in bacteria (Ames), combined with
2. An in vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells (e.g., human peripheral lymphocytes or established cell lines like Chinese hamster ovary—CHO—of fibroblasts—V 79—or lung cells—CHL—cells) *or* an in vitro micronucleus test or an in vitro mouse lymphoma *tk* assay and
3. An in vivo test for chromosomal damage using rodent hematopoietic cells

Option II:

1. A test for gene mutations in bacteria (Ames), combined with
2. An in vivo test for chromosomal damage using rodent hematopoietic cells, preferably integrated into a repeat dose toxicity study and
3. A second in vivo test using a different endpoint and a different tissue, also integrated into a repeat dose toxicity study

Additional tests are needed for compounds which are positive in one or more in vitro tests but negative in the micronucleus test. Such tests could be an ex vivo Unscheduled DNA synthesis test in hepatocytes measuring repair of DNA or the measurement of DNA adducts by chemical/radiolabeling methods, e.g., 32P-postlabeling or transgenic models, e.g., Mutamouse or Big Blue and possibly Comet Assay which detect DNA strand breakage. The UDS test has been preferred formerly but because it is nearly always negative today it is less recommended by the FDA.

Modifications of the standard battery may be necessary for some classes, e.g., antibiotics which are toxic to bacteria or, e.g., for compounds like topoisomerase inhibitors which interfere with the mammalian cell replication system.

The ICH guidance complements the details of study designs outlined in the OECD guidelines. Additionally, they inform about the top concentrations for in vitro tests and the expected levels of cytotoxicity, and about repeat or confirmatory testing and the proof of target tissue exposure to the test article in the in vivo studies.

Support for the interpretation of positive test results and considerations on conditions leading to false positive data can be found.

Genotoxicity results are very important for go/no go decisions. Positive results do not necessarily indicate "no go." Consistent results over the battery of tests provides the greatest level of confidence in predicting hazard as potent genotoxic carcinogens tend to give positive results across a number of in vitro and in vivo assays.

49.5.1.7 Toxicokinetics/Pharmacokinetics (ICH/S3A and S3B)

Table 49.10 cites the toxicokinetic and pharmacokinetic guidelines.

ICH/S3A

The objectives of Toxicokinetics are primarily to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study. And further, to relate exposure levels to toxicological findings, to assess the relevance of these findings to clinical safety and to support the design as the choice of species and treatment regimen in nonclinical studies.

If animals or humans are exposed to pharmaceutical compounds, they will either elicit a pharmacodynamic effect, e.g., show a suppression of blood pressure, or reveal in analytical blood samples exposure levels of the compound. This kinetic information of the parent compound and its metabolites is an important

contribution for the extrapolation of safety data from animal studies to humans. Species differ considerably in regard to their kinetic conditions as C_{max}, T_{max}, Area Under the Curve (AUC), $t_{1/2}$, and ADME (Absorption, Distribution, Metabolism and Excretion).

Therefore, it is important to know what a drug does with the body (what pharmacology and toxicology is induced?); it is also crucial to know what the body does with the drug.

The following toxicokinetic studies are usually distinguished: concomitant toxicokinetics, which are normally integrated in the toxicity studies and other supportive studies which mimic the conditions of the toxicity study.

The focus before IND is on T_{max}, C_{max}, and AUCs, while the complexity of pharmacokinetic characterization (like oral bioavailability, plasma half-life, volume of distribution, mean residence time, absorption, solubility, and concentration) is built up during clinical trials and on the basis of comparable human data. The compound can be bound to plasma proteins, cells, or tissues. Therefore, a distinction between "unbound drug" or "free fraction" is relevant. Distribution studies help to design preclinical studies. Demonstration of accumulation can, e.g., explain toxicity at the site of increased compound accumulation.

Kinetic data should be considered in repeat dose toxicity studies, in genotoxicity studies when there are negative results and exposure can be characterized in the indicator tissue, in teratogenicity studies and in cancerogenicity studies, where monitoring is appropriate but according to the guideline not beyond 6 months duration. Here the author recommends to take blood samples also after 12 and 24 months of treatment, since drug or age induced toxic damage, especially of liver and kidney, may influence the exposure levels of individual animals considerably.

ICH/S3B

Single dose studies provide usually sufficient information about tissue distribution but there may be cases where assessments after repeated dosing may provide better information. Such studies are necessary when:

1. Single dose distribution studies suggest that the half-life of the test compound and/or metabolites in organs or tissues significantly exceeds the half-life of the elimination phase in plasma or
2. Steady-state levels of a compound/metabolite in the circulation, determined in repeated dose pharmacokinetic or toxicokinetic studies, are markedly higher than those predicted from single dose kinetic studies or
3. When histopathological changes were observed that were not predicted from short-term toxicity studies.

This information is provided in the ICH Guideline S3B: "Pharmacokinetics: Guidance for repeated dose distribution studies (CPMP/ICH/395/95)." Other relevant kinetic questions are the investigations of the potential of compounds to penetrate the barriers of placenta, blood-brain, or excretion into milk.

Knowledge about metabolites and their activity is further an important criterion for the assessment of species-specific effects and differences, e.g., the search for the most human-like test model as the best predictor for human reactions, focuses on such differences. Metabolism can lead to pharmacologically active metabolites; such knowledge is desirable early in development. In vitro metabolism studies normally precede in vivo preclinical safety assessments.

For safety reasons, it is important to identify, and perhaps eliminate, drugs from further development if they are subject to polymorphic metabolism or extensive metabolism by key human enzymes. Knowledge about the cytochrome P450 (CYP450) superfamily of drug metabolizing enzymes is of particular interest. Such data today are needed before IND according to the revised guideline M3(R).

49.5.1.8 ICH Guideline S4A, Duration of Non-rodent Repeat Dose Studies

This is a guideline where harmonization was difficult to reach. There was clear agreement that rodent studies were only needed for a maximum duration of 6 months. When a 6 month study in rodents is needed, then continuous application of pharmaceutical compounds will take place. Under such conditions, a long-term carcinogenicity study will usually be performed. Long-term exposure and lifelong observation is therefore possible. For non-rodents, lifelong exposure is normally not required. The EU proposed recommending a common maximum duration of 6 month studies for rodents and non-rodents, but there were 16 cases where unexpected findings appeared after the treatment time of 6 months.

Facing these results, the Guideline S4A recommends the following: For non-rodents, 12 month studies are usually not necessary, in the EU 6 month studies are acceptable based on the Directive 75/318/EEC.

Table 49.11 History of reproductive studies (ICH/S5 A + B) and preclinical evaluation of biotechnology-derived products (ICH/S6)

ICH No.	Title	CPMP Doc. No.	Step
S5A	Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products	CPMP/ICH/386/95	Step 5
S5B (M)	Reproductive Toxicology: Toxicity on Male Fertility (Modification)	CPMP/ICH/136/95	Step 5
S5(R2)	Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility	CPMP/ICH/386/05	Step 5

Accordingly, as a compromise, the Expert Working Group agreed that 9 month studies are recommended in general. In the US Federal Register this guideline was published with an FDA Note on ICH/S4A as follows: 9 month studies are acceptable for most development plans; shorter ones like 6 month studies may be acceptable for some drugs, while longer durations, e.g., 12 months, may be more appropriate for others. This advice is characterized by the FDA as their current line of thinking. In practice, FDA agreed with the sponsors in 50% for 9 month studies and in 32% for 6 month studies, while 12 month studies were only requested for compounds with novel mechanisms, or when only sparse clinical data were available, as in indications like HIV. Aids patients should be provided early on with the new treatment and the lack of complex clinical data is compensated by longer non-rodent studies. Sponsors are advised by the FDA to get in contact with the Agency when the maximum duration needs to be determined for non-rodents.

49.5.1.9 ICH S5(R2) Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility

The Parent Guideline dated from June 1993, the Addendum on Toxicity to Male Fertility dated November 9, 2000, this Addendum was incorporated in November 2005 into the core guideline without any changes.

The special toxicology discipline "Reproductive and Developmental Toxicity" focuses on undesirable adverse effects on male and female fertility, birth defects (developmental toxicity, malformations, teratogenicity), and nonphysiological changes that appear shortly before, during, and after birth, and during the weaning period (Table 49.11).

S5(R) illustrates the testing of all stages for the reproduction cycle: in detail from premating to conception (adult male and female reproductive functions, development and maturation of gametes, mating behavior, fertilization); from conception to implantation (adult female reproductive functions, preimplantation development, implantation). From implantation to closure of the hard palate (adult female reproductive functions, embryonic development, major organ formation). From closure of the hard palate to the end of pregnancy (adult female reproductive functions, fetal development and growth, organ development and growth); from birth to weaning (adult female reproductive functions, neonate adaptation to extrauterine life, preweaning development and growth) and finally from weaning to sexual maturity (postweaning development and growth, adaptation to independent life, attainment of full sexual function).

Accordingly the study types can be divided into the former three segments following the different time periods:

Segment I: The early embryonic development (fertilized egg or zygote, prior to implantation, and prior to formation of the three primary germ layers)

Segment II: The embryo-fetal development (embryo during major organ development, i.e., organogenesis, the fetus in the postembryonic period)

Segment III: The prenatal and postnatal development (neonate or postnatal offspring)

These segments can be tested separately or in a combined manner. All stages of development from conception to maturity and the detection of acute and delayed effects of exposure through one complete life cycle should be examined. The standard species are rodents, rats as the preferred rodent species for all study types and, the rabbit as the second non-rodent species for the embryo-toxicity studies. In some rare cases mice or monkeys are used too, if special conditions—usually kinetic data—justify such species. The route of administration should be similar to the intended human usage.

Part two of S5(R) deals with male fertility. Compounds inducing selective effects on male reproduction are rare; compound affecting spermatogenesis

almost invariably affect postmeiotic stages and weight of testis; mating with females is an insensitive means of detecting effects on spermatogenesis. Histopathology of the testis has been shown to be the most sensitive method for the detection of effects on spermatogenesis. Good pathological and histopathological examination (e.g., by employing Bouin's fixation, paraffin embedding, transverse section of 2–4 μm for testes, longitudinal section for epididymides, PAS, and hematoxylin staining) of the male reproductive organs provides a direct means of detection. Sperm analysis (sperm counts, sperm motility, sperm morphology) can be used as an optional method to confirm findings by other methods and to characterize effects further. Information on potential effects on spermatogenesis (and female reproductive organs) can be derived from repeated dose toxicity studies or reproductive toxicity studies.

For detection of effects not detectable by histopathology of male reproductive organs and sperm analysis, mating with females after a premating treatment of 4 weeks has been shown to be at least as efficient as mating after a longer duration of treatment. Since 2 week study was validated to be as effective as a 4 week study, 2 weeks treatment before mating is also acceptable.

In humans, malformations and changes in development are relatively rare (around 6%) and often caused by accidental genetic errors; some are caused by external factors, e.g., chemical drugs. If a compound is labeled as a developmental toxicant, then the occurrence of structural or functional abnormalities in offspring is significantly increased at a dose level which does not induce severe maternal toxicity. If malformations are developed, then exposure levels of the individual fetuses are important, but also at which time of the development exposure was high. The inclusion of kinetic data helps to support the evaluation.

49.5.1.10 ICH/S6(R) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (June 2011)

The ICH S6—Preclinical Safety Evaluation of Biotechnology-derived Products continues to be implemented and valid, but an Addendum was created which illustrates a number of important details helpful when biotechnology-derived products are being developed and where regulatory advice is needed.

S6 outlines the products for which this guidance is applicable, such as recombinant DNA proteins, vaccines, peptides, plasma derived products, endogenous proteins extracted from human tissues, or oligonucleotide drugs, etc., while heparin, vitamins, and cellular blood components, e.g., are not covered.

In general, for biotech products high flexibility for the developmental scheme is recommended and should be reconsidered on a case-by-case basis.

Usually, as in other toxicity studies, two animal species should be used, but when the biological activity is well understood or when in short-term toxicity studies the effects were similar in both species, then longer-term studies could be run only with one species.

When no relevant species can be identified, one should consider the use of homologous proteins or transgenic animals.

With regard to Safety Pharmacology, testing of the vital function such as cardiovascular, respiratory, and CNS functions is recommended, but S 6 also mentions renal function, which from today's perspective would not be necessary before first administration of a compound in humans if there is no specific concern.

Some information on absorption, disposition, and clearance in the animal models is desirable before clinical trials and systemic exposure of the compound should be monitored as well as the appearance of antibodies and their ability to neutralize the intended effect.

The immune response could also alter the pharmacokinetic or pharmacodynamic effects. Anaphylactic responses tested in the guinea pig, at any rate, are not predictive for humans and therefore not necessary. The same holds true for the standard testing batteries for immunotoxicity, these are not recommended.

Studies with a duration of between 2 weeks and 3 months are often sufficient and it is only with chronic use in humans that 6 month studies should be considered.

Flexibility may allow further reduction of the traditional testing program for the reproductive endpoints. Such studies may not be necessary if a new one, related to well-known compounds, shows similar effects.

The same holds true for genotoxicity. The standard studies are not appropriate, but testing on a case-by-case basis of impurities or promoter studies might be helpful. Long-term carcinogenicity studies are usually not appropriate, but when there is cause for concern, studies with a single rodent species are sufficient.

Cause for concern may have arisen in general toxicity studies, or else a stimulation of the growth of normal as well as malignant cells can be assumed.

The ICH guideline S6 (R1)—Preclinical safety evaluation of biotechnology-derived pharmaceuticals was recommended for implementation as Step 5 on December 2011. This Addendum is complementary to the parent first guideline. Where the addendum differs from the original guideline, the guidance in the addendum prevails. The purpose of the addendum is a clarification and update on following topics: species selection, study design, immunogenicity, reproductive, and developmental toxicity and an assessment of the carcinogenic potential.

As factors for Species Selection consider the comparisons of target sequence homology between species as starting point, further in vitro assays to make qualitative and quantitative cross-species comparisons of relative target binding affinities and receptor/ligand occupancy and kinetics and finally an assessments of functional in species-specific cell-based systems and/or in vivo pharmacology or toxicology studies. Other aspects are: the modulation of a known biologic response or of a pharmacodynamic (PD) marker as evidence for functional activity to support species relevance. The model should be capable of demonstrating potentially adverse consequences of target modulation; when the target is expressed at very low levels in typical healthy preclinical species (e.g., inflammatory cytokines or tumor antigens), binding affinity and activity in cell-based systems can be sufficient to guide species selection.

A new and important change is the value of tissue cross-reactivity in animal tissues, which is today considered being of limited value for species selection.

As described in ICH S6 Guideline, when no relevant species can be identified because the biopharmaceutical does not interact with the orthologous target in any species, use of homologous molecules or transgenic models can be considered.

The question if one or two species should be used when assessing bioproducts is being taken up: If there are two pharmacologically relevant species for the clinical candidate (one rodent and one non-rodent), then both species should be used for short-term (up to 1 month duration) general toxicology studies. If the toxicological findings from these studies are similar or the findings are understood from the mechanism of action of the product, then longer-term general toxicity studies in one species are usually considered sufficient.

The rodent species should be considered unless there is a scientific rationale for using non-rodents. Studies in two non-rodent species are not appropriate. The use of one species for all general toxicity studies is justified when the clinical candidate is pharmacologically active in only one species. Studies in a second species with a homologous product are not considered to add further value for risk assessment and are not recommended.

For chronic use products, repeat dose toxicity studies of 6 months duration in rodents or non-rodents are considered sufficient. Studies of longer duration have not generally provided useful information that changed the clinical course of development.

Immunogenicity assessments are conducted to assist in the interpretation of the study results and design of subsequent studies. Such analyses in nonclinical animal studies are not relevant in terms of predicting potential immunogenicity of human or humanized proteins in humans. Measurement of anti-drug antibodies (ADA) in nonclinical studies should be evaluated when there is: (1) evidence of altered PD activity; (2) unexpected changes in exposure in the absence of a PD marker; or (3) evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.).

Reproductive toxicity studies should be conducted in accordance with the principles outlined in ICH S5(R2) Guideline; however, the specific study design and dosing schedule can be modified based on an understanding of species specificity, the nature of the product and mechanism of action, immunogenicity and/or pharmacokinetic behavior and embryo-fetal exposure. The evaluation of toxicity to reproduction should be conducted only in pharmacologically relevant species. When the clinical candidate is pharmacologically active in rodents and rabbits, both species should be used for embryo-fetal development (EFD) studies, unless embryo-fetal lethality or teratogenicity has been identified in one species.

Developmental toxicity studies should only be conducted in nonhuman primates (NHPs) when they are the only relevant species.

If there is a specific cause for concern for any damage of fertility based on pharmacological activity or previous findings, specialized assessments such as menstrual cyclicity, sperm count, sperm morphology/motility, and male or female reproductive hormone levels can be evaluated in a repeat dose toxicity study.

Table 49.12 Safety Pharmacology (ICH/S/A + B) and Immunotoxicology Studies (ICH/S8)

ICH No.	Title	CPMP Doc. No.	Step
S7A	Safety Pharmacology Studies for Human Pharmaceuticals	CPMP/ICH/539/00	Step 5
S7B	Non-Clinical Studies for Assessing Risk of Repolarisation—Associated Ventricular Tachyarrhythmia for Human Pharmaceuticals	CPMP/ICH/423/02	Step 5

The species-specific profile of embryo-fetal exposure during gestation should be considered in interpreting studies. The following information is important: High molecular weight proteins (>5,000 D) do not cross the placenta by simple diffusion. For monoclonal antibodies with molecular weight as high as 150,000 D, there exists a specific transport mechanism, the neonatal Fc receptor (FcRn) which determines fetal exposure and varies across species. In the NHP and human, IgG placental transfer is low in the period of organogenesis and begins to increase in early second trimester, reaching highest levels late in the third trimester (Pentsuk and van der Laan 2009). Therefore, standard embryo-fetal studies in NHPs, which are dosed from early pregnancy up to Gestation Day 50, might not be of value to assess direct embryo-fetal effects in the period of organogenesis, although effects on embryo-fetal development as an indirect result of maternal effects can be evaluated.

For the assessment of neoplastic potential a number of considerations should be regarded: The mechanism of action of some biopharmaceuticals might raise concern regarding potential for carcinogenicity. If the weight of evidence supports the concern regarding carcinogenic potential, rodent bioassays are not warranted. In this case potential hazard can be best addressed by product labeling and risk management practices. However, when the weight of evidence is unclear, the sponsor can propose additional studies that could mitigate the mechanism-based concern. For products where there is insufficient knowledge about specific product characteristics and mode of action in relation to carcinogenic potential, a more extensive assessment might be appropriate (e.g., understanding of target biology related to potential carcinogenic concern, inclusion of additional endpoints in toxicity studies). Rodent bioassays (or short-term carcinogenicity studies) with homologous products are generally of limited value to assess carcinogenic potential of the clinical candidate.

49.5.1.11 S7A Safety Pharmacology Studies for Human Pharmaceuticals

S7B Non-Clinical Studies for Assessing Risk of Repolarisation—Associated Ventricular Tachyarrhythmia for Human Pharmaceuticals

S7A was implemented in 2001 and the S7B reached step 4 in June 2005 and are implemented now worldwide (Table 49.12).

S7A informs in general about the requirements necessary for testing the vital functions usually in single dose studies in Safety Pharmacology.

S7A differentiates between three types of studies: core battery, follow-up, and supplemental studies. The core battery of tests in S7A consists of an investigation of the effects of a test substance on three vital functions: central nervous system; cardiovascular system; respiratory system, and on other systems when appropriate. The exclusion of a system or function should be justified.

Safety Pharmacology studies carried out as necessary are:

Follow-up studies for core battery (they provide a greater depth of understanding than, or additional knowledge to, that provided by the core battery [e.g., mechanistic studies]).

Supplemental studies evaluate effects of the test substance on systems not addressed by the core battery when there is cause for concern not addressed elsewhere (e.g., in toxicology).

S7A expresses very clearly when such studies should be available and what conditions should be considered in regard to good laboratory procedures (see Table 49.13).

Special focus is given to the cardiovascular system. For the core battery of the cardiovascular system according to S7A blood pressure, heart rate, and electrocardiogram should be assessed, but also in vivo, in vitro, and/or ex vivo evaluations, including methods for repolarization and conductance abnormalities should be considered.

This text was finalized at a time when the details of S7B were not yet outlined. During recent years there has been an increase of regulatory concern. The awareness that non-cardioactive drugs, used for sometimes

Table 49.13

Guideline	Timing
ICH/S7A	Before first administration to humans
	Core battery tests. Follow-up or supplemental studies when appropriate
	During clinical development. Additional studies as required to clarify observed or suspected undesirable effects in animals or humans
	Before approval. Effects on all organ systems, if not covered elsewhere (e.g., toxicology) or in clinical trials
GLP/S7A	Core battery tests should be conducted according to GLP
	Follow-up and supplemental studies should be conducted according to GLP as far as possible deviation should be justified and impact discussed
	Primary and secondary pharmacodynamic studies need not be conducted according to GLP

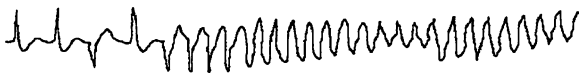

Fig. 49.1 Increased risk of ventricular tachyarrhythmia

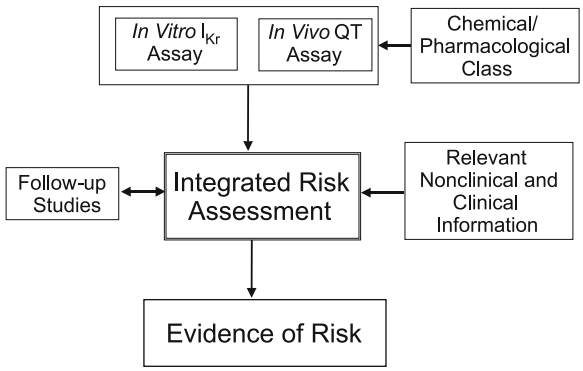

Fig. 49.2 Nonclinical testing strategy

nonlife-threatening diseases, can cause QT prolongation and serious dysrhythmias such as Torsades de Pointes (TdP) was intensified.

A greater number of compounds became known to be associated with QT prolongation and the potential to cause Torsades de Pointes.

Accordingly, the ICH Expert Working Group for S7B was created to work on this specific concern.

ICH S7B—Non-Clinical Studies for Assessing Risk of Repolarisation—Associated Ventricular Tachyarrhythmia for Human Pharmaceuticals

The background of S7B is summarized as follows:

The QT interval (time from the beginning of the QRS complex to the end of the T wave) of the electrocardiogram (ECG) is a measure of the duration of ventricular depolarization and repolarization.

QT interval prolongation can be congenital or acquired (e.g., pharmaceutical-induced)

When the QT interval is prolonged, there is an increased risk of ventricular tachyarrhythmia, including Torsade de Pointes (TdP), particularly when combined with other risk factors (e.g., hypokalemia, structural heart disease, bradycardia), see Fig. 49.1.

The basis for *Integrated Risk Assessment* is in vitro and in vivo assays, supported by any knowledge about the chemical/pharmaceutical class. This first risk assessment may later be modified when results from follow-up studies or relevant nonclinical or clinical information becomes available. The *Evidence of Risk* summarizes the preclinical evaluation of the proarrhythmic potential and should serve as the essential message for clinicians when treating volunteers or patients.

Parallel to the development of S7B a clinical guideline (ICH/E14) was drafted and reached step 4, also in 2005. During the discussion between these two expert groups, the question was raised again and again, if toxicologists could exclude any risk for QT prolongation for humans in their testing strategies. Of course they cannot, as clinicians cannot exclude any such risk for future patient generations based only on their clinical trial results. In addition, the Food and Drug Administration (FDA) is comparing preclinical data with clinical results and seems to have identified a few cases where QT prolongation was observed under clinical conditions while the preclinical tests were negative. This discrepancy is the basis for the diplomatic text in regard to the need for availability of S7B QT studies.

Timing of S7B Non-clinical Studies and Integrated Risk Assessment in Relation to Clinical Development/ Step 4, June 2005, Brussels:

Conduct of S7B non-clinical studies assessing the risk for delayed ventricular repolarization and QT interval prolongation prior to first administration in humans should be considered.

These results, as part of an integrated risk assessment, can support the planning and interpretation of subsequent clinical studies

The term "should be considered" allows flexibility either to do the studies before first time in humans or at a later stage of development. In practice, these S7B studies are most often available before IND, because one wants to cope with this issue in time and wants to provide best safety to volunteers and patients. S7B studies are most often requested by EMA or MHW in Japan.

In conclusion one can state:

S7B proposes a series of nonclinical tests which it is believed can predict the likelihood that a compound will prolong cardiac repolarization in vivo, in animals and in humans.

These data currently seem to have little impact on the clinical development proposals contained in the draft E14 guideline. A thorough analyses or more robust data from animals and humans is needed to arrive at a final testing strategy and at a valid assessment of this risk potential.

49.5.1.12 Immuntoxicology Studies (ICH/S8)

This guideline was implemented as step 5 in 2005 and replaces in Europe all the recommendations expressed in the EU Repeat Dose Toxicity guideline from 2002. In 2010, the Immuntoxicity part was deleted in this regional guidance.

S8 is restricted to unintended immunosuppression and immunoenhancement, excluding allergenicity or drug-specific autoimmunity. The guideline applies to new pharmaceuticals intended for use in humans, as well as to marketed drug products proposed for different indications or other variations on the current product label. The guideline does not apply to biotechnology-derived pharmaceutical products covered by ICH S6 and other biologicals.

Immunosuppression or enhancement can be associated with two distinct groups: (1) Drugs intended to modulate immune function for therapeutic purposes (e.g., to prevent organ transplant rejection) where adverse immunosuppression can be considered exaggerated pharmacodynamics. (2) Drugs not intended to affect immune function but cause immunotoxicity due, for instance, to necrosis or apoptosis of immune cells or interaction with cellular receptors shared by both target tissues and nontarget immune system cells.

Methods include standard toxicity studies (STS) and additional immunotoxicity studies conducted as appropriate. Whether additional immunotoxicity studies are appropriate should be determined by a weight of evidence review of cause(s) for concern.

Findings from standard toxicity studies (STS) pharmacological properties of the drug intended patient population structural similarities to known immunomodulators disposition of the drug clinical information.

Findings from standard toxicity studies (STS) pharmacological properties of the drug intended patient population structural similarities to known immunomodulators disposition of the drug clinical information.

The immunotoxicity evaluation follows a straightforward strategy which is detailed in Figs. 49.3 and 49.4 below.

Alterations in immune system organ weights and/or histology (e.g., changes in thymus, spleen, lymph nodes, and/or bone marrow), changes in serum globulins that occur without a plausible explanation, such as effects on the liver, can be an indication for immunogenic potential. Also increased incidences of infections and increased occurrence of tumors may be viewed as a sign of immunosuppression in the absence of other plausible causes such as genotoxicity.

The assessment of immunotoxicity should include the following:

- Statistical and biological significance of the changes
- Severity of the effects dose/exposure relationship
- Safety factor above the expected clinical dose
- Treatment duration
- Number of species and endpoints affected
- Changes that may occur secondarily to other factors (e.g., stress)
- Possible cellular targets and/or mechanism of action
- Doses which produce these changes in relation to doses which produce other toxicities and reversibility of effect(s)

Additional immunotoxicity testing should be considered:

- If the pharmacological properties of a test compound indicate it has the potential to affect immune function (e.g., anti-inflammatory drugs). If the majority of the patient population for whom the drug is intended is immunocompromised by a disease state or concurrent therapy.

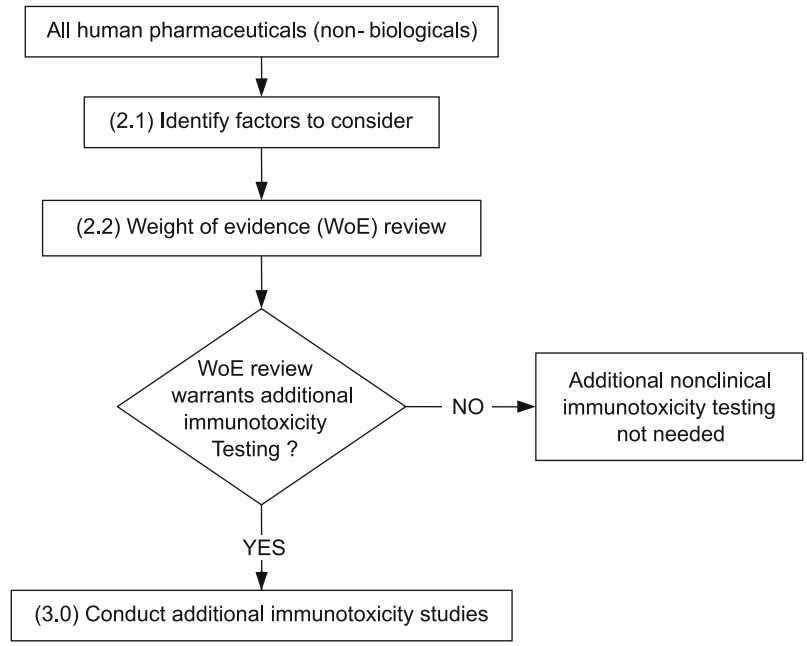

Fig. 49.3 ICH S8: Flow diagram for Recommended Immunotoxicity Evaluation

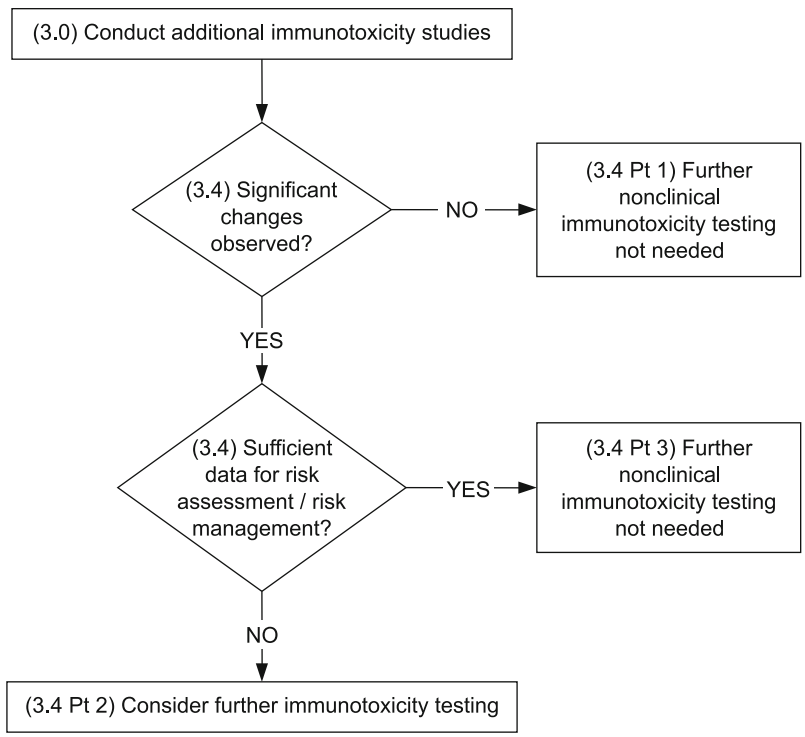

Fig. 49.4 ICH S8

- If a compound is structurally similar to compounds with known immunosuppressive properties.
- If the compound and/or its metabolites are retained at high concentrations in cells of the immune system.
- If clinical findings suggestive of immunotoxicity in patients exposed to the drug occur.

If the weight-of-evidence review indicates that additional immunotoxicity studies are needed, there

are a number of assays which can be used. It is recommended that an immune function study be conducted, such as a T-cell dependent antibody response (TDAR). If specific cell types are affected in STS not involving cells participating in a TDAR, assays that measure function of that specific cell type might be conducted. Immunophenotyping of leukocyte populations, a nonfunctional assay, may be conducted to identify the specific cell populations affected and may provide useful clinical biomarkers.

Generally accepted is the conduct of studies with 28 consecutive daily doses in rodents. Adaptations of immunotoxicity assays have been described using non-rodent species. The species, strain, dose, duration, and route of administration used in immune function assays should be consistent, where possible, with the nonclinical toxicology study in which an adverse immune effect was observed.

Usually both sexes should be used in these studies, excluding nonhuman primates. The high dose should be above the no observed adverse effect level (NOAEL) but below a level inducing changes secondary to stress. Multiple dose levels are recommended in order to determine dose-response relationships and the dose at which no immunotoxicity is observed.

Additional studies may show that no risk of immunotoxicity can be detected and no further testing is needed.

Additional studies may demonstrate a risk of immunotoxicity, but there are no sufficient data for a reasonable risk-benefit decision. In this case further testing may be of benefit to provide sufficient information for the risk-benefit decision. If the overall risk-benefit analysis suggests that the risk of immunotoxicity is considered acceptable and/or can be addressed in a risk management plan (see ICH E2E), then no further testing in animals might be called for.

If the weight-of-evidence review indicates the need for additional immunotoxicity studies, these should be completed before exposure of a large population of patients, usually Phase III. If the target patient population is immunocompromised, immunotoxicity testing can be initiated at an earlier time point in the development of the drug.

The different methods which are recommended for the evaluation of immuntoxic effects are summarized in Table 49.14.

Table 49.14 Standard toxicity studies

Area	Subject
Hematology	Total leukocyte counts and absolute differential leukocyte counts
Clinical Chemistry	Globulin levels and A/G ratios
Gross pathology	Lymphoid organs/tissues
Organ weights	thymus, spleen (optional: lymph nodes)
Histology	thymus, spleen, draining lymph node, and at least one additional lymph node, bone marrow, Peyer's patch[a], NALT[b], BALT[b]

[a]oral administration only
[b]inhalation administration only

Methods to evaluate immunotoxicity:
Additional immunotoxicity studies

- T-cell Dependent Antibody Response (TDAR)
- Immunophenotyping (lymphocyte subsets)
- Natural Killer Cell Activity Assays
- Host Resistance Studies
- Macrophage/Neutrophil Function
- Assays to Measure Cell-Mediated Immunity

This new ICH guideline replaces all guidances from EU, USA, and Japan. It represents a very pragmatic approach and uses studies, e.g., standard toxicity studies, which are conducted anyhow (see Table 49.15). There is great confidence in the prediction of these assays for any potential of new compounds to induce immune suppression or immune stimulation. This guideline helps to reduce the number of animals and requires additional studies only in special cases for concern.

49.5.1.13 ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals

This guideline was implemented as step 5 in October 2009.

The guidance provides recommendations for nonclinical evaluations to support the development of anticancer pharmaceuticals in clinical trials for the treatment of patients with advanced disease and limited therapeutic options. The aim is to facilitate and accelerate the development of anticancer pharmaceuticals and to protect patients from unnecessary adverse effects, while avoiding unnecessary use of animals, in accordance with the 3R principles (reduce/refine/replace), and other resources.

Table 49.15 Recommended duration of repeated-dose toxicity studies to support the conduct of clinical trials

	Recommended minimum duration of repeated-dose toxicity studies to support clinical trials	
Maximum duration of clinical trial	Rodents	Non-rodents
Up to 2 weeks	2 weeks	2 weeks
Between 2 weeks and 6 months	Same as clinical trial	Same as clinical trial
Less than 6 months	6 months	9 months

For anticancer pharmaceuticals, clinical studies often involve cancer patients whose disease condition is progressive and fatal. Therefore, the dose selection may be different and often close to or at the adverse effect dose levels. For these reasons, the type, timing, and flexibility called for in the design of nonclinical studies of anticancer pharmaceuticals can differ from those elements in nonclinical studies for other pharmaceuticals.

The guideline applies to both small molecule and biotechnology-derived pharmaceuticals (biopharmaceuticals), regardless of the route of administration. It describes the minimal requirements to initiate clinical trials in patients with advanced cancer whose disease is refractory or resistant to available therapy, or where current therapy is not considered to be providing benefit. The nonclinical data to support Phase I and the clinical Phase I data would normally be sufficient for moving to Phase II and into second or first line therapy in patients with advanced cancer.

Studies to support clinical trials and marketing authorization:

1. *Pharmacology studies*: Prior to Phase I studies, characterization of the mechanism(s) of action and schedule dependencies as well as antitumor activity of the pharmaceutical should have been made.
2. *Safety Pharmacology studies*: An assessment of the pharmaceutical's effect on vital organ functions (including cardiovascular, respiratory, and central nervous systems) should be available before the initiation of clinical studies. Conducting stand-alone safety pharmacology studies to support studies in patients with advanced cancer is not called for.
3. *Pharmacokinetic information*: The evaluation of limited pharmacokinetic parameters (e.g., peak plasma/serum levels, area under the curve (AUC), and half-life) in the animal species used for nonclinical studies can facilitate dose selection, schedule, and escalation during Phase I studies. Further information on absorption, distribution, metabolism, and excretion of the pharmaceutical in animals should normally be generated in parallel with clinical development.
4. *General toxicity studies*: Phase I assessments can include dosing to a maximum tolerated dose (MTD) and dose limiting toxicity (DLT). Toxicology studies to determine a no observed adverse effect level (NOAEL) or no effect level (NOEL) are not considered essential to support clinical use of an anticancer pharmaceutical. For small molecules, the general toxicology testing usually includes rodents and non-rodents. In certain circumstances alternative approaches can be appropriate (e.g., for genotoxic drugs targeting rapidly dividing cells, a repeat-dose toxicity study in one rodent species might be considered sufficient, provided the rodent is a relevant species.
5. *Reproductive toxicity studies*: Embryo-fetal toxicity studies of anticancer pharmaceuticals should be available when the marketing application is submitted, but these studies are not considered essential to support clinical trials intended for the treatment of patients with advanced cancer. These studies are also not considered essential for the purpose of marketing applications for pharmaceuticals that are genotoxic and target rapidly dividing cells (e.g., crypt cells, bone marrow) in general toxicity studies or belong to a class that has been well characterized as causing developmental toxicity. For small molecules, embryo-fetal toxicology studies are typically conducted in two species as described by ICH S5(R2). For biopharmaceuticals, an assessment in one pharmacologically relevant species should usually be sufficient. A study of fertility and early embryonic development is not warranted to support clinical trials or for marketing of pharmaceuticals intended for the treatment of patients with advanced cancer. A pre- and postnatal toxicology study is generally not warranted to support clinical trials or for marketing of pharmaceuticals for the treatment of patients with advanced cancer.

6. *Genotoxicity*: Genotoxicity studies are not considered essential to support clinical trials for therapeutics intended to treat patients with advanced cancer. Genotoxicity studies should be performed to support marketing (see ICH S2).
7. *Carcinogenicity*: Carcinogenicity studies are not warranted to support marketing for therapeutics intended to treat patients with advanced cancer.
8. *Immunotoxicity*: For most anticancer pharmaceuticals, the design components of the general toxicology studies are considered sufficient to evaluate immunotoxic potential and support marketing. For immunomodulatory pharmaceuticals, additional endpoints (such as immunophenotyping by flow cytometry) might be included in the study design.
9. *Photosafety testing*: An initial assessment of phototoxic potential should be conducted prior to Phase I, based on photochemical properties of the drug and information on other members in the class. This recommendation may be modified by the new safety guideline S10 which is in progress within the ICH activities.
10. *Start Dose for First Administration in Humans*: For most systemically administered small molecules, interspecies scaling of the animal doses to an equivalent human dose is usually based on normalization to body surface area. For biopharmaceuticals with immune agonistic properties, selection of the start dose using a minimally anticipated biologic effect level (MABEL) should be considered. A common approach for many small molecules is to set a start dose at 1/10 the Severely Toxic Dose in 10% of the animals (STD 10) in rodents. If the non-rodent is the most appropriate species, then 1/6 the Highest Non-Severely Toxic Dose (HNSTD) is considered an appropriate starting dose. The HNSTD is defined as the highest dose level that does not produce evidence of lethality, life-threatening toxicities or irreversible findings.
11. *Duration and Schedule of Toxicology Studies to Support Initial Clinical Trials*: In Phase I clinical trials, treatment can continue according to the patient's response, and in this case, a new toxicology study is not called for to support continued treatment beyond the duration of the completed toxicology studies. Results from repeat dose studies of 3 months' duration following the intended clinical schedule should be provided prior to initiating Phase III studies. For most pharmaceuticals intended for the treatment of patients with advanced cancer, nonclinical studies of 3 months duration are considered sufficient to support marketing.
12. *Combination of Pharmaceuticals*: Pharmaceuticals planned for use in combination should be well-studied individually in toxicology evaluations. Data to support a rationale for the combination should be provided prior to starting the clinical study. In general, toxicology studies investigating the safety of combinations of pharmaceuticals intended to treat patients with advanced cancer are not warranted.
13. *Additional aspects*: Examples of Treatment Schedules for Anticancer Pharmaceuticals to Support Initial Clinical Trials are reported and recommendations for nonclinical studies to support trials in Pediatric Populations, conjugated and liposomal products, evaluation of drug metabolites and impurities are being illustrated.

49.5.1.14 ICH Multidisciplinary Guideline M3(R2) Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals

The former Guideline M3 (Timing) was revised and complemented with a great number of new recommendations in June 2009. It continues to focus on the timing of nonclinical studies to support clinical trials and marketing and also on conditions which need to be considered when different patient populations like men or children or women of child bearing potential (WOCBP)are being included into clinical trials.

In more details, recommendations can be found for Timing of preclinical studies (IND, NDA), Duration of safety studies, Options for expediting development (exploratory trials, microdosing, etc.), Inclusion of patient populations, Case-by-case evaluations (e.g., phototoxicity) and development of combination drugs.

The guidelines expresses as goals that development of a pharmaceutical is a stepwise process, that evaluation of animal and human efficacy and safety is needed. Toxic effects should be characterized; there is a need for the identification of target organs, clarification of

dose dependence, relationship of toxicities to exposure, and assessment of potential reversibility.

M3 has contributed considerably to reduce differences between regions; to facilitate timely conduct of clinical trials; to avoid unnecessary use of animals and other resources and to promote early availability of new drugs. M3 (R) will continue to reduce further the differences of regulatory recommendations among regions like Europe, USA, Japan, and other areas of the world. The stress is on the concept, that such a guideline is not a legal requirement, but serves as supportive compendium of recommendations which are scientifically and ethically appropriate.

The core battery of *Safety Pharmacology* is recommended to be conducted prior to first administration in humans. Any follow-up or supplemental studies as appropriate. During clinical development, a clarification of observed or suspected adverse effects in animals or during clinical trials may be needed. Before NDA, an assessment of effects on all systems should be provided, either based on in vitro or animal or human data.

For *kinetics*, an information on exposure data (AUC) in animals prior human clinical trials is needed; while ADME data are needed at completion of Phase I (Human Pharmacology) studies.

The duration for *Repeat Dose Toxicity Studies* is related to the duration of clinical trials and their therapeutic indication. In principle, the duration of animal studies are equal to or exceed the duration of the human clinical trials. Thorough Japanese studies in 2000 compared the utility of routine 4 week toxicity studies with 2 week studies and concluded that in regard to the prediction of toxicities to the male reproductive organs, 2 weeks studies were as valid as 4 week studies. Therefore the former regional Japanese requirement to ask for a minimum duration of 4 week studies before starting trials in men was dropped for a global consensus that the minimum duration of nonclinical studies is 2 weeks in rodents and 2 weeks in non-rodents.

In general, there is a relationship of 1:1 ratio for studies in two mammalian species (one non-rodent) as long as clinical trials are running. The details can be found in Table 49.15.

For marketing the duration of repeat dose studies may be longer as illustrated in Table 49.16.

Table 49.16 Recommended duration of repeated-dose toxicity studies to support marketing

Duration of indicated treatment	Rodent	Non-rodent
Up to 2 weeks	1 month	1 month
>2 weeks to 1 month	3 months	3 months
>1–3 months	6 months	6 months
>3 months	6 months[c]	9 months[c,d]

On the other hand, regional differences continue in regard to *Single dose animal studies* supporting single dose studies in humans: Historically, acute toxicity information has been obtained from single-dose toxicity studies in two mammalian species using both the clinical and a parenteral route of administration. However, such information can be obtained from appropriately conducted dose-escalation studies or short-duration dose-ranging studies that define an MTD in the general toxicity test species. On the other hand, information on the acute toxicity of pharmaceutical agents could be useful to predict the consequences of human overdose situations and should be available to support Phase III.

In the USA, single dose toxicity studies with extended examinations can support single-dose human trials since 1996. This concept encouraged the ICH to offer comparable options with the concept of *EXPLORATORY CLINICAL TRIALS*. This principle may be especially valuable for gaining early data for Go/No go decisions, when several candidates are being developed in parallel. Such streamlined early exploratory approaches can be conducted early in Phase I, may involve limited human exposure, have no therapeutic intent and are not intended to examine clinical tolerability. Table 49.17 reports about the different five options:

Genotoxicity in general: Results from two in vitro genotoxicity studies are recommended to be available prior to first administration to humans, while the standard battery should be completed prior to initiation of Phase II studies.

Carcinogenicity studies do not need to be completed in advance of the conduct of clinical trials unless there is cause for concern (ICH: S1A).

For pharmaceuticals to treat certain serious diseases, carcinogenicity testing, if needed, may be concluded post-approval.

Patient Population: The inclusion of different patient populations reveals regional differences, especially for women with child bearing potential. There is a high level of concern for unintentional exposure of an embryo/fetus.

Table 49.17 Five different exploratory clinical approaches

Single microdose of 100 μg: e.g., investigate target receptor binding or tissue distribution via PET study	*Repeat ≤5 administrations* of 100 μg (a total of 500 μg per subject). Useful for target receptor binding, but with less active PET ligands	*Single dose* studies at subtherapeutic doses or into the anticipated therapeutic range	*Dosing up to 14 days* into the therapeutic range but not intended to evaluate clinical MTD in two species with standard parameters	*Dosing up to 14 days* in rodents and a confirmatory study in non-rodent ($n = 3$) at the anticipated NOAEL exposure in rodent, with duration of a minimum of 3 days

The currently regional differences in the timing of reproduction toxicity studies to support the inclusion of women with childbearing potential are:

1. *Japan and EU*: Definitive nonclinical developmental toxicity studies should be completed before exposure of WOCBP
2. *US*: Assessment of embryo-fetal development can be deferred until before Phase III for WOCBP using precautions to prevent pregnancy in clinical trials.
3. *All ICH regions*: WOCBP can be included in repeated-dose Phase I and II trials before conduct of the female fertility study since an evaluation of the female reproductive organs is performed in the repeated-dose toxicity studies.
4. *All ICH regions*: Nonclinical studies that specifically address female fertility should be completed to support inclusion of WOCBP in large-scale or long-duration clinical trials (e.g., Phase III trials).
5. *In all ICH regions*: The pre-postnatal development study should be submitted for marketing approval.

The inclusion of *children* into clinical trials has gained tremendous interest. The following data are requested:

- Safety data from previous adult human exposure:
  - Most relevant information
  - Necessary before pediatric clinical trials
- Prior trials:
  - Appropriate repeated dose toxicity studies
  - All reproduction toxicity studies
  - Standard battery of genotoxicity tests
  - Juvenile animal studies should be considered
- Carcinogenicity testing:
  - Former: Prior to long-term exposure of children
  - Today: Unless there is a significant cause for concern (e.g., evidence of genotoxicity in multiple tests, or concern for pro-carcinogenic risk based on mechanistic considerations or findings from general toxicity studies), carcinogenicity studies are not recommended to support the conduct of pediatric clinical trials.

Abuse liability: Consider an evaluation of abuse liability for drugs with central nervous system activity by using PK/PD profile to identify the duration of action, similarity of chemical structure to known drugs of abuse, receptor binding profile, and behavioral/clinical signs from in vivo nonclinical studies. Three types of studies to evaluate the potential for abuse liability are often applied mostly as stand-alone tests: drug discrimination, self-administration of the compound, and an assessment of withdrawal.

Combination drug toxicity: M3R provides helpful details how to develop combination drugs of different maturity and data set. Provided complete nonclinical development programs are being conducted on the individual entities and a nonclinical combination toxicity study is warranted to support combination clinical trials, the duration of the combination study should be equivalent to that of the clinical trial, up to a maximum duration of 90 days. A 90-day combination toxicity study would also support marketing.

Summary of M3R

Marketing Approval

The nonclinical safety assessment for marketing approval of a pharmaceutical usually includes:

- Pharmacology studies
- General toxicity studies
- Toxicokinetic and nonclinical pharmacokinetic studies
- Reproduction toxicity studies
- Genotoxicity studies and possibly
- An assessment of carcinogenic potential

Other nonclinical studies conducted on case-by-case:

- Phototoxicity
- Immunotoxicity
- Juvenile animal toxicity
- Abuse liability

First in Man. Studies needed before first administration of a pharmaceutical compound:

Primary PD studies (in vivo and/or in vitro)

Safety pharmacology core battery studies (CV, CNS, respiration)

Kinetics: In vitro metabolic and plasma protein binding data for animals and humans and systemic exposure data from Tox species (AUC, cmax)

Repeat dose studies with minimum duration for 2 weeks in two species

No single dose studies or data on lethality needed

No reproductive studies

Other studies if concern, e.g., phototoxicity

49.5.1.15 Common Technical Document (ICH/M4)

M4 is another very important multidisciplinary guideline, which combines information for the three disciplines within the ICH Process: for Quality, Safety, and Efficacy. The following section focuses predominantly on the preclinical safety issues.

The total document is divided into *five modules*: Module 1 contains regional specific aspects, it provides for the European Union, e.g., the European Community specific data. This module therefore is not harmonized but region specific. Module 2 provides the Summaries for Quality, for Safety and Efficacy. The quality part uses as a headline "Quality Overall Summary," for safety and efficacy the terms "Non-Clinical or Clinical Overview." The different names signal that the quality part is a clear summary, while the nonclinical and clinical part should be critical evaluations. Module 3 provides chemical, pharmaceutical and biological information. Module 4 contains the nonclinical reports and Module 5 provides clinical study reports.

The Objectives of M4 are to assist authors in the preparation of nonclinical pharmacology, pharmacokinetics, and toxicology written summaries in an acceptable format. The CTD is not intended to indicate what studies are required, but provides an appropriate format for the nonclinical data. The Common Technical Document is nothing other than a placeholder for the different parts of a documentation for the market authorization process.

No guideline can cover all eventualities; common sense and a clear focus on needs of regulatory authority assessor are best guides to constructing an acceptable document. Therefore, modify the format if needed with the aim to provide best possible presentation and facilitate the understanding for the evaluation of the results.

Module 1 is regulated by regional requirements while the Modulus 2–4 are structured on an international basis. **Module 1** was agreed upon in the Europe in 2006 as follows:

Module 1 Table of Content

1.0	**Cover Letter**
1.1	**Comprehensive Table of Contents**
1.2	**Application Form**
1.3	**Product Information**
1.3.1	**SPC, Labelling and Package Leaflet**
1.3.2	**Mock-up**
1.3.3	**Specimen**
1.3.4	**Consultation with Target Patient Groups**
1.3.5	**Product Information already approved in the Member States**
1.3.6	**Braille**
1.4	**Information about the Experts**
1.4.1	**Quality**
1.4.2	**Non-Clinical**
1.4.3	**Clinical**
1.5	**Specific Requirements for Different Types of Applications**
1.5.1	**Information for Bibliographical Applications**
1.5.2	**Information for Generic, 'Hybrid' or Bio-similar Applications**
1.5.3	**(Extended) Data/Market Exclusivity**
1.5.4	**Exceptional Circumstances**
1.5.5	**Conditional Marketing Authorisation**
1.6	**Environmental Risk Assessment 1.6.1 Non-GMO 1.6.2 GMO**
1.7	**Information relating to Orphan Market Exclusivity**
1.7.1	**Similarity**
1.7.2	**Market Exclusivity**
1.8	**Information relating to Pharmacovigilance**
1.8.1	**Pharmacovigilance System**
1.8.2	**Risk-management System**
1.9	**Information relating to Clinical Trials**
1.10	**Information relating to Paediatric**
1.11	**Responses to Questions**
1.12	**Additional Data**

The CTD-Safety is organized as follows:

F. Nonclinical summary

1. *Pharmacology*
   a. Written summary
   b. Tabulated summary
2. *Pharmacokinetics*
   a. Written summary
   b. Tabulated summary

3. *Toxicology*
   a. Written summary
   b. Tabulated summary

This organization is kept up in all parts of the dossier; it is repeated for the overview, the summaries, and the reports.

The detailed organization for Pharmacology, Kinetics, and Toxicology are as follows:

1. Pharmacology Written Summary
   - Brief Summary
   - Primary Pharmacodynamics
   - Secondary Pharmacodynamics
   - Safety Pharmacology
   - Pharmacodynamic Drug Interactions
   - Discussion and Conclusions
   - Tables and Figures (either here, or included in text)
2. Pharmacokinetics Written Summary
   - Brief Summary
   - Methods of Analysis
   - Absorption
   - Distribution
   - Metabolism
   - Excretion
   - Pharmacokinetic Drug Interactions (Nonhuman)
   - Other Pharmacokinetic Studies
   - Discussion and Conclusions
3. Toxicology Written Summary
   - Brief Summary
   - Single-Dose Toxicity
   - Repeat-Dose Toxicity
   - Genotoxicity
   - Carcinogenicity
   - Reproduction Toxicity Local Tolerance
   - Other Toxicity Studies
   - Discussion and Conclusions

Examples of detailed advice for sections on discussion and conclusion of Pharmacokinetics: Information should be integrated across studies and across species, exposure in the test animals should be related to exposure in humans given the maximum intended doses.

Similar examples for toxicology: in vitro studies should precede in vivo studies. Where multiple studies of the same type need to be summarized within the Pharmacokinetics and Toxicology sections, studies should be ordered by species, by route, and then by duration (shortest duration first).

The species should be ordered as follows: 1. mouse; 2. rat; 3. hamster; 4. other rodent; 5. rabbit; 6. dog; 7. nonhuman primate; 8. other non-rodent mammal; 9. nonmammals (see also Table 49.18).

It is also recommended to limit the information in the summaries and overview. The overview should contain the essential and critical results on approximately 30 pages. The length of the Nonclinical Written Summaries should in general not exceed 100–150 pages.

The brief Summaries for Pharmacology should be written on 2–3 pages, for Pharmacokinetics the same length and for Toxicology approximately 6 pages.

Module 2 contains, in addition, 34 templates and 31 examples.

The examples have been taken from real dossiers and give good orientation on how to fill out the templates.

For illustration of the templates see Table 49.19 for Pharmacology, Table 49.20 as summary for Toxicology and Table 49.21 with details for a Repeat-Dose Toxicity Study.

Finally, the following list shows examples of recommendations for Module 4.

The appropriate location for individual-animal data is in the study report or as an appendix to the study report.

4.1 Table of Contents

A Table of Contents should be provided that lists all of the nonclinical study reports and gives the location of each study report in the Common Technical Document.

Study Reports

The study reports should be presented in the following order:

4.2 Pharmacology
- 4.2.1 Primary Pharmacodynamics
- 4.2.2 Secondary Pharmacodynamics
- 4.2.3 Safety Pharmacology
- 4.2.4 Pharmacodynamic Drug Interactions

4.3 Pharmacokinetics
- 4.3.1 Analytical Methods and Validation Reports (if separate reports are available)
- 4.3.2 Absorption
- 4.3.3 Distribution
- 4.3.4 Metabolism
- 4.3.5 Excretion
- 4.3.6 Pharmacokinetic Drug interactions (nonclinical)
- 4.3.7 Other Pharmacokinetic Studies

Table 49.18 Recommendations on how to summarize studies and tables

Studies	Recommendations
Repeat-Dose Toxicity (including supportive toxicokinetics evaluation)/CTD-S	Summarize in order by species, by route, and by duration
	Give brief details of the methodology
	Highlight important findings (e.g., nature and severity of target organ toxicity, dose (exposure)/response relationships, no observed adverse effect levels)
	Non-pivotal studies can be summarized in less detail (pivotal studies are the definitive GLP studies specified by ICH Guideline M3)
Carcinogenicity (including supportive toxicokinetics evaluations)/CTD-S	A brief rationale should explain why the studies were chosen
	The basis for high dose selection. Individual studies should be summarized in the following order:
	Long-term studies (in order by species; including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)
	Short- or medium-term studies (including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)
	Other studies
Other toxicity studies (if available)/CTD-S	If other studies have been performed, they should be summarized.
	When appropriate, the rationale for conducting the studies should be provided.
	Antigenicity
	Immunotoxicity
	Mechanistic studies (if not reported elsewhere)
	Dependence
	Studies on metabolites
	Studies on impurities
	Other studies

Table 49.19

1 Pharmacology			**Overview**	**Test Article:**
Type of Study	**Test System**	**Method of Administration**	**Study Number**	**Location Vol. Page**
1.1 Primary Pharmacodynamics				
1.2 Secondary Pharmacodynamics				
Safety Pharmacology				

Table 49.20

3A Toxicology	**Overview**					**Test Article:**		
Type of Study	**Species and Strain**	**Method of Administration**	**Duration of Dosing**	**Doses mg/kg**	**GLP Compliance**	**Testing Facility**	**Study Number**	**Location Vol.**
nsingle-Dose Toxicity								
3.2 repeat-Dose Toxicity								
Genotoxicity								
3.4 Carcinogenicity								
3.5 Reproductive & Developmental Toxicity								
3.6 Local Tolerance								
3.7 Other Toxicity Studies								

Table 49.21

3.2 Repeat-Dose Toxicity	**Report Title:**	**Test Article**						
Species/Strain:	**Duration of Dosing**	**Study No.**						
Initial Age:	**Duration of Postdose**	**Location in CTD: Vol. page**						
Date of First Dose:	**Method of Administration**							
	Vehicle/Formulation							
Special Features:								
No Observed Adverse-Effect Level:								
Daily Dose (mg/kg)	0 Control							
Number of Animals	M	M:	F:	M:	F:	M:	F:	M:
Toxicokinetics: AUC ()								
Noteworthy Findings								
Died or Sacrificed Moribund								
Body Weight (%[a])								
Food Consumption (%[a])								
Water Consumption ()								
Clinical Observations								
Ophthalmoscopy								
Electrocardiography								

4.4 Toxicology
- 4.4.1 Single-Dose Toxicity (in order by species, by route)
- 4.4.2 Repeat-Dose Toxicity (in order by species, by route, by duration; including supportive toxicokinetics evaluations)
- 4.4.3 Genotoxicity
  - 4.4.3.1 In vitro
  - 4.4.3.2 In vivo (including supportive toxicokinetics evaluations)
- 4.4.4 Carcinogenicity (including supportive toxicokinetics evaluations)
  - 4.4.4.1 Long-term studies (in order by species; including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)
  - 4.4.4.2 Short- or medium-term studies (including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)
  - 4.4.4.3 Other studies
- 4.4.5 Reproductive and Developmental Toxicity (including range-finding studies and supportive toxicokinetics evaluations). (If modified study designs are used, the following subheadings should be modified accordingly.)
  - 4.4.5.1 Fertility and early embryonic development
  - 4.4.5.2 Embryo-fetal development
  - 4.4.5.3 Prenatal and postnatal development, including maternal function
  - 4.4.5.4 Studies in which the offspring (juvenile animals) are dosed and/or further evaluated.
- 4.4.6 Local Tolerance
- 4.4.7 Other Toxicity Studies (if available)
  - 4.4.7.1 Antigenicity
  - 4.4.7.2 Immunotoxicity
  - 4.4.7.3 Mechanistic studies (if not included elsewhere)
  - 4.4.7.4 Dependence
  - 4.4.7.5 Metabolites
  - 4.4.7.6 Impurities
  - 4.4.7.7 Other

4.5 Key Literature References

This CTD has been tested in practice for several years. Although not perfect for every case, it has proved its usefulness. Industry knows where to place specific information and data, and regulators know where to find them. This has facilitated the review process tremendously, only one dossier is necessary for international registration, a lot of resources can be diverted to more important issues.

But the CTD is a living document, its weakness is apparent. In order to improve its quality and practicability, suggestions and proposals for improvements are invited from the public. These modifications are being dealt with within the process of "Questions and Answers," the ICH experts publish their conclusions regularly.

49.5.1.16 Outlook and Future of ICH

ICH can look back to more than 20 years of achievements. As the most successful international harmonization initiative in the world, its achievements include: an understanding of innovation; a common regulatory platform based on more than 60 guidelines; guidance of guidelines based on science; facilitation of communication among regulators and industry; effective use of research and development (R&D) resources; greater mutual acceptance of R&D data realized.

In addition, the drafting process of guidelines has identified gaps in science, new studies analyzing problems have been conducted to validate the assays confirming safe use of drug in humans, like validity of 2 weeks studies to assess male fertility or the validity of transgenic mouse models for assessing the carcinogenic potential within the ILSI/HESI evaluation process.

ICH has stimulated a process of creating guidelines, e.g., within Europe, the drafting takes place among regulators but industry is always asked to comment and improve those recommendations. In the following some of the EMA/CHMP guidances are collected as examples of this dynamic process:

CHMP Guidance Documents in Preparation/Released for Consultation:

1. Guideline on Environmental Risk Assessments for Pharmaceuticals
2. Guideline on the Limits for Genotoxic Impurities
3. Guideline on the Need for Pre-clinical Testing of Human pharmaceuticals in Juvenile Animals
4. Guideline on Drug-induced Hepatotoxicity
5. Guideline on the Non-Clinical Development of Fixed Combinations of Medicinal Products
6. Guideline on Risk Assessment of Medicinal Products on Human Reproductive and Development Toxicities: from Data to Labelling1. Guideline on Environmental Risk Assessments
7. Guideline on the Investigation of Dependence Potential of Medicinal Products
8. Guideline on the Assessment of Carcinogenic and Mutagenic Potential of Anti-HIV Medicinal Products
9. Guideline on the Non-Clinical Testing for Inadvertent Germ line Transmission of Gene Transfer Vectors
10. Guideline on Adjuvants in Vaccines
11. Points to Consider Document on Xenogenic Cell Therapy
12. Guideline on the Comparability of Biotechnology Products, Preclinical and Clinical Issues
13. Guideline on the Evaluation of Medicinal Products intended for Treatment of Chronic Hepatitis B.

But ICH will also continue to further draft guidelines on issues where discrepancies exist and where common solutions are desirable. There is a strong need for multidisciplinary guidances, here especially between Quality and nonclinical Safety. As examples the following themes in development should be mentioned: Residues of Metals (Q3D), Genotoxic Impurities (M7), and Phototoxicity (S10).

The enormous success of harmonization justifies the continuation. ICH stimulated the creation of new scientific approaches and data, there are new experiments for optimizing strategies, and there is support for new evaluations for better assessments, helping market authorization processes to be more effective.

ICH stimulated a considerable paradigm change, today there is an open dialogue and transparency among agencies and industries, there is increased consciousness for unnecessary hurdles, there is an optimized handling with limited resources at agencies and industry, there is an ongoing dynamic process via maintenance of guidelines with the implementation of the Questions and Answers options and there is recognition of advantages for drug development on an international basis.

References and Further Reading

From http://www.emea.eu.int/htms/human/ich/safety/ichfin.htm

Pentsuk N, Van der Laan JW (2009) An interspecies comparison of placental antibody transfer: new insights into developmental toxicity testing of monoclonal antibodies. Birth Defects Res Part B 86:328–344

Topic S1A Note for Guidance on the need for Carcinogenicity Studies of Pharmaceuticals

Topic S1B Note for Guidance on Carcinogenicity: Testing for Carcinogenicity of Pharmaceuticals

Topic S1C(R) Note for Guidance on Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addition of a Limited Dose and related Notes
Topic S1C Note for Guidance on Dose Selection for Carcinogenicity Studies of Pharmaceutical
Topic S2A Note for Guidance on Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals
Topic S2B Note for Guidance on Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals
Topic S3A Note for Guidance on Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies
Topic S3B Note for Guidance on PharmacoKinetics: Guidance for Repeated Dose Tissue Distribution Studies
Topic S4A Note for guidance on Duration of Chronic Toxicity Testing in Animals (Rodent and non Rodent Toxicity Testing)
Topic S5A Note for Guidance on Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products
Topic S5B Note for Guidance on Reproductive Toxicology: Toxicity on Male Fertility
Topic S6 Note for Preclinical Safety Evaluation of Biotechnology-Derived Products
Topic S7A Note for Guidance on Safety Pharmacology Studies for Human Pharmaceuticals
Topic S7B Note for Guidance on Non-Clinical Evaluation of the potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals
Topic S8 Immunotoxicity Studies for Human Pharmaceutical
Topic S9 Nonclinical Evaluation for Anticancer Pharmaceuticals

Testing of Medical Devices

50

Felix Chevalier

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A medical device is a product which is used for medical purposes in patients, in diagnosis, therapy, or surgery. In contrast to *pharmaceuticals* which achieve their principal effect by pharmacological, metabolic, or immunological mode of action, a *medical device* acts mechanical, physical, thermal, physicochemical, or chemical means.

Innovations in this sector contribute significantly to improve the efficacy of healthcare and patient's quality of life. Medical devices played a more and more important role in global healthcare in the past years. The global market of medical devices reached roughly 209 billion US Dollar in 2006 and is expected to grow with an average annual rate of 6–9% through 2010.[1]

For market access a risk management assessment approved by the relevant health authority is needed. A medical device is generally subject to pre- and postmarketing control regulated by the health authorities.

The ISO guideline 10993[2] is generally accepted by the health authorities and systematically describes the different steps in classification, risk assessment, and biological evaluation of medical devices.

The focus of the ISO guideline 10993 is the protection of patients by determination of biological reactivity of any leachable substances in a biological system and to demonstrate the hazard potential for the use of a medical device in humans. It was harmonized from numerous international and national guidelines

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[1] Acmite Market Intelligence: World Medical Devices Marked (2007), URL: http://www.acmite.com/market-reports/medicals/world-medical-devices-market.html (01–09–2011)

[2] DIN EN ISO 10993

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and standards and serves as guide for biological evaluation of medical devices in a risk management process.

This extensive risk assessment has to be performed and the intended proper use, but also possible abuse of the device, has to be defined. The device has to be characterized physically and chemically. The biological hazard has to be assessed in an evaluation of the materials and parts used in the production process and of possibly extractable compounds from these materials. If possible, the chemical risk has to be assessed by toxicological data analysis with focus on dose response and mode of toxicity. In this evaluation the extraction rate of potential leachables and the resulting exposure for the patient is significant.

50.1 Nature and Duration of Body Contact

An essential part in the characterization of medical devices according to ISO guideline 10993 is the definition of nature and duration of body contact. (Medical devices that do not contact the patient's body directly or indirectly (so-called noncontact devices) are not included in the scope of ISO 10993.) In case the device may be placed in more than one category, the more rigorous testing requirements shall apply.

In a first step it has to be differentiated whether the device is a:

- Surface-Contacting Device with contact to:
  - Intact skin surfaces only (electrodes, external prostheses, fixation tapes, compression bandages)
  - Mucosal membranes (contact lenses, urinary catheters, intravaginal and intraintestinal devices, dental prostheses)
  - Compromised surfaces (dressings, healing devices, and occlusive patches for ulcers, burns, and granulation tissue)
- External Communicating Device with contact to:
  - The blood path at one point and serves as a interconnection for entry into the vascular system (solution administration sets, extension sets, transfer sets, and blood administration sets)
  - Tissue, bone, or pulp/dentin systems (laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials, and skin staples)
  - Circulating blood (intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, hemoadsorbents, and immunoadsorbents)
- Implant Device with contact to:
  - Bone (orthopedic pins, plates, replacement joints, bone prostheses, bone cements, and intraosseous devices)
  - Tissue and tissue fluid (pacemakers, drug supply devices, neuromuscular sensors and stimulators, replacement tendons, breast implants, artificial larynxes, subperiostal implants, and ligation clips)
  - Blood (pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug-delivery catheters, and ventricular assist devices)

Beside the contact definition the duration of contact has to be defined:

- Limited exposure: Single or multiple use or contact is likely to be up to 24 h.
- Prolonged exposure: Single, multiple, or long-term use or contact is likely to exceed 24 h but not 30 days.
- Permanent contact: Single, multiple, or long-term use or contact exceeds 30 days.

50.2 Materials of Manufacture

The characterization of the different materials used for manufacturing of the device, including intended additives, process contaminants, and residues, is the essential next step in the biologically risk assessment.[3] [4] If the chemical ingredients themselves, their combination and production were proven to be safe in former use, or in a literature research could be shown that materials have a known and acceptable toxicity profile, a further characterization might not be needed. A flow chart in ISO 10993-1 can guide through the decision tree.[5]

[3] ISO 10993–18:2005 Chemical characterization of materials

[4] ISO/TS 10993–19:2006 Physicochemical, morphological, and topographical characterization of materials

[5] ISO 10993–1:2009 Evaluation and testing in the risk management process, picture 1—Flow chart to aid in ensuring a systematic approach to biological evaluation of medical devices

50.3 Leachable Substances and Degradation Products

In consideration of the nature and duration of body contact and other components interacting with the final product (e.g., alcohol for disinfection purpose prior use) the potential occurrence of leachable substances that can be extracted from the medical device has to be evaluated. Where the potential for degradation exists, corresponding tests may determine the processes of absorption, distribution, biotransformation, and elimination of leachables and degradation products of medical devices, or their extracts.[6]

For leachables or degradation products with a sufficient toxicological data profile no further characterization is needed. Potential synergies between the different substances have to be taken into consideration.[7] In case of a potential risk remaining after toxicological data analysis, further biological testing in vitro and in vivo is required.

50.4 Extraction

The purpose of extraction of a medical device is to provide a suitable test sample to determine the biological reactivity of any leachable (and potentially toxic) substance in a biological system. Detailed description are given in ISO 10993-12.[8]

The extraction medium and conditions of extraction used should be appropriate to the nature and use of the final product, but should also cover all possible "worst case scenarios." Extraction conditions and application of the extract to test systems, therefore, should ideally reflect not only actual in-use conditions of the products but also exaggerated-use conditions, if possible.

The extraction is generally performed with polar and nonpolar extraction media, but can be adapted to apply to more rigorous extraction conditions. Examples of extraction media are:

- Polar medium: Water, physiological saline; culture media without serum
- Nonpolar medium: Freshly refined vegetable oil (e.g., cottonseed or sesame oil) of quality defined in various Pharmacopeia
- Additional media: Alcohol/water, alcohol/saline, polyethylene glycol 400 (diluted to a physiological osmotic pressure), dimethylsulfoxide, and culture media with serum

An extraction procedure using a volatile solvent such as acetone, methanol, etc., followed by evaporation of the solvent and application of the evaporation residue to the test system, shall be undertaken for polymeric materials for genotoxicity and delayed-type hypersensitivity testing.

Extractions are commonly performed with agitation. When extraction under static conditions is considered to be appropriate, the method should be justified, specified, and reported.

Liquid extracts shall, if possible, be used immediately after preparation to prevent adsorption to the extraction container or other changes in composition. If an extract needs to be stored longer than 24 h, then the stability of the extract under the conditions of storage should be verified.

The extract should not routinely be processed by filtration, centrifugation, or other methods to remove suspended particulates. However, if such processing is necessary, the rationale must be presented in regulatory documents.

Standard extraction temperatures and times (with permissible ranges) are as follows:

37°C ± 1°C for 72 ± 2 h (extraction at (37 ± 1)°C for (24 ± 2) h in tissue culture media might be acceptable for cytotoxicity testing. See ISO 10993-5)

50°C ± 2°C for 72 ± 2 h

70°C ± 2°C for 24 ± 2 h

121°C ± 2°C for 1 ± 0.2 h

The complex process of extraction is influenced by time, temperature, surface-area-to-volume ratio, and the extraction medium. The effects of higher temperatures or other conditions on extraction kinetics and the identity of the extractant(s) should be considered carefully.

It is preferable to test medical devices in their final product form whenever practical. If this is not possible, the second choice is to use representative portions of the device, or representative molded or extruded test

[6]ISO 10993–9:1999 Framework for identification and quantification of potential degradation products

[7]ISO 10993–17:2002 Establishment of allowable limits for leachable substances

[8]ISO 10993–12:2007 Sample preparation and reference materials

Table 50.1

Thickness (mm)	Extraction ratio (surface area or mass/volume) ±10%	Example
<0.5	6 cm^2/ml	Film, sheet, tubing wall
0.5–1.0	3 cm^2/ml	Tubing wall, slab, small molded items
>1.0	1.25 cm^2/ml	Elastomeric closures
>1.0	3 cm^2/ml	Larger molded items
Irregularly shaped solid devices	0.2 g/ml	Powder, pellets, foam, nonabsorbent molded items
Irregularly shaped porous devices (For testing absorbents and hydrocolloids, the "absorption capacity" of the material has to be determined, i.e., the amount of extractant absorbed per gram of the material is determined. In performing the material extraction, this absorbed volume is added to each 0.1 g or 1.0 cm^2 to the extraction mixture.) (low density materials)	0.1 g/ml	Membranes

specimens of the formulated material, that have been preconditioned by the same processing as the final product, shall be tested.

When test samples and reference materials need to be cut into pieces, the influence of previously unexposed surfaces, e.g., lumens or cut surfaces, shall be considered. Methods to cut medical devices into representative portions for testing should be as clean as possible to prevent contamination.

50.5 Standard Surface Areas and Extract Liquid Volumes

Test samples from sterilized devices and reference materials shall be handled aseptically if appropriate to the test procedure.

A blank control and positive and negative controls shall be used where appropriate.

50.6 Biological Tests (Biocompatibility)

Animal testing can only be justified if an in-depth review of available relevant information of the properties of the test material was performed and animal tests are essential to characterize the test material properly.

After classification of the device, data for possible biological effects should be available for an all-embracing risk analysis.

Detailed information about the planning and conduct of animal tests with focus on animal welfare requirements is given in ISO10993-2 (Table 50.2).[9]

The overall biological evaluation of the device should be performed and documented only by experienced experts in this field.

50.7 Cytotoxicity

The cytotoxicity testing is the first basic step of biocompatibility testing for all kinds of medical devices. With the use of cell culture techniques, these assays determine the lysis of cells (cell death), the inhibition of cell growth, and other effects on cells caused by the medical devices, or their extracts. Cytotoxicity tests are described in ISO 10993-5.[10]

50.8 Sensitization

These tests are essential for the risk assessment, because even single exposure or short contact of potential leachables can result in allergic or delayed-type hypersensitivity reactions. For the evaluation of the sensitizing capabilities of medical devices, or their extracts, the choice of the appropriate test model is a critical point.

[9]ISO 10993–2:2006 Animal welfare requirements

[10]ISO 10993–5:2009 Tests for in vitro cytotoxicity

Table 50.2 Initial evaluation: Tests for consideration[11]

Medical device categorization by			Biological effect							
Nature of body contact		Contact duration								
Category	Contact	A—Limited (<24 h) B—prolonged (24 h to 30 days) C—permanent (>30 days)	Cytotoxicity	Sensitization	Irritation or intracutaneous reactivity	Systemic toxicity (acute)	Subacute and subchronic toxicity	Genotoxicity	Implantation	Hemocompatibility
Surface device	Skin	A	x	x	x					
		B	x	x	x					
		C	x	x	x					
	Mucosal membrane	A	x	x	x					
		B	x	x	x					
		C	x	x	x		x	x		
	Breached or compromised surface	A	x	x	x					
		B	x	x	x					
		C	x	x	x		x	x		
External communicating device	Blood path, indirect	A	x	x	x	x				x
		B	x	x	x	x				x
		C	x	x		x	x	x		x
	Tissue/bone/dentin	A	x	x	x					
		B	x	x	x	x	x	x	x	
		C	x	x	x	x	x	x	x	
	Circulating blood	A	x	x	x	x				x
		B	x	x	x	x	x	x	x	x
		C	x	x	x	x	x	x	x	x
Implant device	Tissue/bone	A	x	x	x					
		B	x	x	x	x	x	x	x	
		C	x	x	x	x	x	x	x	
	Blood	A	x	x	x	x	x		x	x
		B	x	x	x	x	x	x	x	x
		C	x	x	x	x	x	x	x	x

The Guinea Pig Maximization Test (GPMT) of Magnusson and Kligman which uses adjuvant and the non-adjuvant occluded patch test of Buehler Test were given preference over other methods in the past and were usually accepted by health authorities.

Recently, mouse models have been developed that offer the advantages of short duration and minimal animal treatment. The local lymph node assay (LLNA) was established and internationally accepted but some health authorities still consider the tests for some chemicals as a first stage in the assessment of skin sensitization potential and expect confirmation of test results by GPMT or Buehler Test. Sensitization tests are described in ISO 10993-10.[12]

[11]ISO 10993–1:2009 Evaluation and testing in the risk management process, Table 1—Initial evaluation tests for consideration

[12]ISO 10993–10:2010 Tests for irritation and delayed-type hypersensitivity

50.9 Irritation

Tests with focus on the irritation potential of medical devices, or their extracts, using appropriate sites such as skin, eye, and mucous membrane in a suitable animal model. There are in vitro tests for skin irritation available for neat chemicals, but for medical devices these tests are still under validation.

Inflammatory changes in the skin as a parameter for the irritant potential of the material are primary endpoints of the tests. Irritation tests are described in ISO 10993-10, Annex B.[13]

50.10 Intracutaneous or Intradermal Reactivity

These tests are performed on rabbits and assess the localized reaction of tissue to medical device extracts. These tests have to be performed where determination of irritation by topical dermal or mucosal tests is insufficient (e.g., implants, or medical devices having access to the blood path). These tests may also be useful where extractables are hydrophobic. Formation of edema or erythema in the skin is the primary endpoint of the tests.

Intracutaneous reactivity tests are described in ISO 10993-10[12].

50.11 Systemic (Acute) Toxicity

These tests estimate the potential harmful effects of either single or multiple exposures to medical devices used in the intended clinical use, or their extracts in an animal model, during a period of less than 24 h. These tests are appropriate where a contact of the medical device allows potential absorption and systemic exposure of toxic leachables and degradation products.

Pyrogenicity tests might be included to detect material-mediated pyrogenic reactions of the body to medical devices, or their extracts. No single test can differentiate pyrogenic reactions that are material mediated from those due to endotoxin contamination. Systemic toxicity tests can be included in subacute and subchronic toxicity test protocols and implantation test protocols, if possible. Systemic toxicity tests are described in ISO 10993-11.[14]

50.12 Subacute, Subchronic, and Chronic Toxicity

These tests determine the effects of either single or multiple exposures or contact to medical devices, or their extracts for a period not less than 24 h but up to 28 days (subacute), not greater than 10% of the total life span of the test animal, e.g., up to 90 days in rats (subchronic), or during the majority of the life span of the test animal, e.g., up to 6 months in rats (chronic). So they are focused on the effects of a prolonged exposure, accumulation of chemicals leached from the medical device in the intended clinical use of the device.

Inclusion of immunotoxicity tests in these studies should be considered for devices where data from other sources is suggestive of immunotoxicological effects. Chronic toxicity test protocols may be expanded to include endpoints for subacute and subchronic toxicity test protocols and implantation test protocols.

Subchronic toxicity tests are described in ISO 10993-11[14].

50.13 Genotoxicity

These in vitro tests use mammalian or nonmammalian cell culture or other techniques to determine gene mutations, changes in chromosome structure and number, and other DNA or gene toxicities caused by medical devices, or their extracts. For the evaluation of the genotoxic capabilities of medical devices, or their extracts, the choice of the appropriate assay is essential. Due to the existing diversity of genotoxic endpoints it is in contrast to endpoints in general toxicology not (or only very limited) possible to combine different endpoints in one study. Therefore a well defined test strategy with different complementary studies is necessary to cover all endpoints. Genotoxicity tests are described in ISO 10993-3.[15]

[13]ISO 10993–10:2010, Annex B, Tests for irritation and delayed-type hypersensitivity

[14]ISO 10993–11:2006 Tests for systemic toxicity

[15]ISO 10993–3:2003 Tests for genotoxicity, carcinogenicity, and reproductive toxicity

50.14 Implantation

These tests assess the local pathological effects on living tissue, at both the gross level and microscopic level, of a sample of a material or final product that is surgically implanted or in a tissue appropriate to the intended application (e.g., special dental usage tests). These tests are equivalent to subchronic toxicity tests if test protocols are expanded and systemic effects are also investigated. Implantation tests are described in ISO 10993-6.[16]

50.15 Hemocompatibility

These tests evaluate the effects of blood-contacting medical devices or materials on blood or blood components. Specific hemocompatibility tests may also be designed to simulate the geometry, contact conditions, and flow dynamics of the device or material during clinical applications. Hemolysis tests determine the degree of red blood cell lysis and the release of hemoglobin caused by medical devices, or their extracts in vitro.

Hemocompatibility tests are described in ISO 10993-4.[17]

50.16 Carcinogenicity

These tests determine the tumorigenic potential of medical devices or their extracts from either single or multiple exposures or contacts during the majority of the life span of the test animal. These tests may be designed in order to examine both chronic toxicity and tumorigenicity in a single experimental study. Carcinogenicity tests should be conducted only if there are suggestive data from other sources and do not have to be performed if a reasonable biologic risk assessment can be provided, showing that carcinogenic effects of the medical device, or its extracts can be excluded. Carcinogenicity tests are described in ISO 10993-3[15].

50.17 Reproductive and Developmental Toxicity

These tests evaluate the potential effects of medical devices, or their extracts on reproductive function (fertility), embryonic development (teratogenicity), and prenatal and early postnatal development. Reproductive and developmental toxicity tests should only be conducted if there are suggestive data from other sources that the device has potential impact on the reproductive potential of the subject and do not have to be performed if a reasonable biologic risk assessment can be provided, showing that teratogenic effects of the medical device, or its extracts can be excluded. Reproductive and developmental toxicity tests are described in ISO 10993-3[15].

[16]ISO 10993–6:2007 Tests for local effects after implantation

[17]ISO 10993–4:2002/Amd 1:2006 Selection of tests for interactions with blood

In Silico Methods

51

Alexander Amberg

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In drug development, it is very important to evaluate the potential toxicological risk of a drug candidate as early as possible to reduce costs and time in drug development process. In the traditional way the toxicological risk of a compound is investigated with the help of a battery of in vivo and in vitro methods. Since the late 1970s many different *in silico* methods for the prediction of toxicity have been developed. The term *in silico* stems from the computer component silicium; *in silico* methods, therefore, refer to methods or prediction using computational approaches. *In silico* methods have the advantage that they can make fast predictions for a large set of compounds in a high-throughput mode. Another advantage is that *in silico* methods make their prediction based on the structure of a compound even before it has been synthesized. *In silico* methods can, therefore, be used at a very early stage in the drug development process, for compounds planned to be synthesized, for which no or only little compound is available, or also for impurities or degradation products later in the drug development process, for which no synthesis is available. However, good predictivity of an *in silico* method is crucial if the method is to be introduced into the drug development process.

Two principle ways of performing *in silico* predictions of toxicity are the constructions of individual prediction models for a specific endpoint or the use of expert systems with already developed prediction models for several endpoints. Expert systems have been defined as "any formal systems" which make predictions about the toxicity of chemicals. All expert systems for the prediction of toxicity are built on experimental data and/or rules derived from such data (Dearden 2003). The expert systems can be further

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divided into subclasses based on the method of generating rules. Typical prediction methods are knowledge/rule-based, statistically fragment-based or quantitative structure-activity relationship (QSAR)-based systems.

In knowledge- or rule-based systems, experts/toxicologists create rules based on a list of structural features that have been related to a specified toxicity (Durham and Pearl 2001). These systems are based on structure-activity relationship (SAR), which means the qualitative relationship between a specific chemical structure and its biological/toxicological activity. In SAR, the occurrence of specific substructures in a molecule is correlated to be responsible and necessary for a biological/toxicological activity. An example of a typical knowledge- or rule-based expert system is DEREK, which will be described in this chapter later.

For statistically fragment-based systems a large training dataset of compounds with known toxicity is needed to derive structural features/fragments that are statistically highly correlated to the specified toxicity (Durham and Pearl 2001). Examples of statistically fragment-based expert system are MultiCASE and Leadscope, which will also be described in this chapter later.

QSAR methods are based on the quantitative relationship between a chemical structure and its biological/toxicological activity with the help of chemical descriptors that are generated from the molecular structure. The descriptors are statistically analyzed for the development of a model describing the specified activity, such as toxicity (Durham and Pearl 2001). Examples of QSAR methods will also be described later in this chapter; some local QSAR methods are also used by the expert systems MultiCASE and Leadscope as part of their predictions.

There are a number of commercial expert *in silico* systems available for the prediction of toxicity. Examples of knowledge/rule-based expert systems are DEREK (Leeds, UK), Hazard Expert (ComGenex, San Francisco, CA, USA), Oncologic (San Francisco, CA, USA), and COMPACT (University of Surrey, Guilford, UK). Examples of statistically fragment-based expert systems are MultiCASE (MultiCase, Beachwood, OH, USA), TOPKAT (Accelrys, San Diego, CA, USA), ADAPT (Jurs Research Group, Pennsylvania State University, Philadelphia, PA, USA), and common reactivity pattern (CORPEA, University A. Zlatarov, Bourgas, Bulgaria). Additionally, expert systems could be further classified according to their level of molecular structure assessment. Two-dimensional structure systems include DEREK and MultiCASE, whereas three-dimensional systems include COMPACT and COREPA. These systems are further described by Patlewicz et al. (2003). In the following chapters, the QSAR method and the three expert systems DEREK, MultiCASE, and Leadscope will be described in details.

Besides these commercial systems also some freely available expert systems and databases offered by different institutions could be used for the prediction of toxicity. Examples are summarized in Table 51.1 including their link on how to access them.

51.1 Quantitative Structure-Activity Relationship (QSAR)

PURPOSE AND RATIONALE

Quantitative Structure-Activity Relationship (QSAR) is a method that makes predictions by the quantitative description of molecular properties with the use of descriptors of the chemical structure (Dearden 2003). This means QSAR models describe the quantitative or calculated relationship between a chemical structure and their biological activity (e.g., toxicity) with the help of chemical descriptors that are generated from the molecular structure (Durham and Pearl 2001). This relationship is described in from of a mathematical equation (e.g., $\log 1/C = a\,\pi + b\,\sigma + \ldots + \text{const}$). QSAR models generally show better predictivity if all compounds of a dataset involved in the prediction are derived from a congeneric series of compounds, which means that they should all act by the same mechanism of action, since the physicochemical and structural descriptors used in the QSAR reflect the same mechanism of action. Sometimes it is difficult to determine the mechanism of action, so series of compounds involved in a QSAR model are often restricted to a given chemical class with the hope that this will ensure a single mechanism of action (Dearden 2003).

Most published QSAR models have been developed from congeneric series. But with the recent use of large, diverse chemical libraries, there is an increasing interest for QSAR models for a heterogeneous

Table 51.1 Freely available expert systems and databases offered by different institutions for the prediction of toxicity

Institution	Prediction expert systems	Databases
EU	European Computational Toxicology QSAR tools (e.g., Toxtree. . .)	European Chemical Substance Information System (ESIS)
	http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/	http://esis.jrc.ec.europa.eu/
FDA	(Computational Toxicology Program)[a]	Informatics and Computational Safety Analysis Staff (ICSAS), Drug Approvals and Databases (AERS) http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092125.htm
	http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092150.htm	
		http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
EPA	ToxCast	Aggregated Computational Toxicology Resource (ACToR, e.g., DSSTox. . .)
	http://epa.gov/ncct/toxcast/	http://actor.epa.gov/actor/faces/ACToRHome.jsp
OECD	OECD QSAR Toolbox http://www.oecd.org/document/54/0,3746,en_2649_37465_42923638_1_1_1_37465,00.html	
Others	CADASTER http://www.cadaster.eu/	CDD (Collaborative Drug Discovery) https://www.collaborativedrug.com/
	CAESAR http://www.caesar-project.eu/	ChEMBL https://www.ebi.ac.uk/chembldb/
	CHEMBENCH http://chembench.mml.unc.edu/	CPDB (Carcinogenicity Potency database) http://potency.berkeley.edu/
	LAZAR (Lazy Structure-Activity Relationships) http://www.in-silico.de/	IARC (International Agency Research Cancer) http://www.iarc.fr/
	OCHEM (Online Chemical Modeling Environment) http://ochem.eu/	NTP (National Toxicology program) http://ntp.niehs.nih.gov/
	OpenTox (ToxPredict) http://www.opentox.org/	PubMed http://www.ncbi.nlm.nih.gov/pubmed/
	VCCLAB (Virtual Computational Chemistry Lab) http://www.vcclab.org/	ToxNet http://toxnet.nlm.nih.gov/
	Others. . .	Others . . .

[a]Available only as commercial systems in collaboration with DEREK, MCASE, Leadscope, etc.

collection of compounds, a non-congeneric series of compounds. It is often possible to develop QSAR models for non-congeneric series to be used as classification models, for example, for a classification into high, moderate, or low toxicity (Dearden 2003).

QSAR models exist for a wide range of biological and toxicological endpoints, for example, published QSAR models for toxicity cover over 30 different endpoints, from carcinogenicity, mutagenicity, skin sensitization, eye irritation, neurotoxicity to gastric irritancy, etc. Dearden (2003) and Barratt and Rodford (2001) review in their publications some of the recent QSAR models for various endpoints.

For the prediction of toxicity there are also QSAR-based expert systems commercially available, such as TOPKAT (TOxicity Prediction by Komputer Assisted Technology, Accelrys, San Diego, CA, USA). These systems already contain developed and predefined prediction models and cover up to 16 different toxicological endpoints including mutagenicity, carcinogenicity, developmental toxicity, skin sensitization, eye irritation and rat oral LD_{50}, etc. The QSAR models from TOPKAT are built on large, heterogeneous databases with carefully selected data and descriptors (Lemont and Lowell 1999).

PROCEDURE

QSAR modeling generally involves three steps: The first step is the collection of a training set of chemicals. The second step is the selection of descriptors that can properly relate chemical structure to biological/toxicological activity. At least the third step is the application of statistical methods, which correlate changes in structure with changes in biological/toxicological activity (Perkins et al. 2003).

The statistical methods of QSAR modeling are based on the correlation of changes in biological/toxicological activity ($\Delta\Phi$) resulting from certain chemical modifications (ΔC), either directly by structural parameters, called Free Wilson-type relationships, or

by the corresponding changes of molecular properties, called Hansch-type analyses (Kubinyi 2002):

$$\Delta\Phi = f\Delta C$$

The classical QSAR methodology started in 1964 with the publications of Hansch and Fujita (1964) and Free and Wilson (1964), and the statement of Hansch (1969) resulted from a proposal by Fujita. They proposed to combine several physicochemical parameters (π, σ), also called descriptors, in a quantitative model. This Hansch-type analysis is very flexible and describes many different kinds of biological activities, for example, in vitro data such as enzyme inhibition (Kubinyi 2002):

$$\text{Log } 1/C = a\pi + b\sigma + \ldots + \text{const}$$
(C is a molar dose that produces a certain biological response)

In an independent publication, Free and Wilson (1964) formulated their mathematical model. This model describes the biological activity in a logarithmic scale as the sum of the biological activity of the reference compound and the group contributions of all substituents that are attached to various positions of this molecule (Kubinyi 2002):

$$\text{Log } 1/C = \sum a_i + \mu$$

(a_i are the group contribution of the individual substituents X_i to the biological activity values and μ is the calculated biological activity of a reference compound, most often the unsubstituted analogue)

Free Wilson analysis is easy to apply. No physicochemical properties are needed to describe biological activity, just values of 1 or 0, to indicate the presence or absence of a certain position.

On the other hand, Free Wilson analysis is much more restricted than Hansch analysis, because of its many parameters and the corresponding decrease on the number of degrees of freedom of the statistical analysis (Kubinyi 2002).

In 1979, 3D QSARs were developed, which correlate spatially localized features across a chemical series with biological activity. The two primary types are 3D QSARs using lattice-based or surface-based descriptors. Among the lattice-based methods, Comparative Molecular Field Analysis (CoMFA) is the most used 3D QSAR method. In a CoMFA model, all molecules of a training set are aligned together regarding the similarities in their structure and then embedded in a 3D grid. 3D arrangements of molecular features are correlated to the biological activity (Perkins et al. 2003).

The molecular QSAR descriptors can be grouped into three categories: 2D descriptors (e.g., molecular connectivity), 3D descriptors (e.g., molecular surface area), and physicochemical properties (e.g., log P). They can also be categorized according to their nature as well as calculation method, such as constitutional, topological, geometrical, electrostatic, quantum chemical, and thermodynamic descriptors (Perkins et al. 2003). In the "Handbook of Molecular Descriptors" from Todeschini and Consonni (2000), an encyclopedic collection and description is available for all molecular descriptors from the beginning (about 2,000 of different definitions). A number of different commercial systems are available for the development of different kinds of QSAR models from a dataset. Examples are Cerius, Moe, Sybyl, TSAR, Golpe, Pipelinepilot (Scitegic), DRAGON, etc.

In the expert system, TOPKAT-predefined QSAR models are integrated in the system with the main use of topological, sub-structural, and electronic descriptors from the 2-D Kier and Hall type, which are developed from a large, heterogeneous databases with carefully selected data (Lemont and Lowell 1999). Thus, no self-development of QSAR models is necessary, and the structures can be immediately entered into the system and the prediction can be started for the different available toxicity endpoints. Continuous endpoints, such as LD_{50}, are modeled using multiple linear regression QSARs to generate different value predictions (e.g., different LD_{50} values). Other endpoints like carcinogenicity, where the endpoint is either positive or negative, are modeled using two-group linear discriminant regression functions (Greene 2002). The program also provides an estimate of confidence in the prediction that the user can examine the most similar compounds in the training set based upon their chemical descriptors. The program also provides an optimum prediction space (OPS) measurement that determines if the compound being investigated is well represented in the training set (Durham and Pearl 2001).

EVALUATION

To date a number of different QSAR models have been published, for the prediction of drug toxicity QSAR models cover over 30 different endpoints. It is not within the scope of this review to cover all of them here. Many authors have discussed and evaluated different QSAR models in the literature (Dearden 2003; Patlewicz et al. 2003; Tuppurainen 1999).

Good examples of QSAR models can be received for the prediction of mutagenicity with the Ames test (Ames et al. 1973). The Ames test is well suited to develop a robust QSAR because it is standardized, delivers a substantial amount of data within a short period of time, can be used for different chemical classes, covers a similar mechanism of toxicity for all compounds, etc. The mechanism of mutagenicity is the result of cell penetration, bioactivation, interaction, and modification of DNA together with various error-free and error-prone DNA repair processes. Many different types of mutagenic compounds follow this mechanism, which is reflected in the great diversity of chemical structures that have been associated with mutagenicity. Most QSAR studies of mutagenic activity have been based on Hansch-type regression models (Patlewicz et al. 2003). Tuppurainen (1999) reviewed some QSAR models for different congeneric series of mutagenic compounds like aromatic hydrocarbons, aromatic amines, nitrosamine, epoxides, etc. One example of these QSAR models is described from Debnath et al. (1992). He developed a QSAR model with a dataset containing the mutagenic activity of 67 aromatic and heterocyclic amines acting on the Ames stain TA100 after metabolic activation with S9-mix. In this model, the mutagenic activity (log TA100 in revertants/nmol) is linearly dependent on hydrophobicity (log P), the energy of the highest occupied molecular orbital (ε_{HOMO}), and the energy of the lowest unoccupied molecular orbital (ε_{LUMO}). The QSAR model representing the relationship between these parameters and activity is given in the following equation:

$$\log \text{TA100} = 0.92(\pm 0.23)\ \log \text{P} + 1.17(\pm 0.83)\ \varepsilon_{HOMO} - 1.18(\pm 0.44)\ \varepsilon_{LUMO} + 7.35(\pm 6.90)$$

This relationship is interpreted in the way that the toxicological activity is dependent on log P, describing the ability of a compound to penetrate cell membranes to reach the target DNA, and on the molecular orbital energies (ε_{HOMO}, ε_{LUMO}), describing the ability of the metabolic activation to the electrophilic, reactive intermediate nitrenium-ion, which reacts with DNA resulting in a mutation and an increase in the revertants number in the Ames strain TA100.

The result of the internal evaluation of this QSAR model is shown in Fig. 51.1. In this figure, the calculated log TA100 values from the upper equation are plotted against the observed log TA100 values from the Ames tests for each of the 67 compounds. The line represents the regression, and results of the evaluation are expressed by the correlation coefficient $r = 0.887$ and the standard deviation from the regression $s = 0.708$. These data indicate the high potential of this QSAR model for the prediction of the mutagenic activity of aromatic and heterocyclic amines in the Ames strain TA100.

The expert system TOPKAT has been evaluated in several studies (Snyder et al. 2004; Dearden 2003; Patlewicz et al. 2003; Cariello et al. 2002; Durham and Pearl 2001; Richard 1998; Benfenati and Gini 1997; Enslein et al. 1994). In these evaluations, all statistical parameters, such as sensitivity, specificity, positive and negative predictivity, and the overall concordance range from 40% to 64% for the various endpoints.

CRITICAL ASSESSMENT OF THE METHOD

The development of a QSAR model with a good quality or predictivity depends on many factors. An important point is the quality of the dataset as bad data points will corrupt the model. In a qualitative good dataset, the number of chemicals should be sufficiently large to ensure statistical stability (at least 15–20 chemicals), the activity range should span two or more orders of magnitude and should be evenly distributed, the dose-response relationship should be smooth, and the chemicals should possess enough structural diversity. For robustness the right selection of descriptors is very important. The more is known about the biological mechanism, the better the descriptors can be selected to best encode the variation of activity with the structure. And finally, it is also very important to select the right QSAR method, which best describes the correlation between the structure and the biological/toxicological activity to give a QSAR model with a good prediction (Perkins et al. 2003).

Many different types of QSAR models and chemical descriptors for a wide range of endpoints are developed and published over the years. This makes QSAR

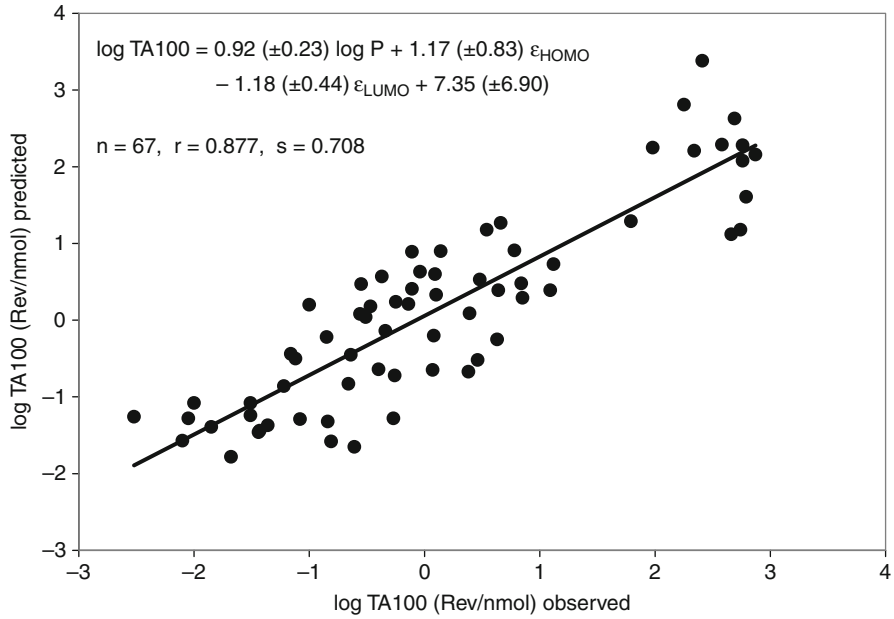

Fig. 51.1 QSAR model for the prediction of the mutagenic activity (in revertants/nmol) of aromatic and heterocyclic amines in the Ames strain TA100. The representing mathematical equation of the QSAR model is shown in the *top left corner*. In the graph, the calculated log TA100 values from QSAR model are plotted against the observed log TA100 values from the Ames tests for each of the 67 compounds of the dataset. The line represents the regression given by the equation. The model is further described in the paragraph evaluation. Abbreviations: *log TA100* mutagenic activity in revertants/nmol, *log P* hydrophobicity, ε_{HOMO} energy of the highest occupied molecular orbital, ε_{LUMO} the energy of the lowest unoccupied molecular orbital, *n* number of compounds from the dataset, *r* correlation coefficient, *s* standard deviation (Debnath et al. 1992)

a very flexible technique to be adapted for many different situations and a quite powerful technique that can provide a wealth of information. This has also a great potential for new QSAR models with every new experimental data.

One restriction of QSAR is that in most cases a good predictivity of a model is limited to a congeneric series or a specific class of compounds, which all act by the same mechanism of action. However, even for non-congeneric series it is often possible to develop classification of QSAR models, for example, for a classification of high, moderate, or low toxicity. So sometimes the QSAR models are not related to a specific mechanism. Therefore, it is not always possible to propose structural changes that will remove toxicity from the compound (Dearden 2003).

For the development of good QSAR models, experience with the method and time is needed, except when using the QSAR-based expert system TOPKAT, since QSAR models are already integrated in the system. TOPKAT has also the advantage that predictions are not limited to a single mechanism or class of chemicals, the program automatically supplies training sets of compounds for different endpoints. An important limitation of TOPKAT is that it is a closed system that does not allow users to expand the training set or to modify the method (Durham and Pearl 2001).

MODIFICATIONS OF THE METHOD

In the theory, it is possible to create new QSAR models with almost all datasets of compounds with known biological/toxicological activity. But practically it is a question of the quality and predictivity of a QSAR model to be applied in prediction of biological/toxicological activity. For this reason evaluation of each QSAR model is extremely important. The evaluation of a QSAR model can be performed either by internal validation (cross-validation) or external validation (use of a test-set). External validation is preferred but is not always possible, because of the small size of a dataset (Dearden 2003).

Unfortunately no modification of the training set or the prediction models is possible for the QSAR-based expert system TOPKAT.

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51.2 DEREK for Windows (DfW)

PURPOSE AND RATIONALE

DEREK for Windows (DfW, *D*eductive *E*stimation of *R*isk from *E*xisting *K*nowledge) is a knowledge-based expert computer system for the prediction of toxicity. The prediction of toxicity is based on structure-activity relationship (SAR) analysis of a chemical structure. It provides a qualitative prediction. The knowledge is stored as rules in the computer system. When the software analyzes a compound, the rules identify features within the structure that have been shown to be responsible for toxicological activity when they have been present in other chemicals. DfW highlights these substructures and give structural alerts for this compound. These toxicological active substructures within the structures are called toxicophores in DfW (LHASA Limited homepage). Since substructures can exist in a variety of molecules, the rules are not chemical-specific, but rather serve as broad generalizations with regard to the chemical structure (e.g., alkylating agent, acid or halogen-containing molecule, Cariello et al. 2002).

The knowledge is based on researching literature data or internal company data with an emphasis on the understanding of mechanisms of toxicity and metabolism required for activation of a compound to

a toxicological intermediate (Patlewicz et al. 2003). It covers a wide variety of toxicological endpoints, which include genotoxicity, carcinogenicity, irritation, skin and respiratory sensitization, hepatotoxicity, hERG channel inhibition, reproductive/developmental toxicity, and other miscellaneous endpoints (LHASA Limited homepage). Its main strengths lie in the prediction of carcinogenicity, mutagenicity, and skin sensitization (Greene 2002).

The DfW software incorporates also a reasoning engine that uses log P (calculated by a ClogP plug-in) and molecular mass to predict the likelihood that a chemical will express its potential toxicity regarding a specific endpoint in the selected species. This feature is used in the evaluation of the skin permeability for the prediction of skin sensitization and photoallergenicity in humans (Patlewicz et al. 2003). This proprietary reasoning engine combines both numerical and non-numerical statements (like selected species, log P, endpoint, toxicophores, etc.) to reach a conclusion about a given event. It is based upon the mathematical framework of the logic of argumentation. The reasoning engine can be adapted to any toxicological endpoint within the DfW system (Greene 2002). The result of the reasoning is that the likelihood of a structure being toxic is then expressed in one of the following terms: Certain, Probable, Plausible, Equivocal, Doubted, Improbable, Impossible, Open, and Contradicted.

The rules are written and maintained by experts from LHASA Limited. LHASA Limited is a non-profit organization that facilitates collaborations between scientists from a wide range of educational, regulatory, and commercial institutions. The rules are regularly updated by LHASA Limited and new versions of the computer software are available every year. Regular collaborative user group meetings are organized with representatives from pharmaceutical, agrochemicals, and regulatory organizations. At these user group meetings, changes in computer software and knowledge base developments are presented and the users provide feedback about the improvements. User suggestions for improvements to knowledge base and software are discussed during the wish list session, and new rules and toxicological information derived from internal company data is presented from the users. This unique system encourages the sharing of toxicological information and knowledge for the benefit of all, without organizations compromising the confidentiality of their proprietary data (Patlewicz et al. 2003).

PROCEDURE

The new versions of DfW are compatible with Microsoft Windows. For the prediction of toxicity the chemical structure of a compound is input into the system with the use of ISIS/Draw (from MDL Information Systems) or by importing a mol-file or sd-file (Fig. 51.2a). After the system has the structure, pushing the "process" button starts the prediction. During processing, the system searches and identifies all known toxicophores in the structure from all the different toxicological endpoints within seconds. After completion of the analysis, the system opens a result window. The result window contains the imported structure, a list of toxicological endpoints that the system has made a prediction for, the number and name of the toxicophores found in the structure (the position of the toxicophores in the structure is highlighted in red), and the level of likelihood for each endpoint in each species (Fig. 51.2b). For each prediction in the result window additional information is available including a reasoning report, an alert description, supporting examples, and processing constraint details. For example by choosing the "Alert Description" button, additional information and a description of the alert including information about the SAR, references, endpoint, and comments are displayed in an extra window for a specific alert (Fig. 51.2c). From the "Alert Description" window users can choose the "Example" button; this opens a new window which displays toxicity data for a number of chemicals that are pertinent to the alert including information about the structure, name, CAS number, and toxic activity. References and comments can be displayed in an extra window (Fig. 51.2d). It is also possible to process a big list of many different structures in the mol-file or sd-file format with one button click by using the Auto DEREK function, making batch processing possible with DfW.

All the results described above can be exported from DfW to other applications by generating a "DEREK for Windows report" in either rich text (rtf), tab delimited text, or modified sd-file format (Fig. 51.3).

EVALUATION

Richardt and Benigni (2002) stated in their paper that there are two common approaches to assess the predictive performance of computer programs for the prediction of toxicity: beta-testing (also called internal

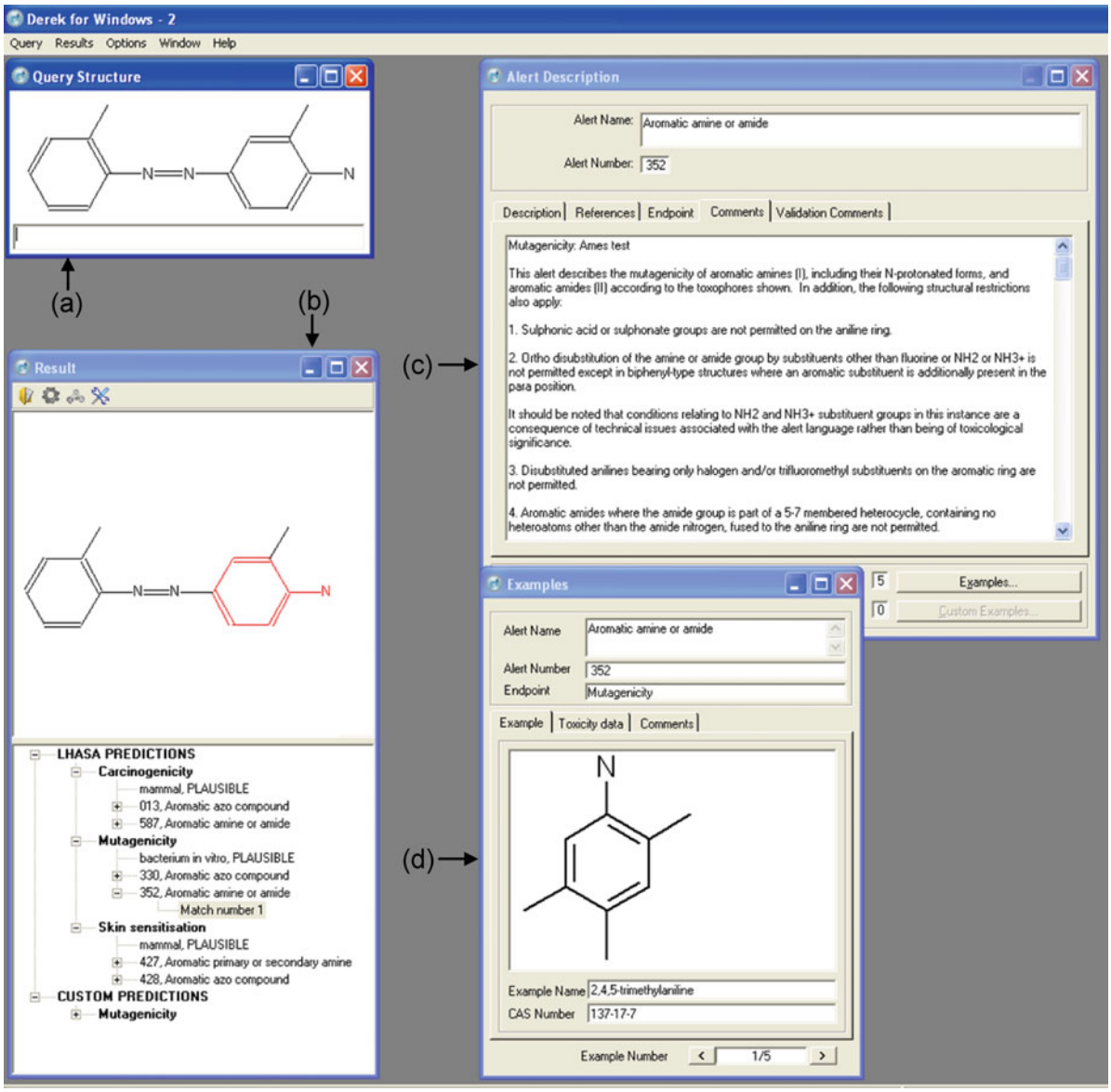
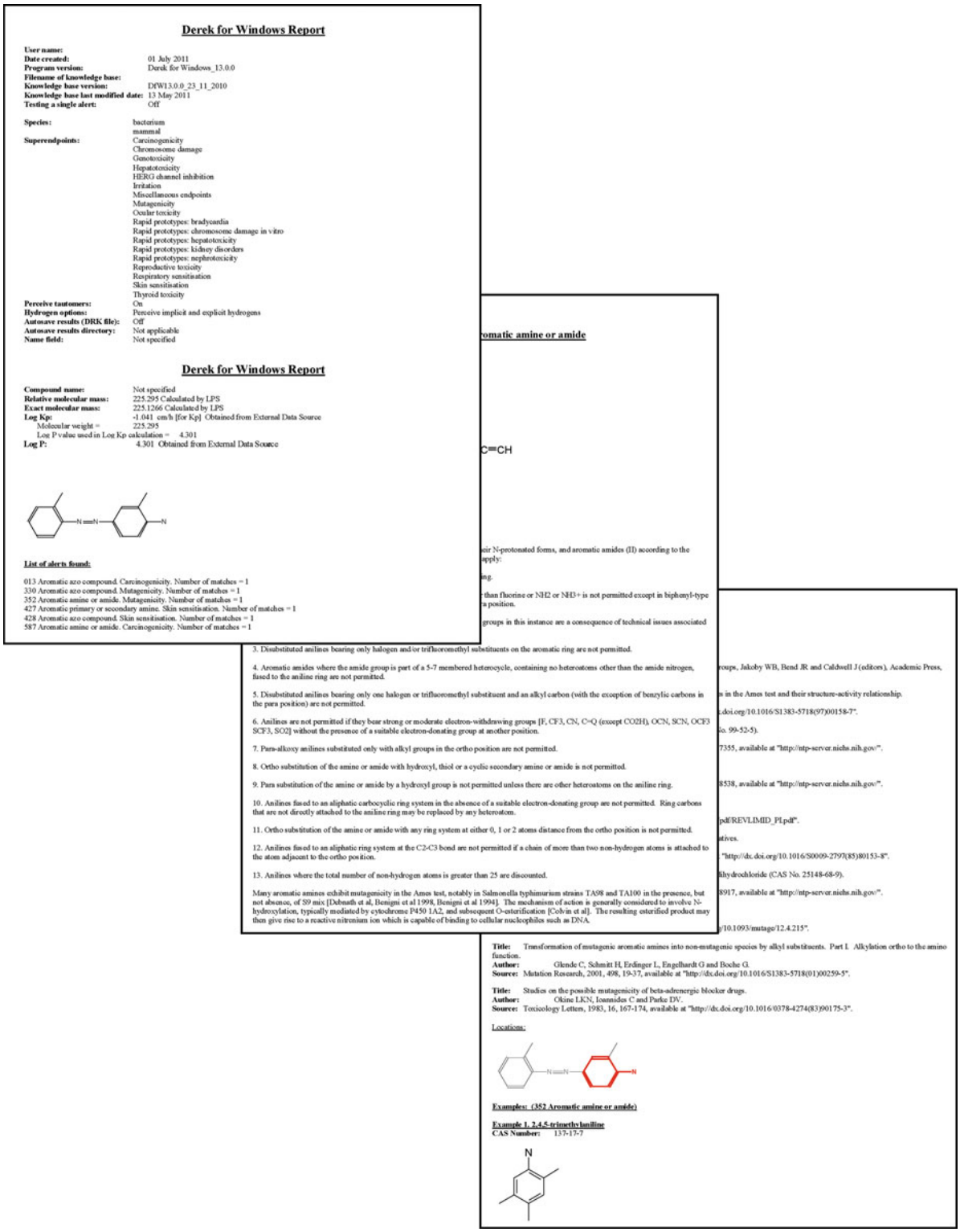


Fig. 51.2 Screen-print from the DEREK for Windows prediction (version 13.0) for the compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine: (**a**) imported structure, (**b**) prediction results window, (**c**) alert description window, (**d**) example compounds window

validation or cross-validation) and prospective prediction (also called external validation). In beta-testing, the available dataset is divided into a training and test set. The model is built upon the training set and the performance is then measured for the test set. In prospective prediction, the model is applied to a novel group of chemical structures where there is often little consistency with training set used to generate the model. A good way to use the prospective evaluation method is for pharmaceutical companies to use their historical, unpublished toxicity data of internal compounds (unavailable to the model developers) to evaluate the prediction systems (Greene 2002).

The Ames test (Ames et al. 1973) is a well-established and fast assay for the prediction of mutagenicity of drug candidates in the pharmaceutical industry. The result of the Ames test is also necessary for approval of a drug. The consequence is that every



Derek for Windows Report

User name:
Date created: 01 July 2011
Program version: Derek for Windows_13.0.0
Filename of knowledge base:
Knowledge base version: DfW13.0.0_23_11_2010
Knowledge base last modified date: 13 May 2011
Testing a single alert: Off

Species: bacterium
mammal
Superendpoints: Carcinogenicity
Chromosome damage
Genotoxicity
Hepatotoxicity
HERG channel inhibition
Irritation
Miscellaneous endpoints
Mutagenicity
Ocular toxicity
Rapid prototypes: bradycardia
Rapid prototypes: chromosome damage in vitro
Rapid prototypes: hepatotoxicity
Rapid prototypes: kidney disorders
Rapid prototypes: nephrotoxicity
Reproductive toxicity
Respiratory sensitisation
Skin sensitisation
Thyroid toxicity
Perceive tautomers: On
Hydrogen options: Perceive implicit and explicit hydrogens
Autosave results (DRK file): Off
Autosave results directory: Not applicable
Name field: Not specified

Derek for Windows Report

Compound name: Not specified
Relative molecular mass: 225.295 Calculated by LPS
Exact molecular mass: 225.1266 Calculated by LPS
Log Kp: -1.041 cm/h [for Kp] Obtained from External Data Source
Molecular weight = 225.295
Log P value used in Log Kp calculation = 4.301
Log P: 4.301 Obtained from External Data Source

List of alerts found:

013 Aromatic azo compound. Carcinogenicity. Number of matches = 1
330 Aromatic azo compound. Mutagenicity. Number of matches = 1
352 Aromatic amine or amide. Mutagenicity. Number of matches = 1
427 Aromatic primary or secondary amine. Skin sensitisation. Number of matches = 1
428 Aromatic azo compound. Skin sensitisation. Number of matches = 1
587 Aromatic amine or amide. Carcinogenicity. Number of matches = 1

romatic amine or amide

C≡CH

eir N-protonated forms, and aromatic amides (II) according to the
pply:

ng.

than fluorine or NH2 or NH3+ is not permitted except in biphenyl-type
a position.

groups in this instance are a consequence of technical issues associated

3. Disubstituted anilines bearing only halogen and/or trifluoromethyl substituents on the aromatic ring are not permitted.

4. Aromatic amides where the amide group is part of a 5-7 membered heterocycle, containing no heteroatoms other than the amide nitrogen, fused to the aniline ring are not permitted.

5. Disubstituted anilines bearing only one halogen or trifluoromethyl substituent and an alkyl carbon (with the exception of benzylic carbons in the para position) are not permitted.

6. Anilines are not permitted if they bear strong or moderate electron-withdrawing groups [F, CF3, CN, C=Q (except CO2H), OCN, SCN, OCF3 SCF3, SO2] without the presence of a suitable electron-donating group at another position.

7. Para-alkoxy anilines substituted only with alkyl groups in the ortho position are not permitted.

8. Ortho substitution of the amine or amide with hydroxyl, thiol or a cyclic secondary amine or amide is not permitted.

9. Para substitution of the amine or amide by a hydroxyl group is not permitted unless there are other heteroatoms on the aniline ring.

10. Anilines fused to an aliphatic carbocyclic ring system in the absence of a suitable electron-donating group are not permitted. Ring carbons that are not directly attached to the aniline ring may be replaced by any heteroatom.

11. Ortho substitution of the amine or amide with any ring system at either 0, 1 or 2 atoms distance from the ortho position is not permitted.

12. Anilines fused to an aliphatic ring system at the C2-C3 bond are not permitted if a chain of more than two non-hydrogen atoms is attached to the atom adjacent to the ortho position.

13. Anilines where the total number of non-hydrogen atoms is greater than 25 are discounted.

Many aromatic amines exhibit mutagenicity in the Ames test, notably in Salmonella typhimurium strains TA98 and TA100 in the presence, but not absence, of S9 mix [Debnath et al, Benigni et al 1998, Benigni et al 1994]. The mechanism of action is generally considered to involve N-hydroxylation, typically mediated by cytochrome P450 1A2, and subsequent O-esterification [Colvin et al]. The resulting esterified product may then give rise to a reactive nitrenium ion which is capable of binding to cellular nucleophiles such as DNA.

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g/10.1093/mutage/12.4.215".

Title: Transformation of mutagenic aromatic amines into non-mutagenic species by alkyl substituents. Part I. Alkylation ortho to the amino function.
Author: Glende C, Schmitt H, Erdinger L, Engelhardt G and Boche G.
Source: Mutation Research, 2001, 498, 19-37, available at "http://dx.doi.org/10.1016/S1383-5718(01)00259-5".

Title: Studies on the possible mutagenicity of beta-adrenergic blocker drugs.
Author: Okine LKN, Ioannides C and Parke DV.
Source: Toxicology Letters, 1983, 16, 167-174, available at "http://dx.doi.org/10.1016/0378-4274(83)90175-3".

Locations:

Examples: (352 Aromatic amine or amide)

Example 1. 2,4,5-trimethylaniline
CAS Number: 137-17-7

Fig. 51.3 Report from the DEREK for Windows prediction (version 13.0) for the compound 2-methyl-4-[(2-methylphenyl)azo] benzenamine

year a lot of unpublished propriety Ames results are produced in every company and are added to each company's historical toxicity database. In 2009, a prospective/external evaluation of DfW was performed at Sanofi-Aventis for the prediction of the endpoint mutagenicity. For this evaluation all proprietary compounds were used that were tested in the Ames assay at Sanofi-Aventis in the year 2008. From these 304 substances in total, 62 compounds (20%) were positive and 242 (80%) were negative in Ames test. For the prediction of these compounds the DEREK version 11.0 optimized with alerts from internal knowledge was used. The results of this evaluation are shown in Table 51.2.

Table 51.2 DfW mutagenicity predictions (version 11.0 + alerts from internal knowledge) for Ames results of 304 Sanofi-Aventis proprietary structures

	DfW prediction			
Ames results	Mutagenicity alert	No alert	Total	
Positive	30	32	62	Sensitivity[a] 30/62 = 48%
Negative	37	205	242	Specificity[b] 205/242 = 85%
Total	67	237	304	Concordance[c] (30 + 205)/304 = 77%

[a]Sensitivity is the percentage of correctly predicted positive compounds
[b]Specificity is the percentage of correctly predicted negative compounds
[c]Concordance is the percentage of correctly predicted positive and negative compounds

The results of this evaluation are summarized with the statistical parameters such as sensitivity, the correctly predicted positive compounds; specificity, the correctly predicted negative compounds; and concordance, the correctly predicted positive and negative compounds. In this evaluation, 30 out of 62 Ames positive compounds were correctly predicted, which gives a sensitivity of 48% for the system. On the other hand 205 out of 242 Ames negative compounds were correctly predicted, which gives a specificity of 85% for the system. The overall concordance, correct positive and correct negative predictions, was 77%.

These results are in good agreement with those found by other authors (Hillebrecht et al. 2011; Matthews et al. 2008; Snyder et al. 2004; Cariello et al. 2002; Greene 2002; Durham and Pearl 2001; Greene et al. 1999; Benfenati and Gini 1997; Sanderson and Earnshaw 1991).

CRITICAL ASSESSMENT OF THE METHOD

Greene (2002) listed some strengths and limitations of DfW in his review article. One of the strengths highlighted in this review is that the rules from DfW are regularly updated by LHASA Limited and peer reviewed by the users. This unique system of collaboration between LHASA Limited and the users is also important for Patlewicz et al. (2003) specifically because it encourages the sharing of toxicological information and knowledge for the benefit of all without organizations compromising the confidentiality of their proprietary data. Another strength for Greene (2002) and Durham and Pearl (2001) is that DfW summarizes, on one screen or with one report, the full list of alerts, the highlighted toxicophores and the justification for its prediction including the alert description, published references, and example compounds credited for the generation of this alert. It is also very easy with DfW to add new company internal rules to the system together with an alert description, literature, and example compounds related to this alert. For Greene (2002) additional strengths are that with the batch-processing feature it is possible to make high-throughput predictions for a large number of compounds for both testing and validation. At least one of the most important strengths of DfW is that the rules are based on scientific knowledge of structure-toxicity relationships and mechanisms (Greene 2002).

For Greene (2002) one of the limitations of DfW is that only a few physicochemical parameters are used; he suggested that their use should be extended to include other 2D and 3D parameters and also their use in predicting other toxicological endpoints should be explored further. Another point is that the activating and detoxification effects of metabolism need to be explored in more detail. For Patlewicz et al. (2003) the primary limitations of DfW relate to the inadequate number of rules for a number of toxicity endpoints and chemical classes reflecting knowledge gaps of the developers and user groups. For Durham and Pearl (2001) the system is limited in that DfW only identifies activation fragments, so the negative prediction is solely based on the lack of positive toxicophores. Therefore, the program is extremely biased toward the prediction of positive compounds. Consequently, DEREK has a low percentage of false negative predictions. The program automatically screens for some simple rules

(e.g., identifying structural exceptions to a rule), but not for other complex rules (e.g., presence of toxicity related to the presence of two or more unconnected toxicophores), even though the program is capable of storing these kind of rules (Durham and Pearl 2001).

MODIFICATIONS OF THE METHOD

Besides the possibility of using the general knowledge base of DfW offered by LHASA Limited, it is also possible to create an internal knowledge base by adding alerts, reasoning rules, and supporting data examples and references in-house. For the creation of new rules a knowledge base editor is available that enables the users to define one or more "patterns" for a new alert. These patterns include the SAR for a new rule and the definition for alerting a fragment within a molecule. The original rules offered by LHASA Limited cannot be modified, but it is possible to duplicate the LHASA Limited rules and to modify these copies to create new rules.

To get a more specific prediction for a special study design it is possible to change the processing constraints in DfW. For example by choosing a species DfW will make a species-specific prediction. The different species that can be selected are human, primate, mammal, rodent, rat, mouse, guinea pig, hamster, and bacterium including *Salmonella typhimurium*. The other processing constraints that can be changed are as follows: consider tautomers or not, perform a DEREK prediction for a single alert, etc.

DfW predictions include information about the mechanism of activity, for example metabolic steps necessary for toxicity. For a more detailed analysis of the toxicity of a compound and its metabolites DfW can be used in combination with an *in silico* program for the prediction of metabolites. LHASA Limited offers such a program with the name METEOR. For the combination of these two systems, first the metabolites of a compound are predicted by METEOR. These metabolites are then sent to DfW for the prediction of toxicity of the compound and its predicted metabolites.

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EXAMPLE

Figures 51.2 and 51.3 show the DEREK for Windows prediction (version 13.0) for the example compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine (CAS-No.: 97-56-3). For this compound many positive mutagenicity and carcinogenicity results are reported in the literature (results from the CCRIS database search). In agreement with the literature, this compound was predicted by DEREK for Windows to be carcinogenic (carcinogenicity alerts 013: aromatic azo compound, 587: aromatic amine or amide) and mutagenic (mutagenicity alerts 330: aromatic azo compound, 352: aromatic amine or amide). Figure 51.2 shows the screen-print and Fig. 51.3 shows the report from the DfW prediction for this compound.

51.3 MultiCASE

PURPOSE AND RATIONALE

MultiCASE (*Multiple C*omputer *A*utomated *S*tructure *E*valuating, MultiCase, Beachwood, OH, USA) is a statistically fragment-based expert computer system for the prediction of toxicity. It consists of a computer system to perform the prediction and different databases (modules). Each database contains a series of diverse non-congeneric chemical structures and their observed activity (quantitative or qualitative) for specific toxicological endpoints, including toxicological active and inactive compounds. Some authors also classify MultiCASE as a hybrid QSAR and artificial expert-structure-based program. The QSAR portion of the program is based upon 2-D chemical descriptors that utilize proprietary statistical analysis developed by Klopman (1984). The artificial expert-structure-based program is based upon the identification of atom fragments that are present in active and inactive molecules and that have a high probability of being relevant or responsible for the observed toxicological activity (Durham and Pearl 2001).

For the prediction of toxicity in a first step each molecule of a database is broken down from MultiCASE into all possible fragments from two to ten heavy (non-hydrogen) atoms including also overlapping fragments. These are then classified statistically as "biophores," fragments associated with toxicity, and "biophobes," fragments not associated with toxicity. In addition to utilizing molecular fragments, MultiCASE also identifies relevant two-dimensional distances between atoms within a chemical structure. MultiCASE then creates organized dictionaries of these biophores and biophobes and develops ad hoc local QSAR correlations that can be used to predict the activity of unknown molecules. The results of this first prediction step are saved, and identified biophores are visible for the users (MultiCASE homepage).

In the second step of the prediction a new molecule is entered into MultiCASE, and then the program evaluates this molecule against this organized dictionary and the appropriate QSARs it has created and makes a prediction of the toxicological activity of the molecule for the corresponding endpoint. To do this, MultiCASE identifies all relevant biophores and biophobes of the unknown molecule, combines these into an equation and calculates the toxicological activity expressed in CASE units with the help of the following equation (Dearden 2003):

$$\text{CASE units} = \text{constant} + \text{a(fragment 1)} + \text{b(fragment 2)} + \ldots$$

The scale of CASE units has linear range from 10 to 99 and normally chemicals with an assigned value of 10–19 are inactive, 20–29 have marginal activity, and 30–99 are moderately active, active, highly active, and extremely active. The system is also capable for identifying fragments that act as modifiers to the activity of each biophores class (Greene 2002).

MultiCASE covers different toxicological endpoints like genotoxicity, carcinogenicity, irritation, developmental toxicity/teratogenicity, adverse effects in humans for the endpoints hepatobiliary, renal/urinary tract and cardiac, and other miscellaneous endpoints. For each of these endpoints one or more databases (modules) containing active and inactive molecules are separately available, and most of the modules were constructed at the Food and Drug Administration (FDA) by the Informatics and Computational Safety Analysis Staff (ICSAS) group.

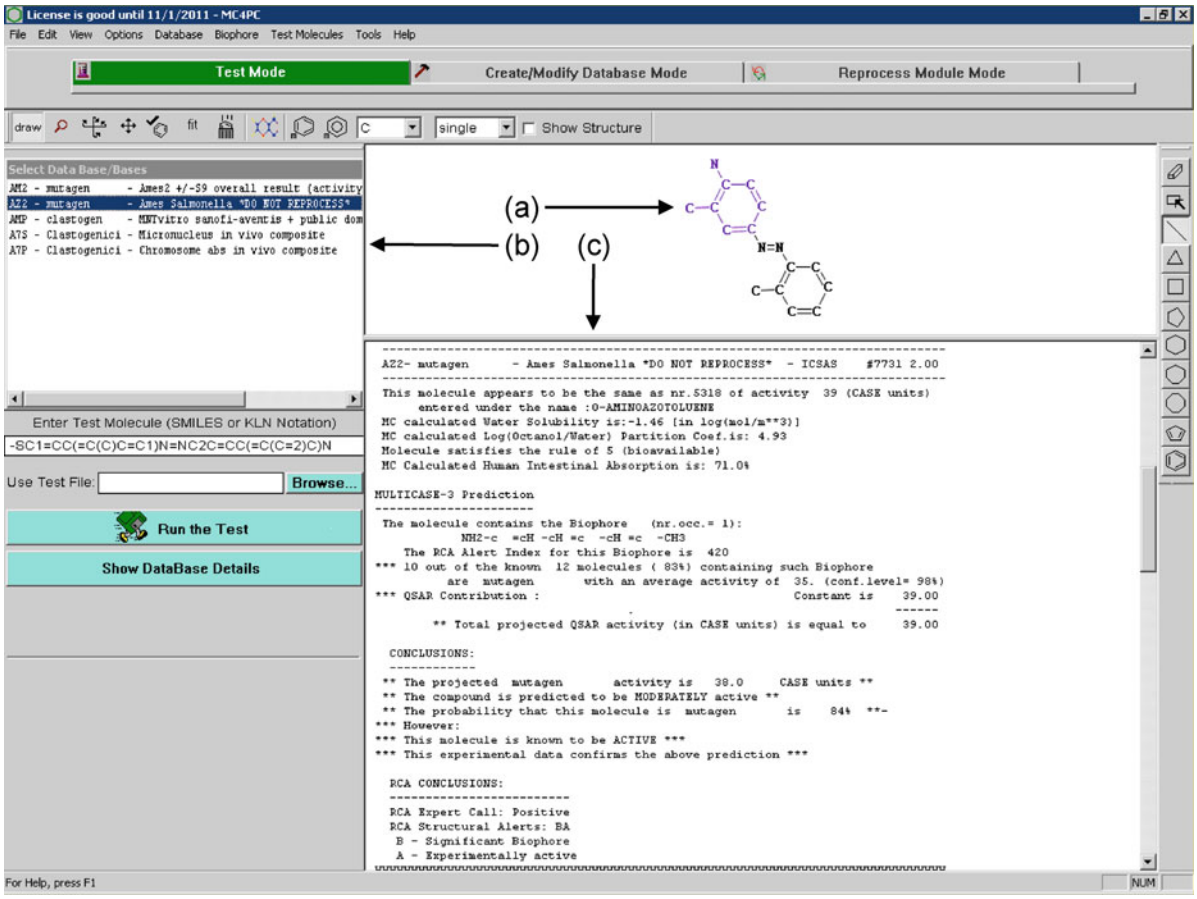

Fig. 51.4 Screen-print from the MultiCASE prediction (version 2.3) for the compound 2-methyl-4-[(2-methylphenyl)azo] benzenamine: (**a**) imported structure, (**b**) chosen modules for the prediction, (**c**) prediction results window

The number of compounds varies from 70 up to 6,000 per module (MultiCASE homepage).

There are also other systems available based on the MultiCASE technology. *C*omputer *A*utomated *S*tructure *E*valuating (CASE) was first developed before the MultiCASE system. It uses the same technology but differs in some ways. The major algorithmic difference in MultiCASE is the use of hierarchy in the selection of descriptors, leading to the concept of biophores and modulators. Another important difference is that only with MultiCASE new internal proprietary data can be used to create new databases.

PROCEDURE

The new versions of MultiCASE (MC4PC) run on Windows 9X/NT/2000 computers. For the prediction of toxicity, the structures of a compound can be easily entered into MultiCASE by using an internal graphical interface from the system (Fig. 51.4a). It is also possible to import structures in mol-file format or to run a set of compounds in an sd-file format. For MultiCASE prediction, one or more databases must be chosen, which should be used for the prediction (Fig. 51.4b). After starting the prediction, MultiCASE utilizes the set of statistically significant fragments and/or distance to find the biophore that has the highest probability of being responsible for the observed toxicological activity. The presence of biophores determines the likelihood of a compound to exhibit toxicity. The prediction then consists of the identification of the biophores responsible for toxicity and the probability (in percent) of a compound being toxicologically active. A compound is presumed to be inactive if it contains no biophore. Within each group of compounds containing a particular biophore, MultiCASE also performs a local QSAR in order to identify molecular

MultiCASE report

RCA Structural Alerts:
B - significant biophore
B? - possibly significant biophore
X - significant unknown fragment alert
X? - possibly significant unknown fragment alert
b - non-significant biophore
x - non-significant unknown fragment alert
D - significant deactivating fragment (biophobe)
d - non-significant deactivating fragment
A - experimentally active
M - marginal experimental activity
I - experimentally inactive
w - unknown fragment warning

Comments:
de - different environment
pos - 2D positional alert
qsar - a significant biophore +B is changed to -B because its total QSAR < 30
deg - highly degenerate fragment
bad alert - the fragment is a biophore for some of the databases and a biophobe for others
SSA - Structural Similar Alerts

		AM2 (Ames II)			AZ2 (Ames)			AMP (MNT vitro)			A7S (MNT vivo)			A7P (CA vivo)				Summary	
		Expert			Expert			Expert			Expert			Expert				Expert	
	Test Chemicals	call	Alert	Cover.	call	Alert	Cover.	call	Alert	Cover.	call	Alert	Cover.	call	Alert	Cover.	#SSA	Call	Comments
# 1	-SC1=CC(=C(C)C=C1)N=NC2C=CC(=C	-		w	+	BA		-	D		+	B?		-	d	w	1	+	

The reasoning method was developed through a Research and Collaborative Activity (RCA) under an approved CRADA with FDA/CDER

Fig. 51.5 Report from the MultiCASE prediction (version 2.3) for the compound 2-methyl-4-[(2-methylphenyl)azo] benzenamine

features, which controls the degree of activity. These features, termed modulators, are selected from the pool of molecular fragments, 2-D distance descriptors, calculated electronic indices (molecular orbital energies, charge densities), and calculated transport parameters (octanol/water participation coefficient, water solubility). These local QSARs are utilized to predict the potency of chemicals containing specific biophore. At least for an overall prediction, the four individual predictions corresponding to the probability of activity and QSAR potencies are combined using Bayes' theorem. For that purpose the predictivity of each of the four SAR models are determined by analysis of the sensitivity and specificity of the model for chemicals not in the database (Rosenkranz et al. 1999).

During the MultiCASE prediction, the output of the prediction is displayed in a text window on the screen (Fig. 51.4c). This text will be automatically saved in several rtf- and xls-files (Fig. 51.5).

EVALUATION

Also for MultiCASE, in 2009 a prospective/external evaluation was performed at Sanofi-Aventis for the prediction of the endpoint mutagenicity. For this evaluation the identical proprietary compounds were used, which were also used for the DEREK evaluation: all proprietary compounds that were tested in the Ames assay (Ames 1973) at Sanofi-Aventis in the year 2008. Again, from these 304 substances in total, 62 compounds (20%) were positive and 242 (80%) were negative in Ames test. For the prediction of these compounds the MultiCASE version 2.0 together with the prediction module "Ames *Salmonella* (AZ2)" optimized with internal compounds was used. The results of this exercise are shown in Table 51.3.

The results of this evaluation are summarized with the help of the statistical parameters such as sensitivity, the correctly predicted positive compounds; specificity, the correctly predicted negative compounds; and concordance, the correctly predicted positive and negative compounds. In this evaluation, 34 out of 62 Ames positive compounds were predicted correctly, which results in a sensitivity of the system of 55%. On the other hand, 215 out of 242 Ames negative compounds were correctly predicted, which gives a specificity of the system of 89%. The overall concordance, correct positive and correct negative predictions, was 82%.

The sensitivity as well as the specificity of MultiCASE is minimally higher compared to the evaluation with DEREK but is overall very similar. In general, a combination of the DEREK and MultiCASE prediction is improving the sensitivity and the identification of the Ames positive compounds, since both systems use different prediction methods (the knowledge/rule-based approach from DEREK combined with the statistically fragment-based approach from MultiCASE).

These results of the MultiCASE evaluation are in good agreement with those found by other authors (Hillebrecht et al. 2011; Matthews et al. 2008; Snyder

Table 51.3 MultiCASE predictions (version 2.0) using the "Ames *Salmonella* (AZ2)" module, optimized with internal compounds, for Ames results of 304 Sanofi-Aventis proprietary structures

Ames results	MultiCASE prediction			
	Positive	Negative	Total	
Positive	34	28	62	Sensitivity[a] 34/62 = 55%
Negative	27	215	242	Specificity[b] 215/242 = 89%
Total	61	243	304	Concordance[c] (34 + 215)/304 = 82%

[a]Sensitivity is the percentage of correctly predicted positive compounds
[b]Specificity is the percentage of correctly predicted negative compounds
[c]Concordance is the percentage of correctly predicted positive and negative compounds

et al. 2004; Greene 2002; Durham and Pearl 2001; Rosenkranz et al. 1999; Cunningham et al. 1998; Benfenati and Gini 1997).

CRITICAL ASSESSMENT OF THE METHOD

In his review Greene (2002) pointed out some strengths and limitations of MultiCASE. One of the strengths of MultiCASE is that it is capable of generating predictive models without knowing the mechanisms of toxicity. The program also utilizes a number of physicochemical properties to modulate its predictions. Finally another strength is that due to the input of FDA data, MultiCASE has substantially increased the applicability to the prediction of rodent carcinogenicity for pharmaceutical type compounds. For Durham and Pearl (2001) some other strength of MultiCASE compared to a knowledge base system like DEREK is that it searches for both activating and deactivating fragments for the toxicity prediction. Another advantage is that it is very easy to add new proprietary data to the databases in order to increase the chemical space and to achieve a more specific prediction for the internal proprietary compounds.

The limitations for Greene (2002) are that the quality of the predictions made by the system is closely linked to the quality of the data used in the training set. The output from the program is also often ambiguous and can lead to misinterpretation of the predictions. Moreover the system often fails to distinguish between molecules containing several small chains within one complex fragment from other molecules containing the same fragments distributed separately.

MODIFICATIONS OF THE METHOD

To perform a prediction with MultiCASE different commercially available databases from the developer of the program can be used. They are updated regularly by adding new compounds to the databases. However, it is also possible to modify the commercial databases as well as to create new databases using internal company data. For this a dat-file first has to be created, containing all compounds for the new database together with their structure code and biological activity. By screening this dat-file with MultiCASE, statistical algorithms are used to create a model capable of predicting the biological activity of the new compounds. A chart-view is available to adjust the indicated properties. After that the dat-file can be stored and other fields for chemical properties calculated by the program, such as boiling point, molecular weight, water solubility, and Log P, are added. The next step is the creation of the new database. The database description must be entered after which MultiCASE starts to calculate the biophores and biophobes of the new database.

There is also a separate expert system for the combination with MultiCASE, which predicts the possible metabolites, formed of a compound. This system is known as META, which was developed to identify molecular sites susceptible to metabolic transformation. The metabolism dictionary associated with META is able to recognize 663 enzyme-catalyzed reaction rules, which have been categorized into 29 enzyme-reaction classes and 286 spontaneous reactions (Klopman and Rosenkranz 1994).

The US Food and Drug Administration (FDA) has adapted the MultiCASE technology and has developed a new system known as MultiCASE QSAR-ES (quantitative structure-activity relationships expert system). This system uses the MultiCASE program and new database modules that were developed under a Cooperative Research and Development Agreement (CRADA) between the US FDA and MultiCASE. It was designed to improve the prediction of the carcinogenic potential of pharmaceuticals. Matthews and Contrera (1998) performed a beta-test evaluation utilizing 126 compounds not included in the test database with 934 compounds. The results demonstrated an improvement in all statistical parameters for the prediction of carcinogenicity compared to the MultiCASE prediction with an overall concordance of 75% (Patlewicz et al. 2003).

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EXAMPLE

Figures 51.4 and 51.5 show the MultiCASE prediction (version 2.3) for the example compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine (CAS-No.: 97-56-3). For this compound many positive mutagenicity and carcinogenicity results are reported in the literature (results from the CCRIS database search). In agreement with the literature, this compound was predicted by MultiCASE to be mutagenic. Figure 51.4 shows the screen-print from the MultiCASE prediction for this compound and Fig. 51.5 shows an example of a MultiCASE report for a dataset of compounds.

51.4 Leadscope

PURPOSE AND RATIONALE

The Leadscope Model Applier (Leadscope Inc., Columbus, Ohio, USA) is an expert computer system using a fragment-based QSAR paradigm. Like MultiCASE, the system consists of a computer software to perform the prediction and different training databases (models) for the prediction of the respective toxicity endpoints. The fragments used for the prediction are predefined in a hierarchically organized dictionary that is closely related to common organic/medicinal chemistry blocks. For binary classification problems, such as the Ames test, the algorithm identifies toxicity-modulating fragments using a $\chi 2$-test. Furthermore, the software is able to build superstructures from smaller fragments if they improve predictivity. Additionally eight global molecular properties are calculated (atom count, hydrogen bond acceptors, hydrogen bond donors, Lipinski score, LogP, molecular weight, polar surface area, and rotatable bonds). These global molecular properties together with the set of fragments are then used as a descriptor set in a partial least squares (PLS) logistic regression model of the activity class. Therefore, the predictions from this algorithm are continuous probabilities of class membership rather than binary outputs, given as the likelihood value between 0 (nontoxic) and 1 (toxic). All probabilities greater than 0.5 are considered as "active" predictions and probabilities smaller than 0.5 as "inactive" predictions. The higher the probability is, the greater chance there is of the test chemical being toxic in a particular endpoint. The program also assesses the applicability domain by measuring

the distance to training set molecules by using two parameters: (1) having at least one feature defined in the model; (2) having at least one chemical in a training neighborhood with at least 30% similarity. Compounds which are annotated as "out of domain" or "missing descriptors" are counted as "not predicted" (Hillebrecht et al. 2011).

Currently, Leadscope offers QSAR models for the prediction of eight different toxicity endpoints. All these QSARs were constructed at the Food and Drug Administration (FDA) by the Informatics and Computational Safety Analysis Staff (ICSAS) group. The training datasets were compiled by ICSAS and the models were built within the Leadscope Prediction Data Miner software using all default settings (Yang et al. 2008).

The QSAR models can be divided into two major groups of endpoints: human clinical endpoints and nonhuman toxicity endpoints. The first group includes three suites of models which predict the effects of compounds based upon human clinical data, including adverse cardiological effects, adverse hepatobiliary effects, and adverse urinary tract effects. The second group includes five different suites, predicting toxicities of compounds based upon results of in vivo animal toxicity and in vitro studies. They include carcinogenicity in rodents, genetic toxicity (i.e., mutagenicity, clastogenicity, and DNA damage), reproductive toxicity in male and female rodents, developmental toxicity (i.e., dysmorphogenesis, fetal development, and survival of the rodent fetus), and neurotoxicity in newborn rodents.

Each toxicity endpoint has many different QSAR models, for some sub-models are constructed to improve the predictive performance of the models. The predictive performance of global models depends highly on the ratio of active (toxic) to inactive (nontoxic) chemicals in a training set. A training set was divided into subsets to maintain optimal active-to-inactive (A/I) ratio to ensure high specificity. This A/I ratio ranges between 0.30 and 0.35 for most training sets. The rationale behind these QSAR models is that predicting true negatives must be maximized while false negatives must be minimized in product safety analysis within the regulatory agencies. In general, more than one sub-model is built for each model, and for best results, Leadscope runs each of the sub-models behind the scene and presents an overall result. The overall prediction results are based on averaging the probabilities (likelihood of being positive) from appropriate sub-models (Leadscope FDA Model Applier Documentation 2008).

PROCEDURE

For the predictions, structure can be imported into Leadscope as files or an entry for a single compound. It handles both MDL mol/SD files and SMILES. Then the individual QSAR models can be accessed via a prediction model wizard. The models are organized hierarchically in a tree view. When clicking the model name, a short description will appear in a "description" box. The wizard allows the review of both the overall average model as well as the sub-models. Then the next window from this wizard will display the cross-validated model results (performed for 10%) for chemicals in the training set. These validation results include concordance (overall accuracy), sensitivity, specificity, receiver-operator constant (ROC) for positives and negatives. After selecting the individual QSAR models, the predictions can be applied simultaneously for the imported structures. Running multiple models is possible and the results of the multiple models are displayed in the "Summary" tab of the prediction results table. The results are tabulated in a "Summary" table. This summary displays all the requested prediction results for each model as well as indicating whether any prediction calls from any of the models were positive. Prediction calls include positive, negative, not-in-domain, or missing descriptors. Also additional model parameters can be retrieved, including number of chemicals found to be within the neighborhood defined by 30% similarity, number of model features used to calculate the predictions, and the average domain distance of the test chemical to the training set structures (based on all chemical features). Once the prediction models are applied and the results are saved, some chemical inference and reasoning can be performed within the Model Applier from the review prediction results wizard. This wizard is also a convenient way to evaluate the external validation results. It displays the contingency table and allows exploration of true positives and negatives as well as false predictions via histograms or a scatter plot.

The last wizard page then correlates the Leadscope hierarchy features with the actual experimental data versus predicted data. The model applier provides ways to perform chemical inferences during the external validation process (Leadscope FDA Model Applier Documentation 2008).

EVALUATION

Also for Leadscope, in 2009 a prospective/external evaluation was performed at Sanofi-Aventis for the prediction of the endpoint mutagenicity. For this evaluation the identical proprietary compounds were used, which were also used for the DEREK and MultiCASE evaluations: all proprietary compounds that were tested in the Ames assay (Ames 1973) at Sanofi-Aventis in the year 2008. Again, from these 304 substances in total, 62 compounds (20%) were positive and 242 (80%) were negative in Ames test. For the prediction of these compounds the Leadscope version 1.3 together with the prediction model *Salmonella* optimized with internal compounds was used. The results of this exercise are shown in Table 51.4.

The results of this evaluation are summarized with the help of the statistical parameters, such as sensitivity, the correctly predicted positive compounds; specificity, the correctly predicted negative compounds; and concordance, the correctly predicted positive and negative compounds. In this evaluation, 36 out of 62 Ames positive compounds were predicted correctly, which results in a sensitivity of the system of 58%. On the other hand, 217 out of 242 Ames negative compounds were correctly predicted, which gives a specificity of the system of 90%. The overall concordance, correct positive and correct negative predictions, was 83%.

The sensitivity as well as the specificity of Leadscope is minimally higher compared to the evaluation with MultiCASE and DEREK but is overall very similar. In general, a combination of the DEREK and Leadscope (or MultiCASE) prediction is improving the sensitivity and the identification of the Ames positive compounds, since both systems use different prediction methods (the knowledge/rule-based approach from DEREK combined with the statistically fragment-based approach from Leadscope or MultiCASE).

Table 51.4 Leadscope predictions (version 1.3) using the *Salmonella* model, optimized with internal compounds, for Ames results of 304 Sanofi-Aventis proprietary structures

Ames results	MultiCase prediction			
	Positive	Negative	Total	
Positive	36	26	62	Sensitivity[a] 36/62 = 58%
Negative	25	217	242	Specificity[b] 217/242 = 90%
Total	61	243	304	Concordance[c] (36 + 217)/304 = 83%

[a]Sensitivity is the percentage of correctly predicted positive compounds
[b]Specificity is the percentage of correctly predicted negative compounds
[c]Concordance is the percentage of correctly predicted positive and negative compounds

These results of the Leadscope evaluation are in good agreement with those found by other authors (Hillebrecht et al. 2011; Valerio et al. 2010; Matthews et al. 2008).

CRITICAL ASSESSMENT OF THE METHOD

Possible strengths and limitations of the Leadscope Model Applier are described in the review article of Valerio et al. (2010). The strengths of this expert system include that it is easy to use and very intuitive with respect to navigating through the screens and the loading of molecules or sets of molecules for predictions. It also allows output to be easily interpreted and exported. Additionally, it shows also a wide variety of models which are available for prediction of different endpoints. The main strength of this expert system is that the chemoinformatics approach of the Leadscope structural feature analysis is unique among other computational methods, thus providing more insight into the chemical landscape. This includes that the predefined structural features are closely related to common organic/medicinal chemistry blocks and that the intuitive chemical interference of these is supported for open training sets.

The limitations for Valerio et al. (2010) include that the location of the array of different prediction models within the various suites is somewhat confusing and presents a rather daunting challenge as to which models should be selected for any given prediction. So are the genotoxicity and carcinogenicity models

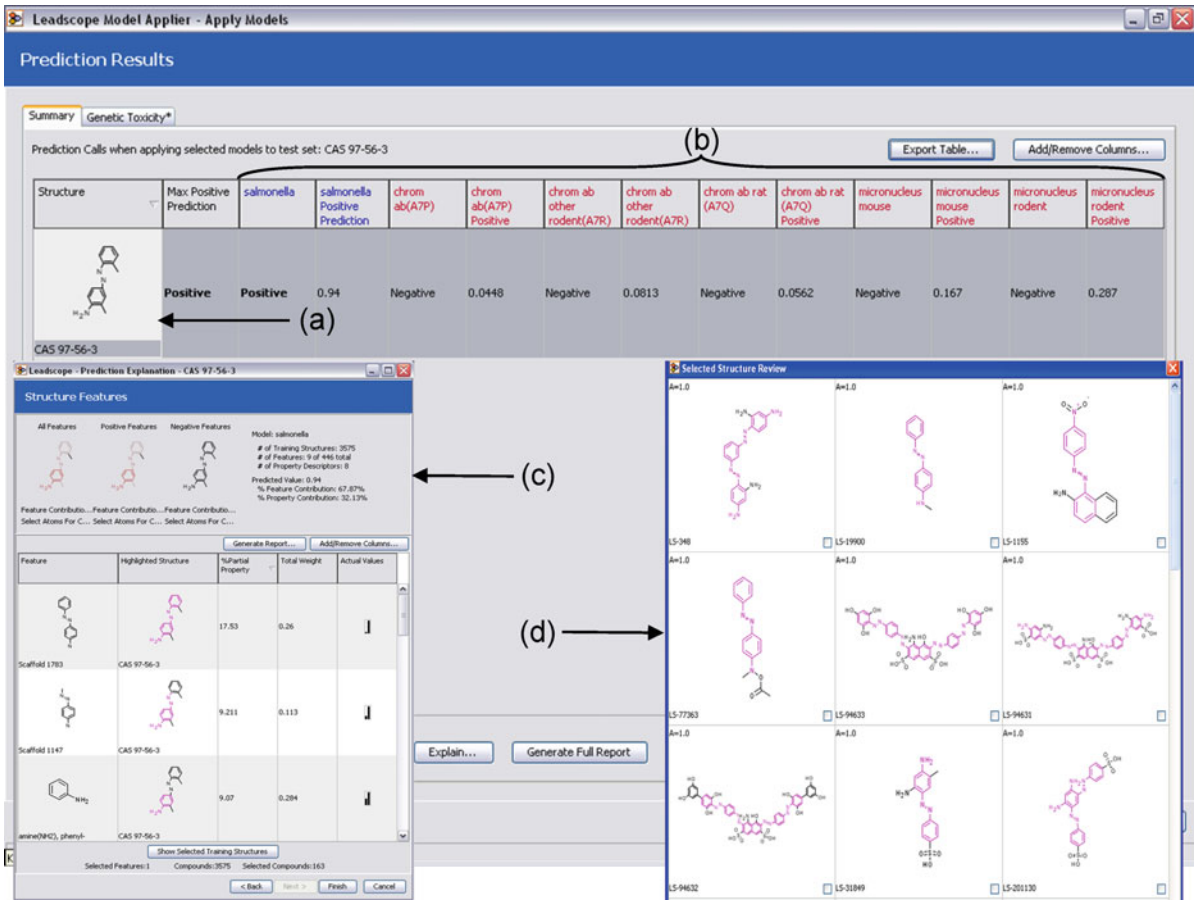
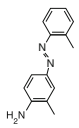


Fig. 51.6 Screen-print from the Leadscope prediction (version 1.3) for the compound 2-methyl-4-[(2-methylphenyl)azo] benzenamine: (**a**) imported structure, (**b**) chosen models for the prediction, (**c**) prediction results window, (**d**) Ames positive compounds from the training dataset on which the positive prediction is based

separated into two suites and therefore, different categories which could mislead users into inappropriate assumptions, organizing all of the toxicity models within their respective phenotype may be better. Also as with any new software package, there are a number of concerns regarding the development of the models (facets of construction and validation techniques used) and the software package, which should be addressed in the next version.

MODIFICATIONS OF THE METHOD

Like described for MultiCASE, also Leadscope offers the possibility to modify the commercially available databases as well as to create new databases using internal company data for the construction of new prediction models with the help of the application "Leadscope Enterprise." This system consists of a server application typically running on a high end computer and one or more client applications running on desktop PCs. All structures and associated data from the central corporate databases are stored on the server or in the personal database embedded in the client running on the PC. The structures and datasets imported are analyzed and then can be used for the construction of the new prediction models using the applications "QSAR analysis." Within these applications, the option "automatically build predictive models" can be chosen by using the default algorithms and parameters coming from the Leadscope system to construct a new prediction model. It is also possible to

Structure	Max Positive Prediction	salmonella	salmonella Positive Prediction Probability	chrom ab(A7P)	chrom ab(A7P) Positive Prediction Probability	chrom ab other rodent(A7R)	chrom ab other rodent (A7R) Positive Prediction Probability	chrom ab rat (A7Q)	chrom ab rat (A7Q) Positive Prediction Probability	micronucleus mouse	micronucleus mouse Positive Prediction Probability	micronucleus rodent
 CAS 97-56-3	Positive	Positive	0.94	Negative	0.0448	Negative	0.0813	Negative	0.0562	Negative	0.167	Negative

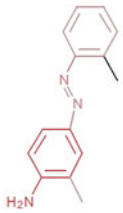
Model: salmonella

Model: salmonella

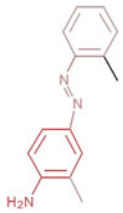
# of Training Structures: 3575
# of Features: 9 of 446 total
# of Property Descriptors: 8

Predicted Value: 0.94
% Feature Contribution: 67.87%
% Property Contribution: 32.13%

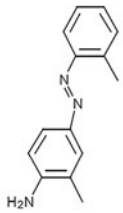
All Features



Positive Features



Negative Features



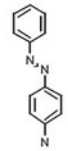
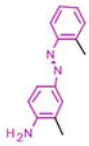
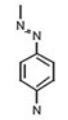
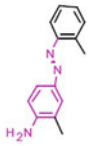
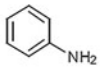
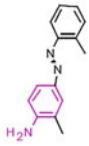
Feature	Highlighted Structure	%Partial Property	Total Weight	Actual Values
 Scaffold 1783	 CAS 97-56-3	17.53	0.26	
 Scaffold 1147	 CAS 97-56-3	9.211	0.113	
 amine(NH2), phenyl-	 CAS 97-56-3	9.07	0.284	

Fig. 51.7 Report from the Leadscope prediction (version 1.3) for the compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine

choose the option "manually build predictive models" with the possibility to change and adapt some of the algorithms and parameters to the specific database.

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EXAMPLE

Figures 51.6 and 51.7 show the Leadscope prediction (version 1.3) for the example compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine (CAS-No.: 97-56-3). For this compound many positive mutagenicity and carcinogenicity results are reported in the literature (results from the CCRIS database search). In agreement with the literature, this compound was predicted by Leadscope to be mutagenic in the *Salmonella* Ames assay. Figure 51.6 shows the screen-print from the Leadscope prediction for this compound, and Fig. 51.7 shows the report from the Leadscope prediction.

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Genotoxicity

52

Ingo Stammberger, Andreas Czich and Knut Braun

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The task of the Genetic Toxicology is to use recognized test methods in order to identify the genotoxic or mutagenic potential of pharmaceuticals and chemicals. The genotoxic or mutagenic potential is what we call the potential health risk of a substance with respect to its ability to cause cell mutations. Substances with this potential are known as mutagens and genotoxicity studies, also called mutagenicity studies, help identify such substances.

Early warnings that a possible new drug product may have a genotoxic effect are of immense importance for drug development. If a potential new drug is proven to have a genotoxic potential, this generally leads to termination of development of that drug, thereby preventing human exposure to drugs with mutagenic effects (except cytostatic drugs).

In addition, in the last decade, the testing and identification of impurities was recognized as important step in the toxicological risk assessment of the drug substance. The identification of a genotoxic potential and the determination of acceptable limits for genotoxic impurities in active substances is a difficult issue and now a days addressed in several publications and Guidelines. The data set usually available for genotoxic impurities is quite variable and is the main factor that dictates the process used for the assessment of acceptable limits. A generally applicable approach was defined by implementing the Threshold of Toxicological Concern (TTC). A TTC value of 1.5 μg/day intake of a genotoxic impurity is considered to be associated with an acceptable risk (excess cancer risk of <1 in 100,000 over a lifetime) for most

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Table 52.1 Overview of genotoxicity assays

Assay	Alternative name/ abbreviation	Genetic endpoint tested	Specific damage detected
Bacterial reverse mutation test	Ames test	Gene mutations	Base pair substitution, addition or deletion
L5178Y tk+/− mouse lymphoma test	MLA	Chromosome aberrations	Point mutations and structural alterations, based on mutations in the tk gene
Micronucleus test in vitro	–	Chromosome aberrations	Induction of micronuclei
Micronucleus test in vivo	–	Chromosome aberrations	Induction of micronuclei
Mammalian chromosome aberration test in vitro	–	Chromosome aberrations	Mainly structural alterations (chromosome or chromatid)
Mammalian chromosome aberration test in vivo	–	Chromosome aberrations	Mainly structural alterations (chromosome or chromatid) including polyploidies
Comet Assay in vivo	Comet in vivo	DNA strand breaks	DNA strand breaks
Unscheduled DNA synthesis test with mammalian liver cells in vitro	UDS in vitro	DNA repair	DNA repair synthesis
Unscheduled DNA synthesis test with mammalian liver cells in vivo	UDS in vivo	DNA repair	DNA repair synthesis

pharmaceuticals (Kroes et al. 2004). From this threshold value, a permitted level in the active substance can be calculated based on the expected daily dose and the duration of treatment (EMEA 2006). However, higher limits may be justified under certain conditions, e.g., based on the therapeutic area.

Numerous test methods are available in order to assess the genotoxic potential of drug substances. There are essentially three main categories of genotoxic damage that can be caused by a substance:

1. Gene mutations
2. Chromosome aberrations and genome mutations
3. DNA damage and DNA repair

For the assessment of potential genotoxic impurities, a combination of in silico analysis (see Chap. 41 "In-Silico ADME Modeling") and the detection of a direct DNA damaging potential of the compound is considered to be sufficient. For the detection of the direct DNA damaging potential, the bacterial reverse mutation test (Ames Test) is recommended by the Guidelines as stand-alone assay.

Gene mutations include base pair substitutions and frameshift mutations, where base pair substitutions arise from the substitution of one or several base pairs in the DNA, and frameshift mutations arise from an insertion or deletion involving a number of base pairs that is not a multiple of three and consequently disrupts the triplet reading frame, usually leading to the creation of a premature termination (stop) codon and resulting in a truncated protein product.

Chromosomal aberrations include both numerical and structural aberrations. Numerical aberrations are changes in the number of chromosomes of the normal number characteristic of the animals utilized (aneugenicity). Structural aberrations are classified into two types, chromosome or chromatid aberrations (clastogenicity). Chromosomal mutations and related events are the cause of many human genetic diseases and there is evidence that chromosomal mutations and related events are involved in cancer development.

DNA damage can happen in several ways. For example, energy production in cells can produce toxic molecules, such as the so-called free radicals. They can react with the bases in the DNA and modify them, thus preventing the genetic code from being used properly. Those DNA damages then need to be repaired. All cells have evolved a system of recognizing DNA errors, fixing them and in special cases repairing them. This DNA repair mechanism can be faulty, which in itself results in a genotoxic effect.

There are specific assays available to detect each of these types of genotoxic damage, and a battery of tests is often used to determine if a substance causes any or all of these three types of damage. An overview of which assays can be used to detect a given type of genotoxic effect is shown in Table 52.1.

These assays are generally performed by applying a test substance to well characterized cell systems (e.g., bacterial or mammalian cell cultures) and evaluating changes in the growth and characteristics of the cells. This may involve assessing the speed to form colonies and the size of the colonies as well as using markers to label chromosome structure or to monitor DNA synthesis.

Usually most bacteria and cell lines do not possess the full capability for metabolizing pro-mutagens and pro-cancerogens. To overcome this deficiency substances are tested in the presence of an exogenous metabolic activation system (S9-mix) as well as in its absence. Adding the S9-mix to the cell culture allows for metabolism of the test substance by enzymes not present in the bacterial cells. The S9-mix is a postmitochondrial supernatant fraction from liver supplemented with a NADP-generating system. It is generally made from rat liver induced with polychlorinated biphenyls, however, livers from hamsters, monkeys, or other species may be used depending on the anticipated metabolism of the substance being tested. An S9-mix has been successfully used in eukaryotic in vitro systems for the metabolic activation of various compounds.

In the last decade, a lot of effort was done to revise the ICH (International Congress on Harmonization) guidelines for genotoxicity testing of pharmaceuticals. Importantly, there are a number of changes to the previous testing strategy that reflects a desire to reduce the false positive rate in the in vitro test battery. For instance, the top dose was lowered for noncytotoxic drugs in the mammalian cell assays. In addition, one testing option eliminates the need to conduct the in vitro mammalian cell assays (and just conduct Ames and an in vivo assay with two endpoints included). New assays and endpoints were introduced (Micronucleus Assay in vitro for regulatory purposes, Comet Assay in vivo) and a few assays might be used as follow-up assays very soon (PigA Assay).

The test guidelines for new pharmaceuticals recommend that for follow-up testing to evaluate positive results in genotoxicity test in vitro, in vivo assays should be conducted in two tissues. In practice this is usually achieved by performing an erythrocyte chromosome-damage test in bone marrow and a genotoxicity assay in liver, because some genotoxic carcinogens are known to be positive in the liver but not in the bone marrow assays. In current regulatory guidance the rodent Comet assay is considered as a useful follow-up test in case of positive results in in vitro genotoxicity assays. The Comet Assay is recognized as a useful tool for the evaluation of genotoxicity in organs/cell types that cannot easily be evaluated with other standard tests, e.g., in skin and stomach.

In addition the implementation of Genetic Toxicology endpoints into repeat dose toxicity studies was evaluated and recommended by the scientific community, to reduce the numbers of animals within toxicological studies. Those endpoint were mainly the Micronucleus Assay and the Comet Assay.

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52.2 Bacterial Reverse Mutation Test

PURPOSE/RATIONALE

The bacterial reverse mutation test (Ames Test) investigates the ability of chemicals and drugs to induce reverse (back) mutations in bacteria, which involves base pair substitutions additions and/or deletions (frameshift mutations) of one or a few DNA base pairs. The bacterial strains used in the test system have mutations in genes coding for enzymes required for the biosynthesis of the amino acids histidine (*Salmonella*

typhimurium) and tryptophan (*Escherichia coli*). If a test substance causes a mutation that restores function to the enzyme gene, then the bacteria will be able to grow and produce colonies. An extensive data base exists based on the use of this test, and it has been found that compounds producing a positive result in the bacterial reverse mutation test have a high potency to induce cancer in animal studies.

PROCEDURE

The commonly used strains for *S. typhimurium* are TA100 and TA1535 (base pair substitution), TA98 and TA1537 (frame shift mutations) and TA102 for cross-link mutations. The preferred strain of *E. coli* to detect base pair substitution mutations is WP2.

Bacteria of an overnight nutrient broth culture are mixed with the test compound or solvent (as a negative control), S9-mix or buffer, and molten top agar and poured into a petri dish containing a layer of minimal agar. After incubation for approximately 48 h at approx. 37°C in the dark, colonies (representing the number of revertants) are counted by hand or an automatic colony counter. The method can be modified by including a preincubation step or by using a higher amount of S9-mix in the test system. Preincubation would involve incubating the test compound, S9-mix or buffer, and bacteria for a short period before pouring this mixture onto plates of minimal agar. According to the current ICH S2R Guideline, one experiment is sufficient to determine the mutagenic potential. The assay can be conducted as plate or preincubation assay.

52.2.1 Rational for the Dose Selection

The highest dose level in each experiment should correspond:

- either to 5,000 μg/plate
- or to a dose level corresponding to the highest soluble concentration in the solvent
- or to a dose level producing a precipitation in the plates or to a dose level which induces bacteria toxicity, whatever the solubility

EVALUATION

To evaluate the result of a test compound, the number of revertant colonies has to be determined for each concentration used in the test system as well as for the negative (solvent or untreated) and positive control. Bacterial toxicity is expressed as a thinning of the bacterial lawn or in a reduction in the number of colonies compared to the negative control. Bacterial toxicity and precipitation should be taken into consideration when assessing the mutagenicity of a substance. The following criteria should be met to consider a bacterial reverse mutation test as valid.

- The number of revertant colonies on both negative (solvent or untreated) control plates should be in a historical control range described in literature or determined in the laboratory.
- The positive control should induce a significant increase in the number of revertant colonies.
- At least five dose levels for the test article are analyzable (i.e., number of revertant colonies can be determined), for each strain in each experimental condition.
- The highest dose level fulfills the rational for the highest dose level selection

A test compound is classified as inducing point mutations if it causes at least two of the following criteria:

- It produces at least a two- to threefold increase in the mean number of revertants per plate depending on the strain used of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control at complete bacterial background lawn
- It induces a dose-related increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control in at least two to three concentrations of the test compound at complete bacterial background lawn
- The increase number of revertant colonies is reproducible
- The number of revertant colonies exceeds the upper value of the range of the historical negative control data

Moreover, biological significance needs to be discussed, taking into consideration the criteria mentioned above.

If the test substance does not achieve either of the above criteria, it is not considered as showing evidence of mutagenic activity in this system.

CRITICAL ASSESSMENT OF THE METHOD

The advantages of the bacterial reverse mutation test are the ease with which it can be performed, the short amount of time required and the low cost. The test should be regarded as the first test in the GLP testing

strategy for the detection of a genotoxic potential of a test compound. The test is as well required as part of the nonclinical testing strategy before the start of Phase I in the clinical development.

MODIFICATIONS OF THE METHOD

52.2.2 Mini-Ames and Ames II

The bacterial reverse mutation test does not detect all compounds with the potential to induce point mutations. For some chemical series, modifications of the test system are necessary. For example, the potential for azo compounds to induce point mutations can only be detected by using an S9-mix prepared by hamster liver. To get an indication early in the development of a test compound for its potential to induce point mutations, modified test systems of the standard bacterial reverse mutation test are performed as a screening test. These are the so-called Mini-Ames test, performed on smaller agar dishes, or the Ames II^{TM} test, performed with microtiter plates. The test design of the screening method should be as much as possible related to the standard method, e.g., same top dose, to reach highest comparability between both methods. Both screening tests have the same principle as the standard test, and use *S. typhimurium* strains. The advantages of both are that they need much less compound and have a higher throughput in the number of tests per week.

52.2.3 The Bacterial Reverse Mutation Test in the Light of Genotoxic Impurity (GTI) Testing

Genotoxic impurities need to be investigated as well for their potential to induce point mutations. Currently, the need for testing is triggered by in silico alerts. If a compound shows an in silico alert, testing in the Ames Assay is required. If the Ames Assay is positive, the compound is classified as a Genotoxic impurity (GTI) In this case the so-called staged TTC (Threshold of Toxicological Concern) will trigger the further handling of the GTI and no further testing is required as described in Müller et al. (2006) and McGovern and Jacobson-Kram (2006). A new ICH Guidance dealing with Genotoxic impurities is under evaluation (ICH M7).

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52.3 L5178Y tk+/– Mouse Lymphoma Test (MLA)

PURPOSE/RATIONALE

The MLA is used for the detection of point mutations, structural aberrations, and aneugenicity. The principle

of the assay is that cells deficient in thymidine kinase (TK) due to the $tk^{+/-}$ or $tk^{-/-}$ mutation are resistant to the cytotoxic effects of the pyrimidine analogue trifluoro-thymidine (TFT). TK proficient cells are sensitive to TFT. This influences the cellular metabolism and leads finally to an inhibition of further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain TK, are not. The major advantage of the assay is its ability to detect a broad range of mutagenic events represented by optimal detection of both large and small colonies.

The assay was described by Clive and coworkers (Clive et al. 1972) as a mutational assay system using the TK locus in mouse lymphoma cells. In the following years, he and his coworkers undertook a large-scale of investigation of the potential and optimal conduct of the assay. This included the use of the above-mentioned TFT to select tk mutants, a comparison of the hypoxanthine guanine phosphoribosyl transferase (hprt) and tk loci, an analysis of the best expression time for tk mutant selection and a description of distinct "large" and "small" colony tk mutants (Moore et al. 1985).

Originally, the assay was performed mainly in soft agar plates. In 1986, a protocol variation was successfully applied using cloning cells in liquid medium in 96-well microtiter plates instead of soft agar in Petri dishes (Cole et al. 1986). As a result, a better discrimination between small and large colonies was possible. Using a variety of chemicals, Doerr and Moore conducted an extensive evaluation of the correlation between gross chromosome aberration induction in mouse lymphoma cells and small colony tk mutant induction (Moore and Doerr 1990). From these studies it was clear that chemicals that induce small colony tk mutants also induce gross aberrations in the mouse lymphoma cells. Such mutants could result from alterations that: (a) affect expression of the tk and linked loci (chromosome 11 events); (b) affect the tk and other single essential loci in the genome, i.e., multiple point mutations; (c) affect the tk locus and have a chromosome event (other than chromosome 11) that results in slow growth.

In 1998, it was demonstrated that L5178Y cells contain two mutant p53 alleles (Clark et al. 1998). It is possible that the dysfunctional p53 protein in L5178Y cells may account for the sensitivity of these cells to mutagens and for the assay's capability to detect the chromosomal rearrangements and mitotic recombination often seen in the later stages of cancer development.

A number of recommendations and comments were made on the conduct of the MLA (Sofuni et al. 1997), including heterogeneity and the use of single or duplicate cultures, cytotoxicity parameters, relative survival and relative total growth, strategies for dose range finding, and the statistical analysis of MLA data. From the International Working Group of Genotoxicity Testing a very detailed description of the protocol, the validity criteria and the evaluation was published in 2003, 2006, and 2007 (Moore et al. 2003, 2006, 2007). A detailed discussion on the selection of the top dose can be found in Moore et al., 2011.

PROCEDURE

The genotoxic potential of a compound in the MLA is generally evaluated with and without metabolic activation by S9 liver homogenate obtained from rats pretreated with Aroclor or Phenobarbital/ßNaphtoflavone. The addition of the S9-mix allows for metabolism of the test compound by enzymes not present in mouse lymphoma cells. The cells were exposed to the compound for 3–4 h or 24 h without a metabolic activation system and for 3 h with metabolic activation. Cells were then grown for a 48-h phenotypic expression period (two cultures per concentration). The phenotypic expression period is the time required for the mutant (TK deficient) phenotype to be expressed, i.e., loss of preexisting TK and depletion of the preexisting TMP pool. Cells were then cloned and incubated for 10–13 days with the selection agent (TFT) while their cloning efficiencies were checked in nonselective medium.

The ICH4 committee concluded in 1995 that there is the capability of detecting point mutations and most substances that induce chromosome aberrations (including aneugens). Therefore the chromosome aberration assays and the Mouse Lymphoma assay are currently considered interchangeable, if the Protocol recommendations of the ICH S2 documents are fulfilled. That means: (a) in the case of a negative result following 3–4 h (treatment with and without S9), a continuous treatment of 24 h without activation was considered advisable; (b) a requirement for the use of the microwell cloning protocol rather than agar cloning to better discriminate small and large colonies; and (c) the use of an appropriate positive control inducing a higher proportion of small colonies. However, further validity criteria

were described in the OECD Guideline 476 and the ICH2b Guideline. One major point for the interpretation of the data is the fulfillment of the toxicity criteria (approximately 20%). In addition, in 2007, the Global evaluation Factor (GEF) was introduced, to judge if a compound is positive. This factor is based on the Induced Mutant Frequency (IMF), which is defined as the increase above the spontaneous background mutant frequency. For the microwell technique, the GEF has been defined to be equal to 126×10^{-6} (Moore et al. 2007). If the IMF exceeds the GEF, a compound is judged as positive.

EVALUATION

Small colony mutants have been shown to predominantly lack the TKb allele as a consequence of structural or numerical alterations or recombinational events whereas large colonies are the consequence of point mutations. Based on the recommendations of the ICH2b the following points should be considered in general for the interpretation of the results:

- Does the Induced Mutant Frequency (IMF) exceed the Global Evaluation Factor (GEF)? For microwell technique, the GEF has been defined to be equal to 126×10^{-6}.
- Is the increase in response over the negative or solvent control background regarded as a meaningful genotoxic effect for the cells?
- Is the response concentration-related?
- For weak/equivocal responses, is the effect reproducible?
- Is the positive result a consequence of an in vitro specific metabolic activation pathway/in vitro specific active metabolite?
- Can the effect be attributed to extreme culture conditions that do not occur in in vivo situations, e.g., extremes of pH; osmolality; heavy precipitates especially in cell suspensions?
- Is the effect only seen at extremely low survival levels/ high cytotoxicity?

In addition, the use of statistical methods is recommended for the evaluation of the MLA. Two methods should be used, one to evaluate the statistical difference between the different groups and, more importantly, the statistical significance of the dose-response curve. For the evaluation of the results the consideration of the biological relevance is the most important. A detailed discussion of parameters indicating a biological relevance is published in the ICH2B Guideline (1997) and in the recommendation of the International Working Group of Genotoxicity Testing (IWGT; Moore et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

It is apparent that the MLA has some advantages compared to other mutation assays including: (a) rapid growth in suspension culture to high cell density, which provided for the very large numbers of cells necessary for a statistically valid test and (b) the relatively short time (48 h) required for the expression of newly induced mutants.

However, the assay is only really capable of detecting large increases in mutation frequencies. This is because very small increases in mutation frequencies can often be seen at high cytotoxicity levels. At very low survival levels in mammalian cells, mechanisms other than direct genotoxicity per se can lead to "positive" results that are related to cytotoxicity and not genotoxicity (e.g., events associated with apoptosis, endonuclease release from lysosomes, etc.). It has long been recognized for all in vitro systems that mutations induced under these circumstances would not normally occur in vivo. Thus, these responses, while perhaps statistically significant by some methods, are not considered to be biologically relevant. A careful discussion of such responses is needed.

In addition, while the spectrum of mutations detected by the assay is very broad, it should be noted that not all such events are always detected with equal efficiency following treatment with particular test substances. Especially, for the detection of structural aberrations and aneugenicity the recommended conditions of the protocol should be kept in detail.

MODIFICATIONS OF THE METHOD

The test could be performed with different cell lines (e.g., TK6 cells) and different metabolic activation systems.

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52.4 Micronucleus Test In Vitro

PURPOSE/RATIONALE

The purpose of the micronucleus test in vitro is to evaluate the potential of compounds to induce micronuclei (formation of small membrane-bound DNA fragments) in different cell lines or primary cultures, with and without metabolic activation by S9 liver homogenate.

The test system allows to discriminate between a clastogenic and aneugenic potential of a test compound by using an immunochemical labeling of the kinetochores or staining the DNA fragments with FISH (fluorescence in situ hybridization) technique.

PROCEDURE

The cells are treated with the test article in 96-well microplates for a short treatment period with metabolic activation (e.g., 3 h) and for a long treatment period without metabolic activation (e.g., 24 h) and harvested 24 h (recovery time) after the end of the treatment. Cells are then fixed and stained. The cytotoxicity of the test compound is evaluated by the relative cell growth, expressed as a percentage of the negative control. The highest evaluated concentration should produce approximately 50% cell survival or should be the first concentration where precipitation is observed. Duplicated cultures should be performed at each dose level. A very detailed description of the protocol, the validity criteria, and the evaluation was published by the IWGT in 2003 (Kirsch-Volders et al. 2003).

EVALUATION

Structural/numerical chromosome damage is evaluated by the increase in the number of micronucleated cells, scored out of 1,000 cells in three analyzable concentrations. The compound is considered positive if either:

- The increase of micronucleated cells is statistically significant compared to the negative (solvent or untreated) control, or
- The number of micronuclei is dose dependent and showed a biological relevance compared to the negative control.

The positive control must show a clear statistical significant effect compared to the negative control.

CRITICAL ASSESSMENT OF THE METHOD

The micronucleus test in vitro is easy and rapid to perform, inexpensive, and needs much less test compound compared to the mammalian chromosome aberration test. In addition no detailed training of the personnel for the light microscopical evaluation of the slides is necessary. In fact, the micronucleus test in vitro is currently the only in vitro test which allows the differentiation between a clastogenic or aneugenic effect, and is becoming ever more important in the genotoxic testing strategy. The finalization of the OECD Guideline for this assay and the implementation into the ICH S2R Guidance was key to use this assay for regulatory purposes. As the evaluation of micronuclei is much easier and less time consuming, this assay will replace the chromosome aberration assay in vitro in the future.

MODIFICATIONS OF THE METHOD

An alternative method for the evaluation of the induced micronuclei is measurement by Flow cytometry. This method allows the discrimination between micronuclei that contain one or several acentric chromosome fragments (clastogenic action) or one or several whole chromosomes (aneugenic action, interference with the mitotic spindle apparatus) or even a combination of both. To discriminate between the actions an additional staining of the micronuclei with, e.g., CREST antibodies is necessary.

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52.5 Micronucleus Test In Vivo

PURPOSE/RATIONALE

The in vivo mammalian micronucleus test is used to assess the mutagenic hazard in consideration of factors like in vivo metabolism, pharmacokinetics, and DNA repair processes, although these may vary among species and among tissues. This assay is an important part of the gentox testing battery, applied for pharmaceuticals and chemicals.

During erythropoiesis, nuclei are expelled during the formation of polychromatic erythrocytes while micronuclei are retained in the cells. This fact is used for the detection of micronuclei. A significant increase in the number of micronucleated polychromatic erythrocytes is usually considered as indicative of structural and/or numerical chromosome damage caused by exposure to a clastogenic and/or aneugenic substance. The identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei is needed to discriminate a clastogenic effect from an aneugenic effect. The incidence of micronuclei in the polychromatic erythrocytes is scored 24 h after the end of treatment to take into account the time interval between the last mitosis and the formation of polychromatic erythrocytes (at least 8 h) and the lifespan of polychromatic erythrocytes, which is approximately 24 h.

PROCEDURE

The bone marrow of rodents (rats and mice) is routinely used in this test since polychromatic erythrocytes are produced in that tissue, it is a highly vascularized tissue and it contains a population of rapidly cycling cells that can be readily isolated and processed. The assay was developed by Schmid (1975) and modified by Salamone et al. (1980). Recent protocols and recommendations are published in the OECD Guideline 474 and Hayashi et al. 2000. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated. The evaluation of micronuclei in peripheral blood is an easy method integrated the micronucleus assay into General toxicity studies (Rothfuss et al. 2011).

The route of administration of the test compound should ensure a relevant target exposure and in the case of pharmaceuticals it should consider the application route in humans. Each treated and control group must include at least five analyzable animals per sex. It is possible to use only one gender if it could be demonstrated that no substantial differences in metabolism, toxicity, and pharmacokinetics between genders was

observed. Test substances could be administered as a single treatment. Repeated treatment up to 28 days could is also possible. If a single treatment is used the sampling time should be between 24 and 48 h. If repeated treatment is used, the sampling time is 24 h after the last treatment.

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Usually, cells are removed from femurs or tibias in a suitable medium such as fetal serum, and are prepared and stained using established methods. DNA specific stains (e.g., acridine orange) are preferred instead of conventional stains like Giemsa.

The likelihood that the test substance or its metabolites reach the general circulation or the target tissue (e.g., systemic toxicity) should be demonstrated. Preferably, experimental evidence of systemic or target tissue exposure should be presented (e.g., blood level or bone marrow concentration analysis), especially in the case of a negative result with an agent that does not induce observable toxicity.

EVALUATION

The proportion of immature among total (immature + mature) erythrocytes as measure for target organ toxicity is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1,000 erythrocytes for peripheral blood. At least 2,000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes to determine the clastogenic/aneugenic potential of the test compound. The automatic analysis of micronucleated erythrocytes was developed by Romagna and Staniforth (1989).

For a discussion of the result of the micronucleus assay a comparison of the data from the treatment group versus concurrent negative control data and historical control data as well as a statistical analysis of the experimental data using trend analysis or pair-wise comparison (treatment group versus control) need to be considered. It is also recommended to check the variance between the animals and gender. However, for the final assessment, biological relevance of the results should be considered.

CRITICAL ASSESSMENT OF THE METHOD

There are compounds for which standard in vivo tests do not provide additional useful information. This is particularly true for compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues. Examples of such compounds are some radioimaging agents, aluminum-based antacids, and some dermally applied pharmaceuticals. In those cases other tests systems should be considered to be more relevant.

In addition, parameters like decreased body temperature may lead to an indirect induction of micronuclei that is not biologically relevant.

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52.6 Mammalian Chromosome Aberration Test In Vitro

PURPOSE/RATIONALE

This in vitro cytogenetic test is a clastogenicity test system for the detection of chromosomal aberrations in cultured mammalian cells or primary cultures. Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyze cells at their first

posttreatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetic assay. The best estimate of aberration frequency is the first cell division after the start of treatment. Structural aberrations are of two types: chromosome or chromatid aberrations.

Chromosome-type aberrations are induced when a compound acts in the G_1 phase of the cell cycle. Chromatid-type aberrations are induced when a chemical acts in the S or G_2 phase of the cell cycle.

- Chromosome-type aberrations are structural chromosome damages expressed as breakage, or breakage and reunion, of both chromatids at an identical site
- Chromatid-type aberrations are structural chromosome damages expressed as breakage of single chromatids or breakage and reunion between chromatids
- Numerical aberrations are changes in the number of chromosomes of the normal number characteristic of the animals utilized

The best estimate of aberration frequency is the first cell division after the start of treatment.

PROCEDURE

In this test, cultured cells are seeded onto slides and the cells, which have been treated with and without metabolic activation for a short-time period (e.g., 3 h). Where negative or equivocal results are obtained, an independent experiment is conducted in which cells are treated for long-time period (e.g., 20 h) in the absence of metabolic activation alone and then sampled and examined for chromosome analysis. In both experiments the cells are sampled 20 h after the start of treatment as are the concurrent solvent and positive control cultures. Colcemid is added to each culture 2 h before sampling in order to arrest cell division. Chromosome preparations are made, fixed, stained, and examined. However, if clearly positive results are obtained in the first experiment, those from the second assay are not examined. If equivocal or negative results are obtained in the first experiment, modifications to the testing procedure are included in order to clarify the result.

EVALUATION

The set of chromosomes is examined for completeness and the various chromosomal aberrations are assessed and classified. The metaphases are examined for the following aberrations: chromatid gap, chromosome gap, chromatid break, chromosome break, chromatid acentric fragment, chromosome acentric fragment, chromatid deletion, chromosome deletion, chromatid exchanges including intrachanges, chromosome exchanges including intrachanges, dicentrics, pulverization, and ring formation. Metaphases including five or more break events are scored as multiple aberrant. Furthermore the incidence of polyploid metaphases is determined for each cell culture. The quantity of cells is determined by counting the number of cells in, e.g., 10 fields of vision per slide as an indicator of toxicity. The survival of cells is expressed as a percentage. Additionally the mitotic index should be determined by counting the number of cells undergoing mitosis in a total of, e.g., 1,000 cells. The mitotic index is also expressed as a percentage. For each experiment the results from the dose groups is compared with those of the control group and the positive control at each sampling time.

The assay is considered *valid* if both of the following criteria are met:

- The solvent control data are within the laboratory's normal control range for the number of cells carrying structural chromosomal aberrations
- The positive controls induce increases in the number of cells carrying structural aberrations which are statistically significant and within the laboratory's normal range.

A test substance is classified as *non-clastogenic* if either of the following is met:

- The number of induced structural chromosome aberrations in all evaluated dose groups is in the range of our historical control data.
- No significant increase of the number of structural chromosome aberrations is observed.

A test substance is classified as *clastogenic* if both the following are met:

- The number of induced structural chromosome aberrations is not in the range of our historical control data.
- Either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

When evaluating the findings, both biological and statistical significance should be considered together. If the criteria mentioned above for the test item are not clearly met, the classification with regard to the historical data and the biological relevance should be discussed and/or a confirmatory experiment should be performed.

CRITICAL ASSESSMENT OF THE METHOD

Care should be taken to avoid conditions (pH, high cytotoxicity, osmolality, etc) that would lead to positive results but which do not reflect intrinsic mutagenicity. Mammalian carcinogens are often positive in this test. Nevertheless, there is not a perfect correlation between this test and carcinogenicity, which depends on the chemical class. Some chemicals may test positive in this test because they appear to act through other mechanism than direct DNA damage, e.g., apoptosis.

MODIFICATIONS OF THE METHOD

The test could be performed with different cell lines (permanent and primary) and different metabolic activation systems.

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52.7 Mammalian Chromosome Aberration Test In Vivo

PURPOSE/RATIONALE

Like the in vivo micronucleus assay, the in vivo mammalian chromosome aberration assay is especially relevant for assessing the mutagenic hazard while taking into consideration factors like in vivo metabolism, pharmacokinetics, and DNA repair processes, although these may vary among species and among tissues. In the gentox testing battery this assay is mainly used for further investigation of mutagenic effects detected by an in vitro test. In addition, the assay can be used for the detection of compounds that induce polyploidies. An increase in the number of polyploidy cells may indicate that a compound has the potential to induce numerical aberrations.

The induction of structural chromosome aberrations is classified in two types, chromosome or chromatid aberrations. The majority of induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosomal mutations and related events are the cause of many human genetic diseases and there is evidence that chromosomal mutations and related events are involved in cancer development.

In addition, the assay can be used for the detection of compounds that induce polyploidies. An increase in the number of polyploidy cells may indicate that a compound has the potential to induce numerical aberrations.

PROCEDURE

Rodents (rat, mice, Chinese hamster) are routinely used in this test. Although chromosome aberrations can be detected in various tissues, the most common methodologies are available for investigations of bone marrow (Preston et al. 1987), peripheral blood, and female and male germ cells (Russo 2000). Bone marrow is the normally used target tissue in this test, since it is a highly vascularized tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed (Tice et al. 1994). However, methodologies are available for investigations of other tissues and cells.

The route of administration of the test compound should ensure a relevant target exposure and in the case of pharmaceuticals it should consider the application route in humans. Each treated and control group must include at least five animals that can be analyzed per sex. It is possible to use only one gender if it can be demonstrated that no substantial differences in metabolism, toxicity, and pharmacokinetics between genders was observed. Test substances are preferably administered as a single treatment; however, repeated treatment up to 28 days could also be performed. If a single treatment is used two sample times (12–18 h and 36–44 h) should be used for bone marrow. If repeated treatment is used, the sampling time is 6–24 h after the last treatment. If species other than rodents or other tissues are used, the sampling time must be scientifically justified.

Prior to sacrifice (3–5 h), animals are treated with a metaphase-arresting agent (e.g., colchicine). Chromosome preparations are then made from the respective tissues and stained with an appropriate method. For a better discrimination of the chromosomes and to detect translocations, the FISH technology can be used (Natrajan and Boei 2004). Metaphase cells are microscopically analyzed for the occurrence of structural and numerical chromosome aberrations.

The likelihood that the test substance or its metabolites reach the general circulation or the target tissue (e.g., systemic toxicity) needs to be demonstrated. Preferably, experimental evidence of systemic or target tissue exposure should be presented (e.g., blood level or bone marrow concentration analysis), especially in the case of a negative result with an agent that does not induce observable toxicity.

EVALUATION

At least 100 metaphase plates per animal should be scored per animal based on the use of at least five animals per gender per treatment group. The minimal classes of aberrations to score and categorize would be chromosome type versus chromatid type. Within these two categories gaps, breaks, and rearrangements should be differentiated. To describe the toxicity in the target organ, the mitotic index is determined in at least 1,000 nucleated cells per animal. However, for the detection of polyploidies at least 22 metaphase cells should be scored. Due to differences in the mechanism of development, endoreduplicated cells should be scored separately.

For a discussion of the result of the chromosome aberration assay the following parameters need to be considered: (a) comparison of the data from the treatment group versus concurrent negative control data and historical control data (b) statistical analysis of the experimental data using trend analysis or pair-wise comparison (treatment group versus control). It is also recommended to check the variance between the animals and gender. However, for the final assessment, biological relevance of the results should be considered.

CRITICAL ASSESSMENT OF THE METHOD

There are compounds for which standard in vivo tests do not provide additional useful information. This is particularly true for compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues. Examples of such compounds are some radioimaging agents, aluminum-based antacids, and some dermally applied pharmaceuticals. In those cases other tests systems should be considered to be more relevant (p32 Postlabeling, Comet Assay in vivo).

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52.8 Comet Assay In Vivo

PURPOSE/RATIONALE

The in vivo Comet assay (single-cell gel electrophoresis assay) is increasingly used in regulatory genotoxicity testing for the evaluation of DNA damage and repair in various tissues of mice and rats that cannot easily be evaluated with other standard tests. Protocols are established for liver, skin, stomach, gut, kidney, retina, and nasal tissues. The Assay is used as follow-up assay for in vitro positive results and is discussed as second endpoint for a combined in vivo protocol (micronuclei in bone marrow/Comet assay in liver) Rothfuss et al. 2010. The advantage of the model is that different target organs can be used to detect a genotoxic potential.

The test is in general conducted based on the method described by Singh et al. (1988) then modified by Hartmann et al. (2003), Tice et al. (2000) and the validation management team of the International Validation of the In Vivo rodent alkaline COMET assay. Protocols for conducting the in vivo Comet assay were developed by different expert panels, e.g., at the 2nd and 4th International Workshops on Genotoxicity Testing, Burlinson et al. (2007) and the 4th International Comet assay Workshop.

PROCEDURE

The Comet assay is used to visualize and measure DNA strand breaks in individual cells by microscopy. Animals are treated between 2 and 28 days. In general, the last compound administration is 3 h before the animals are anesthetized and euthanized. After necropsy of the specific organs, the cells are isolated by organ perfusion or mincing the cell tissues. It is in general recommended that at least three slides will be prepared per tissue and animal.

In the alkaline version, the isolated cells are embedded in an agarose gel on a microscope slide, immersed in a lysis solution to remove lipids and proteins. The slides will remain in the buffer for 20 min. Using the same buffer, electrophoresis will be conducted in general at a constant voltage of 0.7 V/cm. The current at the start of the electrophoresis will be adjusted to 300 mA, a weak electric field to attract broken negatively charged DNA toward the anode.

After electrophoresis, the DNA is stained using a fluorescent dye and viewed using a fluorescence microscope. When viewed under a microscope, the cell containing DNA strand breaks has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands. Individual images are analyzed for quantifying DNA migration parameters such as the percentage of DNA that has migrated, named tail intensity. These measurements give an indication of the incidence of strand breaks present in the cell.

Coded slides must be scored in blind fashion. Slides will be stained with a DNA stain prior to scoring (SYBR-gold, propidium iodide, or any appropriate DNA stain).

EVALUATION

DNA effects will be assessed by the software system by measuring Comet tail migration, % tail intensity, and Olive tail moment. Tail migration is the distance from the perimeter of the Comet head to the last visible point in the tail; % tail intensity the percentage of DNA fragments present in the tail; and Olive tail moment is the product of the amount of DNA in the tail and the mean distance of migration in the tail. For each sample, 150 cells (three slides per sample with 50 cells per slide or two slides per sample with 75 cells per slide, if possible) will be scored for DNA damage.

Generally, all cells including heavily damaged cells are scored as long as the image analysis system can properly discern a head. Cells without discernable head and large diffuse tail which cannot be scored by the image analyzer (ghosts) are excluded from analysis. The frequency of such ghosts should be determined per sample, based on the visual scoring of 100 cells per sample.

As an example Liver Comet Assay data will be accepted if the following criteria are met: Negative control: Means of %DNA in tail are 1–8%.

Positive control: Ratio of means of %DNA in tail between groups of positive and vehicle control is twofold or higher.

Those values need to be verified for each organ, as there is high variability between organs.

CRITICAL ASSESSMENT OF THE METHOD

The assay is rapid and easy to conduct, but the laboratory needs experience in preparation of the cells. The quality of the assay is highly depending on that. The advantage of the assay is that the assay can be applied to any tissue and cell division not required for the detection of DNA damage. The analysis is conducted in individual cells; therefore low number of cells is sufficient. However, the assay is resource intensive and time consuming, in particular the scoring of comets takes time if not automated. Apoptosis/necrosis as indicators of tissue cytotoxicity needs to be controlled in the histopathology part of the study, and positive results in the presence of toxicity are difficult to interpret. At the moment there are no guidelines available. Different statistical methods are available but should be carefully applied for the interpretation of the results.

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52.9 Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vitro

PURPOSE/RATIONALE

The UDS test detects DNA repair synthesis after excision of damaged segments. DNA repair synthesis is demonstrated by autoradiographic measurement of tritium-labeled thymidine incorporation. Liver cells are routinely used for the UDS test because cells in S-phase are rare and easily distinguished from cells undergoing DNA repair and because hepatocytes exhibit a high metabolic activity that enables the detection of pro-mutagens.

PROCEDURE

Liver cells are isolated by a two-step perfusion procedure from anesthetized rats.

A medio-ventral incision is made from the pubic symphisis to the thorax. A catheter is inserted into the portal vein and the liver is perfused with collagenase solution. At the end of the perfusion, the liver is taken out, the liver capsule is removed, and the cells are mechanically dispersed by gentle shaking. After dispersion of the liver cells in medium, the vessels and remaining conjunctive tissue are removed and collagenase activity is neutralized. Cell viability is assessed by the percentage of refringent cells. Only cell suspensions with more than 70% viability should be retained.

Liver cells are then exposed in vitro to the test compound and incubated with tritium-labeled thymidine for about 18 h. At the end of the incubation, the cells are fixed on slides and prepared for autoradiography. For that the slides are first exposed to liquid photographic emulsion, air-dried and following a 7-day exposure in the dark, exposed to developing solution.

EVALUATION

Cells undergoing DNA repair are identified by the increase in the number of silver grains in the nuclei, i.e., the net nuclear grain count. Only normal-appearing nuclei are scored; occasional nuclei blackened by grains are excluded since these are nuclei undergoing replicative DNA synthesis. One hundred cells per concentration and control are analyzed. For each cell, the number of silver grains in the nucleus and the number of silver grains in three adjacent nucleus-equivalent areas on the cytoplasm are measured. For each cell, the following parameters are calculated:

- The nuclear grain count (N)
- The mean of the three cytoplasmic grain counts (C)
- The net nuclear grain count (NG), i.e., the difference between the nuclear grain count and the mean of the three cytoplasmic grain counts

A cell is considered undergoing DNA repair if the value of the net nuclear grain count is greater than five.

For each slide, the following parameters are calculated:

- The mean and standard deviation of nuclear and cytoplasmic grain counts, and of net nuclear grain count
- The percentage of cells undergoing DNA repair and mean value of net nuclear grain count for these cells.

The test article is considered positive in the UDS assay if the mean net nuclear grain count is greater than five and if the percentage of cells undergoing DNA repair is greater than 20%.

CRITICAL ASSESSMENT OF THE METHOD

The biological significance has to be taken into consideration for positive evaluation (i.e., cytotoxicity can artefactually lead to an increase in the value of net nuclear grain count). The positive control should induce a clear increase in the mean net nuclear grain count higher than the threshold value of five.

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52.10 Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vivo

The principles of this method are the same and the procedure similar as for the above described method "Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells in vitro." The differences between the in vivo and the in vitro method are related to the test compound exposure. In the in vivo assay, rats are first treated in vivo with the test compound and the liver cells are isolated 14 h after treatment. Then the liver cells are incubated for 4 h with a medium containing tritium-labeled thymidine, followed by 18 h in a medium containing non-labeled thymidine. The incorporation of tritium-labeled thymidine is measured by autoradiography. Cells undergoing repair are identified by the increase in the number of silver grains in the nuclei.

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Reproductive Toxicology

53

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53.1 General Considerations

Developmental and reproductive toxicity is a special part of toxicology which comprises any effect of chemicals and active substances on mammalian reproduction (fertility, weaning) and development (embryonal and fetal development). The investigations and the interpretation of the results should be related to all other pharmacological and toxicological data available to determine the risk of the test compound to humans. To allow detection of immediate and latent effects of exposure, the observations should cover one complete life cycle from the mature adult through all stages of development from conception of one generation to conception of the following generation(s).

Animal use in preclinical studies. It is ethically mandatory to conduct preclinical safety studies in animals with new pharmaceutical products before their administration to humans, as laid down in the Declaration of Helsinki. Regulatory approval is required before the start of the first clinical trial and comprises the assessment of the intrinsic hazard of the compound as well as the assessment of the potential risk that such a hazard may pose to humans. Currently this process necessitates the use of animals.

53.2 New Pharmaceuticals: The ICH Guideline

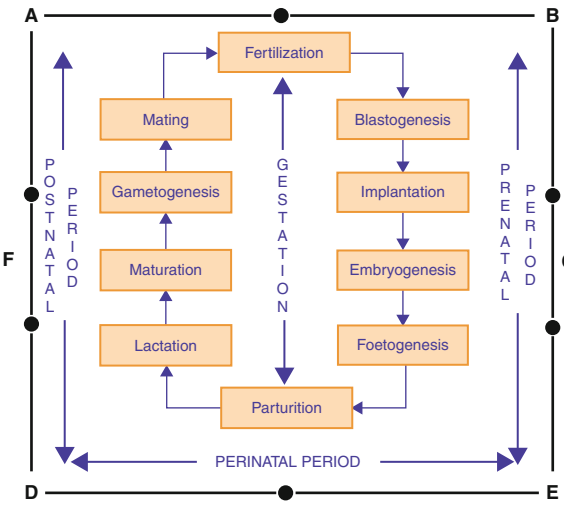
The first fundamental introduction in reproductive toxicology was given by Wilson and Warkany in 1965 (Wilson and Warkany 1965). The first test guideline was published by the FDA in 1966 (Food and Drug Administration 1966), followed by the Committee on Safety of Medicines (1974), MHW of Japan (Ministry of Health and Welfare (MHW) 1975) and many other countries and temporarily ending by the ICH Harmonized Tripartite Guideline "Detection of Toxicity to Reproduction for Medicinal Products" in June 1993, which was initiated by the IFTS (International Federation of Teratology Societies), pharmaceutical industry and the health authorities of EEC, Japan, and USA (Bass et al. 1991; ICH Harmonized Tripartite Guideline 1993).

The ICH tripartite guideline on detection of toxicity to reproduction for medical products comprises the fertility study (ICH 4.1.1), the pre- and postnatal toxicity study (ICH 4.1.2), and the embryo toxicity study (ICH 4.1.3). The former "three-segment classification" (original FDA: segment I: fertility; segment II: embryo toxicity; segment III: peri- and postnatal toxicity) was not transferred into the ICH guideline, but is still commonly used. The ICH guideline was designed to detect the four manifestations of developmental toxicity: death, growth retardation, malformation, and functional defects.

53.2.1 The Reproductive Cycle

The reproductive cycle of a mammalian individual consists of a sequence of various periods and interrelated events and is defined in the ICH guideline as follows:

A. Pre-mating to conception (adult male and female reproductive functions, development and maturation of gametes, mating behavior, fertilization)
B. Conception to implantation (adult female reproductive functions, pre-implantation development, cleavage, morula, blastula, implantation)
C. Implantation and organogenesis (adult female reproductive functions, embryonic development, major organ formation)
D. Fetal development (until the end of pregnancy adult female reproductive functions, fetal development and growth, organ development and growth)
E. Birth and pre-weaning development (adult female reproductive functions, parturition, lactation, neonate adaptation to extrauterine life, pre-weaning development, and growth)
F. Post-weaning development up to sexual maturity (growth, maturation, adaptation to independent life, attainment to full sexual function).



53.2.2 Testing Strategy

The testing strategy consists of various study designs according to the state of the art covering all stages of the reproductive cycle which can be used on a case by case basis.

In planning the studies, they must reflect human exposure to the medicinal product and should allow specific identification of stages at risk. Furthermore, the anticipated drug use especially in relation to reproduction, the physical nature of the test substance, and route of administration or exposure should be taken into account. Also any existing data on toxicity, pharmacodynamics, kinetics, mechanisms of reproductive toxicity in humans or known from previously conducted studies, and similarity to other class-related compounds in structure and activity should be taken into consideration.

The experimental strategy can be adapted on a case by case basis. However, according to the various events during the reproductive cycle, the following most probable option of study designs has been ascertained:

- Studies for effects on fertility and early embryonic development
- Studies for effects on pre- and postnatal development
- Studies for effects on embryo-fetal development

On principle, it is imperative to leave no gaps between the various stages, and thus to allow direct and indirect evaluation of all stages of the reproductive process. When an effect on reproduction is detected, further studies may be necessary to characterize the nature of the response on a case by case basis.

All these studies should be conducted according to the Principles of Good Laboratory Practice.

For basic drug discovery in the first stage of development of the test compound, the study program usually starts with testing for effects on embryo-fetal development including teratogenicity and maternal toxicity. The next step is to study the effects on fertility and early embryonic development. Testing for effects on pre- and postnatal development will be started at a later stage of preclinical and clinical development of the test compound.

53.2.3 Species Selection

Species selection for the different study types should be based on following considerations: Pharmacological activity in the species, together with comparative physiology to that in humans, similar toxicokinetic profile to that in humans, and proven susceptibility of the chosen species to teratogenic effects. The rat is the predominant rodent species, and the rabbit is the predominant non-rodent species, particularly since thalidomide was shown to be teratogenic in this animal species first. If the rabbit is not suitable as a second species in the embryo-fetal toxicity studies, alternative animal species should be considered on a case by case basis, e.g., mice, minipigs, or monkeys.

53.2.4 Number of Animals per Group

For all but the rarest events such as malformations, abortions, or total litter loss, the evaluation of between 16 and 20 litters for rodents and rabbits tends to provide consistency between studies. Below 16 litters per group for evaluation, the study results become inconsistent, whereas more than 20–24 litters per group do not greatly enhance the consistency and precision of the study data (ICH Harmonized Tripartite Guideline 1993). Therefore, and considering a certain non-pregnancy rate, generally 20–24 animals per sex are used per group.

53.2.5 Selection of Doses

In practice, the results from previous pharmacology and acute and repeated-dose toxicity studies of at least 1 month's duration are considered for dose selection. Additionally, preliminary or range-finding-studies are conducted using small amounts of test substance and low numbers of animals (three to ten per dose group) for justification of the doses to be tested in the main studies.

It makes sense to conduct preliminary or range-finding-studies to find out an adequate high dose for the definitive study, inducing maternal, embryo-fetal, or reproductive toxicity. Generally, at least three doses should be studied in the main studies. Ideally, the lowest dose should represent the one (or manifold) pharmacologically active—and presumably—daily therapeutic dose for humans, or that dose which is active in the test animal at which no toxic effects are expected in the parent animals and conceptuses. At the highest dose, signs of toxicity are expected to appear in

the parent animals and/or conceptuses. The geometric mean between the low and high dose is often used as intermediate dose.

The route and frequency of administration should be similar to those of the prospected human use considering the kinetic data. Kinetics should also be determined in the pregnant animal.

In each experiment, a simultaneous group of control animals is treated with the vehicle only in the same manner as the animals in the dose groups. If it is expected that the control preparation may cause effects, a group of same-treated or untreated animals should be added to the study.

The dose of 1,000 mg/kg/day can be used as an upper limit dose.

53.2.6 Data Presentation and Statistics

Good reporting includes tabulation of all individual data in a clear concise manner. Group summary tables should be presented in a biologically plausible manner. Presentation of individual fetal and pup data, especially structural changes, should clearly identify the litters containing the abnormal fetuses and pups, and also identify the individually affected fetuses and pups. All abnormalities should be attached to the individual offspring. As far as it is biologically acceptable, all the data and the occurrence of all abnormalities should be compared statistically with the simultaneous control group data. It should also be compared with the data of the previous relevant control groups (accumulated historical control data) on using scientifically well-defined methods. The litter should be used as the experimental unit, when statistical evaluation is performed.

53.2.7 Study for Effects on Fertility and Early Embryonic Development

This study covers ICH phases A and B of the reproductive cycle and is usually performed in rats. The aim of the study, often called "fertility study," is to assess adverse effects of the test compound on maturation of gametes, mating performance, fertilization, preimplantation stages of the embryo, and implantation. Adverse effects can be detected on the estrus cycle, tubal transport, implantation, and development of the pre-implantation stages of the embryo. In males, functional effects on libido and epididymal sperm maturation may be detected which cannot be observed by histological examination of the male reproductive organs.

Young adult male and virgin female animals, preferably rats, are assigned to groups randomly by a computer-generated algorithm and are treated or exposed with the prepared test compound. Males and females are treated for at least 14 days prior to the cohabitation period covering the developmental stages of the germ cells relevant for copulation, and both through the mating period at a 1:1 mating ratio. After successful mating, the females are treated during the 1st week of assumed gestation. Treatment of the males continues until the end of the study. During the premating treatment period and cohabitation period, vaginal smears are taken from all females to determine the estrus cycle. If spermatozoa are found in the vaginal smear on the following morning, the females are considered to be on Day 0 of pregnancy, and separated from the assigned male. In cases of no spermatozoa, the vaginal smears and mating attempts can be continued with the same partner or another male of proven fertility from the same group. The fertility of those males which did not impregnate can be checked with untreated females not belonging to the study. The investigator decides which intensity the fertility of the animals should be tested by further mating attempts will lead to a most meaningful evaluation of this parameter. Pregnancy can be confirmed by the uterine status of the females at dissection.

Females which are found to be mated are killed at or after midpregnancy (usually Day 14), preferably on the same day of pregnancy to allow meaningful evaluation. After outwardly inspection the uterus is opened and examined for its contents. The live and dead conceptuses are counted. The embryo-fetal primordia undergoing resorption and the number of corpora lutea in the ovaries are also determined. The conceptuses in the amniotic sac after removal from the uterus can be placed in physiological saline and examined under a magnifying glass for gross anomalies if females are euthanized at a later stage of gestation (e.g., Day 17). The uterus of those females exhibiting no conceptuses can be stained in ammonium sulfide solution in order to identify those empty implantation sites which cannot be observed in the unstained uterus wall. Pregnant females without observed insemination

are either euthanized 14 days after end of cohabitation or sooner if the animal appears pregnant. In those females, the number of live and stillborn pups are likewise counted and the uterus contents and number of corpora lutea graviditates examined. Preimplantation loss (number of corpora lutea—number of implantations) is calculated as absolute numbers and as percent per litter.

Parental toxicity, such as the animal's behavior, clinical signs, mortality and general physical condition, food consumption, and body weight are also examined during the study. Euthanasia of males should not occur before completion of the majority of uterine dissections. After euthanasia, the males and females are dissected and the internal organs examined macroscopically for pathological changes. Organs showing conspicuous macroscopic changes may be removed and preserved for possible histopathological examination. Ovaries (with oviduct), uterus, vagina, testes, and epididymides are collected for possible histopathological examination.

Optionally, spermatological examinations can be carried out. For this purpose, the males are euthanized, and samples of sperm from one cauda epididymis are collected for evaluation of percent motionless, locally and progressively motile sperm and the sperm concentration in the sperm sample. Motility can be assessed in samples of sperm obtained by incision of a tubule in the distal part of the cauda epididymis, insertion of a ball pipette, and transfer of the sample in HF-10 medium. Additionally, the number of sperm in a defined amount of liquid is determined by weighing a sample of liquid obtained after incision of a tubule in the distal part of the cauda epididymis, diluting with HF-10 medium, and counting the sperm content using a hemocytometer. To quantify spermatogenesis, testicular spermatid head count is determined by removing the tunica albuginea and homogenizing the testis. Homogenization-resistant spermatid nuclei can be counted after dilution using a hemocytometer. Alternatively to using a hemocytometer, spermatological parameters can be assessed by a computer assisted sperm analyzer (CASA).

53.2.8 Study for Effects on Embryo-Fetal Development

This study covers ICH phase C of the reproductive cycle and is usually performed in rats as rodent species and additionally in one non-rodent species, usually the rabbit. The aim of this study, often called "teratogenicity study," is to detect adverse effects of the test compound on the pregnant female and on the development of the embryo and fetus, from implantation of the blastocyst to closure of the hard palate. Adverse effects to be assessed are: enhanced toxicity to the pregnant organism in relation to the nonpregnant, death of the embryo or fetus, altered growth and structural changes in the conceptus.

Young adult females are used, mature for breeding and virgin. Rats in the estrus phase (observed by vaginal smears) are mated overnight with untreated sexually mature males at the ratio of one male to one female. The following morning, vaginal smears are examined for spermatozoa. Rabbits in the estrus phase—observed by slight external reddening and swelling of the vulva or provoked by hormonal induction of ovulation by injection of estrogen—are mated individually once or twice a day at an interval of approximately 5 h with an untreated male of proven fertility. Ovulation takes place approximately 10 h after copulation or hormonal injection. Copulation is confirmed by detection of spermatozoa in the vaginal smear after the mating attempt. Artificial insemination after hormonal induction is also possible. The day on which spermatozoa are detected is taken as Day 0 of pregnancy. Pregnancy is confirmed by the detection of implantation sites in the uterus or corpora lutea in the ovaries. The animals are assigned to groups simultaneously after spermatozoa detection. It is assumed that for rats and rabbits implantation occurs on Day 5–6 of pregnancy and closure of the palate on Day 16–19 of pregnancy. Other timing conventions of pregnancy are equally acceptable but must be defined. Therefore, the mated female rats are treated with or exposed to the prepared test compound from Day 6–17 and rabbits from Day 6–18/19 of pregnancy (depending on time of closure of the hard palate). The inseminated rats are killed and delivered by cesarean section and dissected on Day 20 or 21, and rabbits on Day 29 of pregnancy. Delivery by cesarean section is necessary to ensure examination of malformed fetuses which could otherwise be cannibalized by the mothers after delivery.

Maternal toxicity, such as altered behavior, clinical symptoms, mortality and general physical condition, food consumption, and body weight, is continuously examined during the study. All mated animals are autopsied and checked macroscopically for outward

appearance and outwardly visible organ changes, with emphasis on the uterus. The implantation sites in the uterus are counted macroscopically (rabbits) or after staining with ammonium sulfide (rats). The latter procedure, together with counting the number of corpora lutea in the ovaries, allows the detection of early embryonic deaths not visible in the unstained uterus.

The uterus is opened and the implantation sites and live and dead fetuses, dead embryo-fetal primordia undergoing resorption and the respective placentas, as well as the corpora lutea in the ovaries are counted and examined macroscopically. The fetuses are assessed for signs of life, sex, outward appearance and outwardly detectable anomalies, and their body weight and, optionally, crown-rump-length are measured. Then the rat and rabbit fetuses are euthanized.

In rats, approximately half of the fetuses from each litter are fixed in alcohol, dissected for macroscopic organ abnormalities (necropsy or micro-dissection), eviscerated, and the carcasses are then cleared in 1% caustic potash solution. The skeletons are stained in alizarin red S solution, then preserved in glycerol, and examined for anomalies with the aid of a stereomicroscope. The skeletal examination distinguishes between malformations (probably adversely affecting postnatal growth and development and probably irreversible), minor anomalies (variations, probably not adversely affecting postnatal growth and development and/or probably reversible), and ossification findings (usually reversible). Examination of both bones and cartilage can be performed by double staining with alizarin red (bones) and alcian blue (cartilage). The remaining rat fetuses of each litter can be fixed in Bouin's solution and examined in body cross sections for visceral anomalies according to the method of Wilson (1965). Alternatively, the fetuses may also be examined for organ abnormalities by using micro-dissection methods, e.g., according to Barrow and Taylor (1969). The examination of the internal organs also distinguishes between minor anomalies and malformations, with or without the aforementioned consequences. All rabbit fetuses are likewise examined for abnormalities of the internal organs (usually by fresh dissection with the aid of a stereomicroscope) and for skeletal abnormalities (with the aid of a magnifying glass) using the same procedures mentioned for the rat fetuses. Additionally, the skull cap is opened and the brain is removed and cross-sectioned according to the method of Wilson.

53.2.9 Study for Effects on Pre- and Postnatal Development Including Maternal Function

This study covers ICH phase C to F of the reproductive cycle and is usually performed in rats in case of development of small molecules. In case of development of biologicals (e.g., antibodies) the enhanced pre- and postnatal study design in monkeys (see below) is often used. The aim of this study is to detect adverse effects on the pregnant and lactating female, and on the development of the conceptus and the offspring following exposure of the female from implantation through weaning. Adverse effects to be assessed are: adverse toxicity relative to that of nonpregnant females, pre- and postnatal death of offspring, altered growth and development, functional deficits of offspring including behavior, maturation (puberty), and reproduction (F1-generation).

Young adult virgin females, preferably rats, are mated with untreated males of proven fertility. They are then assigned to groups randomly by a computer-generated algorithm. Then they are treated or exposed with the prepared test compound from implantation, through pregnancy and parturition, up to the end of the 3-week lactation period. Before implementation of the ICH Test Guideline, treatment or exposure of the animals started with closure of the hard palate of the fetuses on Day 17 of pregnancy. The females are allowed to deliver spontaneously and to rear their offspring up to weaning.

As in the other studies, maternal toxicity, such as animal's behavior, clinical signs, mortality and general physical condition, food consumption, and body weight, are examined during the study. Duration of pregnancy, birth process, and body weight gain until weaning are recorded. The number of live and dead pups, their sex, body weight at birth, and abnormalities are also obtained. During the lactation period, the behavior and body weight gain of the pups, and the number of surviving pups up to weaning, are evaluated. After weaning, the females are killed and dissected for macroscopic changes of the internal organs. The uterus is removed and stained in ammonium sulfide solution in order to count the number of all implantations and for detection of supernumerary implantation sites.

In order to find out adverse effects on postnatal development of the offspring, a great battery of physical landmarks, reflex and behavioral tests, was

established. Generally, the onset of the following physical landmarks can be tested (examples): pinna detachment, fur development, incisor eruption, eye opening, righting reflex, and pupil reflex to light. These parameters are studied in the lactation period. Sexual maturation of the offspring is assessed by the time of preputial separation and vaginal patency. Behavior is tested after weaning by activity, open field (often studied during lactation), photophobotaxis, maze test, social interactive test, electric current avoidance, passive avoidance, FOB including grip strength and/or landing food spread. Further studies are fore- or hind limb hanging, or rotarod. Learning behavior, such as trainability, memory, and re-trainability, as well as motor activity, coordination, and sense of balance, can be checked in a water-maze-test. It has to be decided which test should be used in a routine test battery and which should be used in special cases of test substances. How many pups from each litter and dose group should be examined to provide a meaningful evaluation must also be fixed. Sixteen to 20 litters per groups should be available for evaluation.

After behavioral testing, fertility of offspring is assessed using the procedures described under "Effects on Fertility and Early Embryonic Development" above. Alternatively, females may be necropsied with their pups on Day 4 postpartum.

53.2.10 Alternative Study Designs

One study design: This study design covers ICH stages A to F. Consequently it is a very large study and difficult to handle. An embryo-fetal toxicity study in a second species is needed.

Two-Segment design: These study protocols combine the design of a fertility study and of a pre- and postnatal toxicity study. The study design has the same limitations as the one study design.

Combined fertility and embryo toxicity study design: The treatment of females in the fertility study is prolonged until closure of the hard palate. This design is relatively easy to handle from a logistical point of view and considered as the most advantageous alternative design.

Combination of segments is not advisable if toxicokinetic parameters change over time (e.g., enzyme induction or accumulation). Study designs may furthermore be adapted in special cases, in which subgroups of pregnant animals are dosed in different "windows" of embryo-fetal development, e.g., gestation days 6–9, 10–13, 14–17. This design enables higher (adequate) maternal exposure, higher exposure ratios of animal/human, and a higher chance to detect teratogenic effects. This protocol is confined to special questions and not routinely used.

53.2.11 The "Enhanced Pre- and Postnatal Study" Design

In case of biopharmaceuticals (e.g., monoclonal antibodies) the Cynomolgus monkey is often preferred for preclinical safety testing since the pharmacological activity of the antibody is only present in this species. This enhanced pre- and postnatal study design combines the classical embryo-fetal toxicity study and the pre-postnatal toxicity study into one single study. A comprehensive review of this study design is given by Stewart (2009).

Pregnancy rate is low in Cynomolgus monkeys and is detected by ultrasound at gestation day 18–20. Dosing starts after detection of pregnancy, and therefore implantation and early embryogenesis are not covered in this species. However, since exposure of the fetus to monoclonal antibodies during organogenesis is limited, there is lesser concern of teratogenicity during this period compared to small molecules. Exposure to antibodies increases after organogenesis, and would therefore not be adequately addressed by a classical embryo-fetal toxicity study design (treatment from gestation day 20–50). In the enhanced pre- and postnatal toxicity study design, pregnant female Cynomolgus monkeys are treated from detection of pregnancy ($\sim$ Day 20 post coitum) until birth. Fetal growth is determined by ultrasound on gestation days 50, 90, and 130. Since monkeys do not eat stillborn offspring, their evaluation is possible in absence of a cesarean section. Exposure to the antibody is measured in mother and offspring at birth. Live offspring is weaned after birth. The length of the postnatal phase is variable and depends on the endpoints of concern. If offspring viability and growth is the primary endpoint, then the postnatal phase may be limited to 1–2 months (highest mortality occurs within the first week after birth). The postnatal phase is extended in case functional tests are needed like phenotyping and function of the immune system (e.g., lymphocyte

subpopulations, T-cell dependent antibody response, natural killer cell activity), behavior, learning, and memory. Further endpoints in offspring comprise ophthalmologic examinations, antibody exposure (may be prolonged), skeletal examinations (X-Ray), hematology, clinical chemistry, urinalysis, necropsy, organ weights, and histopathology.

The group size in this study design should be 20–24 animals to take into account abortions, stillbirths, and early postnatal deaths.

53.2.12 Timing of Studies

The requirement of non-clinical reproductive toxicology studies before enrolment of men or women in clinical trials is regulated in by ICH guideline M3(R2) (ICH International Conference on Harmonisation 2009).

Men can be included in Phase I and II trials without reproduction toxicity studies since evaluation of male reproductive organs is performed in repeated-dose toxicity studies. A male fertility study should be completed before initiation of large-scale or long duration clinical trials (e.g., Phase III trials).

Women not of childbearing potential can be included in clinical trials devoid of reproduction toxicity studies since evaluation of female reproductive organs is performed in repeated-dose toxicity studies.

For women of childbearing potential there is a concern for embryonic exposure. Therefore the risk of unintentional exposure has to be minimized (e.g., conduction of reproduction toxicity studies, precautions to prevent pregnancy, pregnancy testing (β-subunit of HCG), use of highly effective methods of birth control, study entry after confirmed menstrual period only). Women of childbearing potential can be enrolled in early Phase I clinical trials without reproductive toxicology studies under the following considerations: The disease is predominant in women; effective precautions to prevent pregnancy are used; duration of treatment is short (e.g., 2 weeks); the mechanism of action is known; the amount of embryo-fetal exposure can be estimated and is negligible. For inclusion of women of childbearing potential in limited phase II studies (up to 150 patients, up to 3 months duration) at least "extended preliminary reproduction toxicity data" are required, i.e., data of embryo-fetal toxicity studies in two species, consisting of at least six animals per group, with full external and visceral examination of the fetuses. In the USA definitive embryo-fetal studies are needed for Phase III whereas they are needed generally in EU and Japan except the cases mentioned above. Female fertility studies are required before long duration or large-scale clinical trials (e.g., Phase III) are initiated. Pre-postnatal toxicity data are needed for submission.

If pregnant women are planned to be included in clinical trials, all female reproductive toxicity studies are needed. In addition, evaluation of safety data (previous human exposure) is required.

53.3 OECD Guidelines

Besides the study designs described above for reproductive toxicity testing especially developed to examine medicinal products, a battery of further reproductive and developmental toxicity test guidelines was published by the OECD. The first OECD reproductive toxicity test guideline, especially developed for testing chemicals and agricultural products, was adopted in 1981.

Usually, three dose levels and a concurrent control group are used in these studies. The highest dose level should be chosen with the aim to induce some developmental and/or maternal/paternal toxicity (clinical signs or a decrease in body weight development) but not death or severe suffering. The dose of 1,000 mg/kg/day is generally accepted as an upper limit dose level. However, human exposure may indicate the need for higher dose levels.

53.3.1 OECD Guideline 414: Prenatal Developmental Toxicity Study

This guideline is similar to the ICH design for effects on embryo-fetal development. As a difference the administration period is at least from implantation through the last day before cesarean section. When appropriate, administration of the test compound through the entire gestation period is encouraged. As in the ICH guideline, the most relevant species should be used. The preferred rodent species is the rat, the preferred non-rodent species is the rabbit. The number of animals should result in approximately 20 pregnant animals, groups with less than 16 implantation sites

may be inappropriate. The high dose level should produce maternal toxicity (e.g., reduction of body weight gain), but mortality should not exceed 10%. In non-rodents, the heads of one half of each litter should be evaluated for soft tissue alterations (eyes, brain, tongue, and nasal passages) (OECD Guidelines for Testing of Chemicals 2001).

53.3.2 OECD Guideline 415: One-Generation Reproduction Toxicity Study

This study is usually conducted with rats or mice. Males are dosed for at least one spermatogenic cycle (70 days in rats, 56 days in mice). Females are treated for at least two complete estrous cycles (14 days) in order to detect adverse effects on estrous cyclicity. Treatment continues to both sexes during cohabitation, and thereafter to females during pregnancy and nursing of F1 animals. Females are allowed to litter normally. At least 20 females per group are needed at or near parturition. Litter size standardization to four males and four females on Day 4 postpartum is optional. Observations and measurements include clinical signs, body weight, food consumption, duration of gestation, number and sex of pups, still births, live births, and gross anomalies. Additionally, macroscopic examinations are performed at necropsy, and reproductive organs of P animals are preserved for possible microscopic examination. Microscopic examination is mandatory if these organs have not been examined in repeat-dose toxicity studies.

This Guideline is rather old. It was adopted in 1983 (OECD Guidelines for Testing of Chemicals 1983) and is usually replaced by OECD416 or the extended One-Generation Reproduction Toxicity Study (see below).

53.3.3 OECD Guideline 416: Two-Generation Reproduction Toxicity Study

In that study design, the exposure of the experimental animals to the chemical is continuously extended over two complete generations (P- and F1-generation), including weaning of the F2-generation (OECD Guidelines for Testing of Chemicals 2001). Assessment of sperm parameters (homogenization-resistant sperm heads in the testis, sperm reserves in epididymis (cauda), and sperm motility and morphology) are endpoints for P and F1 animals. The two-generation toxicity reproduction toxicity design is the only one that exposes the F1 animals from conception onward and would cover effects on fertility during this early period. Due to the high animal number used, discussions are being carried on to replace the OECD 416 with the extended one-generation reproductive toxicity test.

53.3.4 OECD Guideline 443: Extended One-Generation Reproductive Toxicity Study

This study design has been discussed as a replacement for the Two-Generation Toxicity Study design, as it requires considerably less animals. Pre-cohabitation treatment is 2 weeks in males and females. Dosing is continued during the mating period and until weaning. Dosing of F1 starts at weaning and continues until necropsy. After weaning, F1 animals are assigned to three cohorts, reproductive toxicity, neurotoxicity and clinical endpoints, and immunotoxicity; necessity of the latter two is dependent on available information as well as regulatory purposes. A second mating of P animals might be triggered in case of equivocal effects during the study and should be done preferably with untreated females (OECD Guidelines for Testing of Chemicals 2011).

Endpoints are similar to the pre- and postnatal toxicity study. In addition, developmental immunotoxicity is assessed by IgG and IgM antibody response to Keyhole Limpet Hemocyanin or sheep red blood cells. If antibody response is not or only marginally affected, cellular immune response and delayed type hypersensitivity response, cell mediated cytotoxicity, or natural killer cell assay are determined in the remaining animals. If antibody response is affected, the remaining animals are immunized with sheep red blood cells, and phenotypic analysis of splenocytes is carried out to identify affected cells. Assessment of reproductive toxicity (triggered) is done in case of equivocal effects on reproduction in P animals. The F1 animals of cohort I can be maintained until Day 90 and then cohabitation similar to P animals, but termination of F2 animals is on PND4.

Sperm parameters comprise testes and epididymis weight in all P and F1 males, histological examination

of one testis and epididymis, absolute and relative amount of different germ cell stages, and epididymal sperm count, motility, and morphology.

Organ weights are determined in P and F1 animals, and full histopathology is carried out in 10 randomly chosen rats per sex of the control and high dose group of the P generation and reproductive toxicity F1 cohort, respectively (low and mid dose animals are examined in case of compound-related effects). In F1 animals of the neurotoxicity cohort, CNS is fixated by instillation, and separate sections are immunostained for glial fibrillary acidic protein and a synaptic marker. Furthermore, quantitative morphometric evaluations are recommended to be carried out. Neurohistopathology should be consistent with OECD 426 (see below).

53.3.5 OECD Guideline 421: Reproduction/ Developmental Toxicity Screening Test

In this test, which uses 10 animals per sex and dose group (usually rats), males are treated at least for 4 weeks and females 2 weeks before cohabitation. Then the animals are mated 1:1. Treatment continues during cohabitation and in females during pregnancy until 4 days postpartum. Observations and measurements include clinical signs, body weights, food consumption, and gestation length, number of live and dead pups, sex distribution, and pup weights at birth and postnatal Day 4. Further examinations comprise number of corpora lutea and implantation sites, weights of testes and epididymides, macroscopic examinations at necropsy, and histopathological examinations of ovaries, testes, and epididymides with special emphasis on stages of spermatogenesis and histopathology of interstitial testicular structure (OECD Guidelines for Testing of Chemicals 1995).

53.3.6 OECD Guideline 422: Combined Repeated-Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test

This test system combines the repeated-dose toxicity study (OECD Guideline for Testing of Chemicals 2008) and the reproduction/developmental toxicity screening test described above. The design includes detailed clinical observations (outside the cage in a standard arena) and neurological examinations (sensory reactivity to auditory, visual, and proprioceptive stimuli, grip strength, assessment of motor activity) (OECD Guidelines for Testing of Chemicals 1996).

53.3.7 OECD Guideline 426: Developmental Neurotoxicity Study

This study has to be carried out in case of hints for neurotoxicity (e.g., based on results of other studies). Groups of 20 rats are dosed from implantation through lactation, with adjustment of litter size on postnatal Day 4. During the pre-weaning phase, subsets of pups are assessed regarding behavioral ontogeny, brain weight, neuropathology, and optionally brain morphometry. Likewise, subsets of pups are assigned to behavioral/functional tests, motor activity, sexual maturation, auditory startle response (as described in more detail above in the pre- and postnatal toxicity study), brain weight, neuropathology, and optionally brain morphometry during the post-weaning phase (OECD Guidelines for Testing of Chemicals 2007).

53.4 Alternative Test Systems

There are many alternative in vivo mammalian, nonmammalian, and in vitro test assays. However, at present no alternative test can totally replace the existing reproductive toxicity tests using live mammalian animals. The major drawback of these test systems is that the maternal component and its interaction with the conceptus is missing. However, these alternative methods can be used for screening of drug candidates for potential embryo toxicity. Commonly used alternative test systems are the frog embryo teratogenesis assay *Xenopus* (FETAX), the Zebra fish assay, the whole embryo culture test (WEC), and the mouse embryonic stem cell test (EST).

The FETAX test uses mid blastula stages of *Xenopus*, which are incubated with the test compound at different concentrations for 96 h. In the Zebra fish assay, embryos are exposed to the test substance 6–120 h after fertilization. In both assays, prediction of teratogenicity is based on the ratio of the concentration causing a distinct percentage of lethality and the

concentration causing a distinct percentage of larvae with malformations (Bantle et al. 1989; Fort and Paul 2002).

In the WEC test, rat embryos are removed from the mother at Day 10 of gestation, cultured, and exposed for up to 48 h to the test compound (Piersma et al. 2004). This period covers development of major organ systems, including closure of the neural tube, heart, limb buds, eyes, and ears. Endpoints are growth (head length, crown-rump length) and malformations.

The mouse EST uses cells derived from inner layer blastula cells, which develop to embryoid bodies. Originally the test used the development of contracting differentiated myocardial cells in relationship to cytotoxicity in 3T3 cells as an endpoint. Today, EST cells can also be developed into CNS, blood, bone, and cartilage cells to assess potential effects in these tissues. A further improvement of this test is the use of nonhuman primate EST cells instead of mouse EST cells, since the former are more similar to human EST cells (Davis et al. 2010). Recently, implementation of transcriptomics has been shown to improve the predictivity of this test system (Van Dartel et al. 2011).

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In Vitro Toxicology

54

An Integrated Tool for Early Safety Evaluation

Mostafa Kabiri

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In recent years, considerable progress has been made in developing in vitro test systems. In vitro assays are ideal tools for rapid screening with high reproducibility and increased sensitivity, and they are relatively inexpensive, especially compared with in vivo studies. In pharmaceutical industry, there has been a dramatic increase in interest in in vitro toxicology and in the search for in vitro methods for predicting toxicological effects. The industry needs early, rapid, and robust preclinical lead optimization technology screening assays, which allow a lead series of compounds to be ranked for desirable or undesirable characteristics.

A better lead candidate selection for preclinical drug development should reduce the high rate of attrition and the involved increasing costs. Outside the industry the prominent driving forces for the development and application of in vitro toxicity systems was (and still is) the societal criticism of using animals in toxicological studies as well as the growing demands for greater safety. Broad spectrums of in vitro systems, with individual techniques associated with particular classes of compounds, have already been tested in validation studies and their usefulness has been proved for the assessment of organ toxicities. The non-animal screening tests are now performed prior or in parallel to regulatory in vivo studies either to exclude potentially severe effects in vivo or to provide mechanistic data (Bugelski et al. 2000; Peters 2005; Dorato and Buckley 2007). Importantly, the screening assays should be predictive, sufficiently rapid, and cost-effective. This chapter describes the most in vitro approaches in molecular toxicology applied to drug development that fulfill all these criteria and that are based upon essential cellular, molecular, and biochemical processes. The central idea is that

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the investigation of toxic reactions should be expanded to multiple parameters, different cellular models and different end points combined with a detailed concentration-response relationship, to achieve a complex system closed to in vivo conditions. These in vitro data are then regarded as basic toxicological information that are used together with other data (in silico, target activity) for the final evaluation, hazard and risk identification (Atterwill and Steele 1987). Starting with in silico, the available knowledge and computer models/methods can be utilized to identify likely toxicity with significant scores. In the next tier, cell-based assays are conducted to unravel effects of the test compounds on cultured and primary cells. Using animal and human cell types may allow better estimates of risk for human situation. Finally, exploratory in vitro studies (probably supplemented by in vivo studies) are conducted to generate mechanistic data that can be used to decipher the processes associated with the observed target-organ toxicity. There have been many diverse sets of assays described in the literature that cover almost all targets of side effects including models for rare toxicities (e.g., thyroid gland). However, the in vitro methods described here focus mainly on hepatotoxicity, cardiotoxicity, and nephrotoxicity that build the most organ toxicities caused by side effects of therapeutics. The concerned action in each organ model is a multisystem approach with sequential examination in different cells and species applying multiple physiological end points (Gross and Kramer 2007; McKim et al. 2005).

54.1 General Insights into Cellular Systems

Since the development of cell culture techniques and the evolution of the culture media, almost all cell types can effectively be cultured. Cells from most mammals as well as lower vertebrates and insects are routinely cultivated and grown in vitro for short or long lasting periods. The cultivation period depends mostly on whether the cells are immortalized (transformed) or normal cells isolated from corresponding organs (primary cells). While the latter cells can be cultured just for a few days or weeks, transformed cells can be maintained in culture for many months or even years. Primary cells can be isolated using intact organs of experimental animals as well as humans. Immortalized cells are either isolated from malignant organs and are sequentially adapted to culture conditions, or are normal cells that are transformed in culture by virus infections. This origin and molecular programming of the cells determines the requirements for successful cultivation in vitro. The cells grow either without cell contact floating in the culture medium (suspension culture) or need cell contact and attachment to a suitable substrate and grow on that substrate (inert surface such as plastic). The design of culture dishes is fundamental for the culture of many primary cells and cell lines. While, for example, primary cultures of hepatocytes require collagen-coated dishes, fibroblasts can attach and grow in untreated culture dishes. In order to achieve a tissue-like cell architecture and function, three-dimensional matrices are developed that represent more complex in vitro tools. The matrix in this approach serves as a skeleton and directs a three-dimensional cell assembly. Differentiated cells in this culture method maintain their phenotype and functional characteristics. The matrix-assisted culture technique was fundamental for the development of some in vitro models, for example, the cholestasis model that is described below.

Unwanted contamination with microorganisms like bacteria is a permanent and serious issue in handling cells in culture. Therefore, it is of paramount importance to complying with laboratory hygiene to protect cultures from contamination with microorganisms. Supplementation of media with antibiotics prevents bacterial contaminations. However, the cross-reaction of antibiotica with test compounds should be appraised. Temperature and humidity are other important factors that need adaptation to culture needs (Waymouth et al. 1981; Walum et al. 1990).

54.1.1 Available In Vitro Models to Investigate Hepatotoxicity

Hepatotoxicity is one of the most important drug side effects. Therefore, in vitro hepatotoxicity assays are applied early in drug development to screen development compounds for their potential to induce hepatotoxicity. Generally, three different hepatic in vitro models are used to examine the toxic effects on the hepatic system: (a) The most frequently used model is the cell culture model of primary hepatocytes that can be isolated either from conventional laboratory

Table 54.1 Hepatocytes cultivation media

Williams E (500 ml) (Gibco, No. 22551-022)	Attachment medium	Culture medium
	Supplements	
Glutamine (200 mM)	1%	1%
Pen./Strep. (10.000U/10.000 µg)	1%	1%
Dexamethasone (1 mg/ml in PBS)	1%	1%
ITS supplement (Gibco, No. 41400-045)	1%	1%
FBS	5%	–

animals (rat, mouse, dog, rabbit) or human liver specimens. (b) Permanent hepatic cell cultures of animal (H4IIE) or human origin (HepG2) represent the second approach. (c) As an alternative to the cell-based approaches, precision-cut liver slices are applied to the studies of liver toxicity (Groneberg et al. 2002). Intact hepatocytes represent a complex biological system with high capacity for drug metabolism (Seddon et al. 1989; Bayliss et al. 1994). Working with permanent cell lines is easy, time-saving, compatible to high-screening modes, and they are accessible to genetic manipulations (Alley et al. 1988). Preparation and working with liver slices is quick and easy to handle. The more relevant usefulness of liver slices is that they are accessible to classical toxicological techniques and can be investigated as tissue-like in accord with histopathological examinations of the native organ (Ekins et al. 1996; de Kanter et al. 2002). In addition, they exhibit high conservation of drug-metabolizing enzyme activities (Sohlenius-Sternbeck et al. 2000). Nevertheless, each of these models has advantages and limitations. Therefore, depending on the toxicological aspects under investigation, one or all of these models can be applied (Guillouzo 1998).

54.1.1.1 Isolation and Culture of Primary Hepatocytes

For the isolation of primary hepatocytes, a rat is anesthetized with Hostaket/Rompun® (i.p.). The abdomen is washed with 70% ethanol and a ventral midline incision is made from the xiphisternum to the pupic bone. The hepatic portal vein and vena cava are exposed and a loose ligature is placed around the vena cava inferior under the departure of the left kidney. Next, the vena cava superior is clamped near by the diaphragm and the vena cava inferior is catheterized, and the ligature is tightened. The catheter is now attached to the perfusion tubing containing Gibco BRL Liver Perfusion Medium (Gibco, No. 17701-038) and at the same time the portal vein is cut. The liver perfusion starts with 100 mL Perfusion Medium at a flow rate of 20 mL/min. Following the first perfusion and to digest the liver, the liver is perfused with Gibco BRL Liver Digest Medium (Gibco, No.17703-034) until the liver is considered to be sufficiently digested. The liver is then excised and transferred into a sterile glass dish containing Digest Medium. The fully digested liver is gently teased with a sterile fork to free the cells from the connective tissue. In the next step the mashed liver is filtered through two layers of sterile gaze and washed up with cold Gibco BRL Hepatocyte Wash Medium. To harvest the primary hepatocytes the cell suspension is kept on ice for approximately 10 min to allow sedimentation of hepatocytes. After the sedimentation of the cells, the supernatant is discarded and the pellet is washed three times. After the last washing, the pellet is recovered in cold Attachment Medium (See Table 54.1). Yield and viability is immediately determined, and cell preparations with the viability of $\geq$80% are then seeded in culture plates coated with Collagen I in Attachment Medium. Cultures are incubated now for 4 h to allow them to attach to the substrate. Next, the Attachment Medium is removed and the cells are incubated for a further 20 h in Culture Medium (see Table 54.1). The overnight cultures are then used for in vitro experiments (Berry and Friend 1969; Seglen 1976; Quistorff et al. 1989; Le- Cluyse et al. 1996a, b; Mudra and Parkinson 2001). The graph in Fig. 54.5 represents the application of primary hepatocytes in cytotoxicity experiments. The application of primary hepatocytes is not restricted and a broad range of end points can be analyzed with primary hepatocytes (Eckl and Bresgen 2003).

For the isolation of primary hepatocytes from other mammals (rabbit, dog, monkey, and human) the liver is excised and the whole (rabbit) or a piece of liver (dog, monkey, human) is perfused ex vivo (Jamieson et al. 1988; Coulet et al. 1998). All other steps are according to the described method with some adjustments depending on the size of the liver or liver pieces. In this way, isolated primary hepatocytes in monolayer culture keep their metabolism for several days, but lose their tissue-like physiology over time. Therefore, it is important to start experiments immediately after

cell adaptation to in vitro cell culture conditions. Matrix-supported culture models may be considered when primary hepatocytes cultures are intended for longer experimental duration or if formation of hepatocytes-specific structures is desired (see formation of canaliculi in the cholestasis model bellow). Non-static cultures in bioreactors with continuous fluidic flow would also provide an alternative (Zeilinger et al. 2002; Allen et al. 2005; Novic et al. 2009; Kidambi et al. 2009).

Preparation and Culture of Liver Slices

Precision-cut liver slices are prepared from the liver obtained from a rat of 6–7 weeks of age. After sacrifice (Refer to hepatocytes isolation protocol), the liver is removed and the liver lobes are separated and stored in Washing Medium (Krebs-Henseleit-Bicarbonat buffer, containing HEPES buffer and gentamicin) on ice until the preparation of slices. Slices of dog, monkey, rabbit, and human liver can be prepared accordingly from samples of ca. 1 cm thickness. From the liver lobes cores of ca. 8 mm diameter are prepared using a motor-driven coring toll. The cores are stored in Washing Medium until preparation of slices. Slices of ca 250–100 μm thickness are prepared in medium at 2–8°C using a krumdieck tissue slicer (Krumdieck AR&D). Slices are washed in Washing Medium and stored in the same medium on ice until start of the culture or cryopreservation. To culture liver slices, three slices are transferred to a 20 mL glass Erlenmeyer flask containing 5 mL Culture Medium (Williams Medium E supplemented with glutamine [292 μg/mL], gentamicin [50 μg/mL], and insulin [0.1 μM]) and pre-incubated for approximately 1 h at 37°C in a shaking water bath. After pre-incubation, the medium is replaced with 5 mL fresh supplemented medium containing the test compound. Stock solution of test compounds are prepared in DMSO or other solvents and added to the culture in a broad range of concentrations. The cultures are kept under a carbogen atmosphere with 95% O_2 and 5% CO_2. The exposure period depends on the experimental design, but is normally 24 and 48 h. If cytotoxicity is measured, a positive reference compound, for example, 0.5% Triton, is tested in parallel. Cytotoxicity is measured either through the LDH leakage or MTT conversion. Liver slices can also be subject to histopathological examination. After the incubation time, slices are cut in half. One half is used for the determination of MTT conversion. The other half is fixed in 4% buffered formalin. Next, slices are horizontally embedded in paraffin and 8 μm sections are cut using a microtome. Sections are stained with H&E and evaluated microscopically. The so prepared slices are as nearest to in vivo situation a valuable tool in mechanistic studies, for example, apoptosis, cross-species comparisons, and metabolism (Bach et al. 1996; Hashemi et al. 1999; Glöckner et al. 2002; Jewell and Miller 1999).

54.1.1.2 Culture of Human Cell Line HepG2

HepG2 is a human hepatic carcinoma cell line and commercially available (HB-8065, ATTC, Manassas, VA).

HepG2 cells are cultured in high-glucose Dulbecco's modified Eagle's medium ([DMEM, Invitrogen, No. 11995-065] containing 25 mM glucose and 1 mM sodium pyruvate and supplemented with 5 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], 10% heat inactivated FBS, and penicillin-streptomycin [pen-strep; 500 μg/mL final concentration]) and kept in 5% CO_2 at 37°C. Cells are maintained on collagen-coated flasks and seeded onto 96-well plates for individual experiments. Culture of cells in collagen-coated dishes ensures a better cell growth and proliferation. To allow them to adhere HepG2 cells are incubated for 24 h at 37°C with 5% CO_2 prior to treatment. For culture of cells, different plate formats are available. The choice of white or transparent well plates depends on choice of biochemical assays. For cytotoxicity assays, LDH or NRU (see Sect. 54.1.4.1 Cytotoxicity Assays) clear well plates (e.g., Greiner No. 655098) are required, and for CellTiterGloTM assay, white well plates (e.g., Greiner No. 655183) are recommended.

54.1.1.3 Culture of Rat Cell Line H4IIE

H4IIE is a rat hepato carcinoma cell line and commercially available (CRL-1548, ATTC, Manassas, VA).

H4IIE cells are cultured in MEM Eagle medium ([Sigma No. M 5650] supplemented with 10% [5% in treatment medium] heat inactivated FCS, 2% glutamin, and 1% pyruvic acid sodium). Cells are expanded in 175 cm^2 culture flasks and subcultured in micro-well plates thereafter. To subculture H4IIE, cells are detached from the substrate by enzymatic digestion. Prior to enzyme treatment, media are removed, flasks are rinsed two times with 10 mL prewarmed phosphate buffered saline deprived

of Ca^{2+}/Mg^{2+} (PBS Sigma No. 20012-019). In the next step, 5 mL Accutase is added and the culture is incubated at 37°C for ca. 10 min. The reaction is terminated with addition of culture medium (about 25 mL) and continuous resuspension with a pipette. Cell number is determined and 1×10^4 cells per well are seeded into a 96-well plate and incubated for 24 h at 37°C with 5% CO_2 prior to treatment.

54.1.2 Available In Vitro Models to Investigate Cardiac Toxicity

The most common in vitro models for assessment of cardiac toxicity are cardiac cell lines or primary cardiomyocytes isolated from laboratory animals. The prominent cardiac cell lines in toxicity studies are H9c2 from rat embryonic ventricular myocardium and the human cell line AC16. H9c2 cells exhibit many cardiac muscle characteristics like electrophysiological activity, automatic receptors, and ion channels (Wang et al. 1999). Freshly isolated cardiomyocytes stand out due to having in vivo physiological properties like receptor and enzymatic activities. Embryonic stem cells represent an alternative source for cardiomyocytes. However, the physiological characteristics of these cells are not well defined yet.

54.1.2.1 Isolation of Primary Cardiomyocytes from Neonatal or Adult Rats

Primary cardiomyocytes can either be isolated from neonates or adult rats. For the isolation of neonatal rat cardiomyocytes, cardiac ventricles from 1- to 2-day-old rat neonates are excised, washed with PBS buffer to remove blood, and gently minced. The minced ventricles are then digested with collagenase H in Ca- and Mg-free HBSS medium. Dissociated cells are then filtered through 200-μm mesh and collected by centrifugation. The isolated cells are cultured in DMEM medium supplemented with fetal bovine serum. After a 24 h culture and adherence of cells, Cytosine 1-β-D-arabinofuranoside at a concentration of 10 μM is added to retard the growth of fibroblasts (Boerma et al. 2002; Fu et al. 2005). The isolated cultures are incubated for several days, before starting experiments. Even though the isolation and culture of fetal cardiomyocytes provides some technical advantages, there are a few drawbacks that limit their utilization. Cardiomyocytes derived from newborn animals are different in their physiology and morphological characteristics compared to cardiomyocytes isolated from adult animals.

For the isolation of primary cardiomyocytes, the heart of a rat is excised and stored in PBS buffer lacking $MgCl_2$ and $CaCl_2$. Before mounting the heart on the Langendorff apparatus for perfusion, the aorta is exposed and a cannula is inserted. Washing Solution ([Pure Joklik HEPES medium, PAN BIOTECH, Germany] supplemented with 1% Penicillin/Streptomycin, 250 μM EDTA, 15 mM 2,3-butandione monoxide [BDM]) is perfused with a fluid pressure of not more than 80 mm HG until all the blood is washed out. Then the heart is perfused with Digest Solution ([Washing Solution] supplemented with 20 μM $CaCl_2$, 1% BSA, 5 mg liberase™ [Roche] in 50 mL Washing Solution) for ca. 40 min. The digested heart is then transferred into a sterile glass dish containing Powell-Gradient Solution I (40 μM $CaCl_2$ in 120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM $NaHCO_3$, 5.6 mM Glucose, 15 mM BDM, 5 mM Taurine, 5% BSA) and gently teased with a sterile scalpel. In the next step, the mashed heart is filtered through a sterile cell sieve and washed up with Powell-Gradient Solution I. To harvest the primary cardiomyocytes the cell suspension is centrifuged at 400 U/min for 3 min. The supernatant is discarded and the cell pellet is suspended in Powell-Gradient Solution II (200 μM $CaCl_2$ in Powell-Gradient Solution I). In the next step, cells are washed up with Powell-Gradient Solution III (400 μM $CaCl_2$ in 120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM $NaHCO_3$, 5.6 mM Glucose, 15 mM BDM, 5% BSA) and finally the pellet is resuspended in Incubation Medium I. After the yield and viability of cells are determined, cell are seeded in culture plates coated with Laminin in Incubation Medium I and incubated for 1 h at 37°C to allow them to attach to the substrate. Next, the Medium is changed and the incubation is continued for next 1 h. At the end the culture medium is removed, and the cells are incubated in Incubation Medium II (Medium 199 [PAN BIOTECH] supplemented with 1 mM $CaCL_2$, 1% Pen/Strep). The cells isolated with this protocol can be cultured at 37°C at 5% CO_2 and used for the planned experiments within 4–8 days (Mitcheson et al. 1998; Thum and Borlak 2000, 2001; Zhou et al. 2000; Louch et al. 2011). This protocol can also be applied to other experimental models like mouse and rabbit. Considering this very complex and time-consuming procedure, these isolated

Table 54.2 Major classes of therapeutic agents that induce nephrotoxicity

Acute tubular necrosis	Interstitial nephritis	Medullary necrosis	Papillary necrosis	Glomerular damage
Amphotericin B	*Antibiotics*	Ibuprofen	Indomethacin	Doxorubicin
Bacitracin	Aminoglycosides	Indomethacin	Ketorolac	Puromycin
Gentamicin	Penicillins	Ketorolac	Ifosfamide	Adriamycin
Acetaminophen	Indinavir			Mitomycin
Metthoterexate	Sulfonamides			Heavy metals
Cisplatin/cab oplatin	Vancomycin			*d*-penicillamine
Heavy metals	*Diuretics*			Methimazole
Radiog. Contrast media	Acetazolamide			Heroin
	Furosemide			Captopril
	Thiazides			
	Neuropsychotric			
	Carbamazepine			
	Phenobarbital			
	Phenytoin			
	NSAIDs			
	Ibuprofen			
	Indomethacin			
	Ketorolac			
	Naproxen			
	Miscellaneous			
	Acetaminophen			
	Omeprazole			
	Warfarin			

primary cardiomyocytes can mainly be used for investigative and mechanistic studies. For screening purposes, cell lines may be more beneficial.

54.1.2.2 Culture of Myocyte Cell Line H9c2

H9c2 is a rat cell line that was derived from the ventricular part of a 13th-day rat heart embryo and is commercially available (CRL-1446, ATTC, Manassas, VA).

H9c2 cells have been extensively used in cytotoxicity studies also because of their cardiac properties, for example, electrophysiological activity, ion channels, and autonomic receptors (Shi et al. 2009; Pereira et al. 2011). H9c2 cells express CYP1A1, 1B1, CYP2B1, 2B2, 2E1, and 2 J3 (Zordoky and El-Kadi 2007).

H9c2 cells are cultured in DMEM medium at 37°C at 5% CO_2. For planned experiments, they are seeded into 96-well micro well plates at 3×10^4 cells per well and incubated overnight. On the following day they can be exposed to test compounds.

54.1.3 Available In Vitro Models to Investigate Renal Toxicity

Kidney is a highly differentiated organ that possesses many specialized regions harboring very specialized cell types (Table 54.2). Nephrotoxicity following drug therapy is a common phenomenon that is mostly limited to a well-defined region of this organ. Hence, for the investigation of region-specific toxicities, culture of concerned regions is mandatory. Because of their role in secretion and reabsorption processes and their metabolic capacity, proximal tubular cells are the primary target of many nephrotoxic agents. In proximal nephron, metabolizing enzymes are expressed and higher concentration of GSH and GSH-dependent enzymes are present. The proximal tubular cells have the potential to accumulate high concentration of chemicals by active transport (by a factor of 100–1,000).

Protocols are developed to isolate and culture primary cells from different parts of the kidney, but

due to their importance most attempts relied on development of protocols to culture proximal tubules (Boogaard et al. 1989; Boom et al. 1992; Valente et al. 2011).

54.1.3.1 Isolation of Primary Tubular Cells from an Adult Rat Kidney

For the isolation of primary tubular cells a male or female rat is anesthetized with Hostaket/Rompun® (i.p.). The abdomen is washed with 70% ethanol and a ventral midline incision is made from the xiphisternum to the pupic bone. The aorta below and above the renal arteries are freed. After closing the celiac and mesenteric arteries, aorta is cannulated, renal veins are cut, and perfusion started immediately with 150 mL carbogen saturated calcium-free Hank's buffer (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 26 mM $NaHCO_3$, 25 mM HEPES, 0.5 mM EGTA) with a flow of 8 mL per minute at 37°C. After a second perfusion with 25 mL Hank's buffer without EGTA, the kidney is digested with collagenase (0.12% w/v) in Hank's medium containing 4 mM $CaCl_2$ for 20 min in recirculating system. Subsequently, the digested kidney is perfused with 10 mL Hank's medium containing 2.5% (w/v) BSA to wash out collagenase. Immediately after perfusions, the capsule is removed and the cortex is dispersed in cold Hank's buffer with 2.5% (w/v) BSA and 1.8 mM $CaCl_2$ (HB-BSA). The mashed kidney is then filtered through two layers of nylon gauze (first 135 μm and then 60 μm). The filtrate is centrifuged (80 × g, 4°C, 3 min) and washed with HB-BSA. The cell pellet is resuspended in Culture medium ([DMEM/F12] supplemented with 5% FCS, 1% penicillin/streptomycin, 5 mg/mL insulin, 0.5 μg/mL hydrocortisone, 10 μg/mL transferrin, 0.01 μg/mL hEGF, 0.5 μg/mL epinephrine, and 6.5 ng/mL triiodothyronine) and the cells are seeded into Collagen I-coated culture plates. The regular morphology check and marker analysis (e.g., NHE3) ascertain the identity of isolated proximal tubular cells. This protocol can principally be used to isolate tubular cells from mouse, rabbit, and with some modifications for the isolation of human proximal tubular cells.

The primary tubular cells isolated with this protocol can be used both for screening and for investigative studies. However, for a wide range of chemical series suspected to exhibit kidney toxicity the first line of cytotoxicity assays may include kidney-driven cell lines such as LLC-PK1 to sort out cytotoxic compounds or rank them based on their inhibitory properties. This cell line and MDCK cell line that have been regularly used in toxicity studies and proved to be useful are given bellow and their cultures are described.

54.1.3.2 Culture of Porcine Kidney Cell Line LLC-PK1

LLC-PK1 is a porcine cell line from proximal nephron and commercially available (CL-101 ATTC, Manassas, VA).

These cells are incapable of gluconeogenesis with low or no expression of CYP3A and CYP2B isotypes. LLC-PK1 cells are cultured in DMEM medium with 10% heat inactivated FBS and 1% glutamine at 37°C and 5% CO_2. For planned experiments they are seeded into 96-well micro well plates at 3×10^4 cells per well and incubated overnight. On the following day they can be exposed to test compounds. This cell line has been shown to be a valuable test system for prediction of kidney-specific toxicities (Fig. 54.1).

54.1.3.3 Culture of Canine Kidney Cell Line MDCK

MDCK is a canine cell line from collecting duct and is the most representative of the distal tubule, commercially available (CCL-34, ATTC, Manassas, VA).

MDCK cells are cultured in DMEM medium (supplemented with 10% heat inactivated FBS, 1% glutamine, 1% natrium pyruvat, and 1% NEAA) at 37°C and 5% CO_2. For planned experiments, they are seeded into 96-well micro well plates at 3×10^4 cells per well and incubated overnight. On the following day they can be exposed to test compounds.

54.1.4 Available Cytotoxicity and Functional Assays

54.1.4.1 Cytotoxicity Assays

Cytotoxicity assays can be used as ranking tool and for an organ or non-organ-specific early hazard identification (Xu et al. 2004; McKim 2010). Ranking test compounds allow selecting candidates with least toxicity potential. Cytotoxicity data determined in in vitro experiments applying a multisystem and multi-endpoint approach can provide a good estimate of the plasma concentration where toxicity in vivo

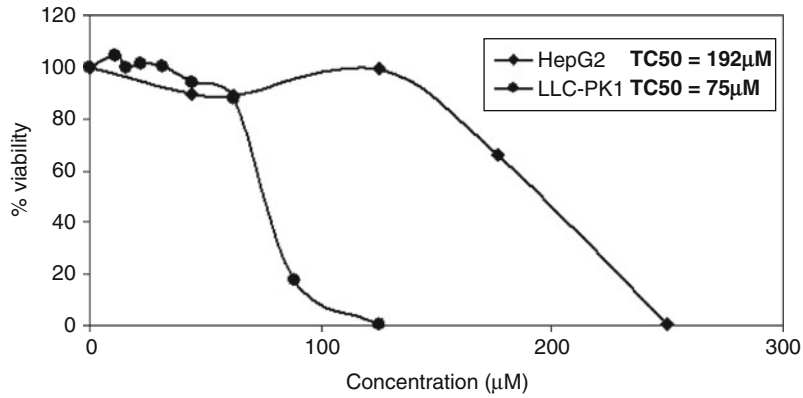

Fig. 54.1 Cytotoxicity of nephrotoxic 4-aminophenol hydrochloride on LLC-PK1 and HepG2 cells. The toxicity was measured by neutral red assay. The kidney LLC-PK1 cells exposed to 4-aminophenol hydrochloride display a specific response with much lower toxicity (TC50 = 75 μM) compared with human hepatoma HepG2 cell (TC50 = 192 μM)

Table 54.3 The cytotoxicity of therapeutic drugs tested on hepatocellular models compared to the no observed adverse effect level (NOEL) in plasma of patients

Compounds	TC50 (μM)	NOEL (μM)
Ketoconazol	18	30
Camptothecin	0.23	0.1
Acetaminophen	2,000	1,000
Chloramphenicol	1,000	300
Cyclosporin	6	1
Nitrofurantoin	45	4

(considering animal or human plasma data) would be expected (NIH publication 2001; Evans et al. 2001). Correlation of in vitro and in vivo data may provide a good rationale for using cytotoxicity assays as ranking tool and as a first step in hazard identification (Table 54.3). Combining primary hepatocytes from rat, dog, and man may allow the evaluation of cross-species differences and to estimate the relevance for human situation. The most sensitive evaluation timepoints for each assay (early response versus late response) should be determined prior to planned experiments. However, in many experimental settings, cultures are incubated for 6, 24, and 48 h as a single point or combined for kinetic determinations. To test the cytotoxicity or other potential effects of test compounds on the cell physiology several concentrations are tested, covering a concentration range of $\leq$1 μM to 1,000 μM. It is advisable to use six technical replicates per test concentration. The scheme in Fig. 54.2 represents a plate setup for such analysis.

To assess compounds for their cytotoxicity, basically any assay that can record cell perturbation could be used (Barile 1994; O'Hare and Atterwill 1995). However, a portfolio of physiological end points has been proved to be very useful (Ehrich and Sharova 2000). They have been tested thoroughly according to strict quality guidelines and regarding their performance, for example, reproducibility, sensitivity, organ expression and homogeneity, and of course ease of use. Some of physiological parameters that are used as markers of toxicity are described here (Fig. 54.3).

54.1.4.2 ATP Content, Measure of Energy Status

The catabolic pathways for the breakdown of glucose to produce energy in the form of ATP (adenosine triphosphate) and the replication process of DNA are very similar in all organisms. Consequently, ATP has been proved as a marker for cell viability because it is present in all metabolically active cells. The ATP concentration declines rapidly when cells undergo necrosis or apoptosis. The total intracellular ATP is assessed through an assay that is based on the generation of a luminiscent signal proportional to the amount of ATP present in the supernatant after cell lysis. The glow-type luminiscent signal is produced by a luciferase reaction and is detected in a device equipped with a luminescent signal detector (CellTiterGlo™ Luminescent Cell Viability Assay, Promega No. G7571).

In this assay, treated cells are lysed, which results in ATP release from cells. ATP present in the supernatant after cell lysis is then measured by a luminescent reaction. The quantified ATP is proportional to the viable

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
C	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
D	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
E	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
F	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
G	Blank	N	C8	C7	C6	C5	C4	C3	C2	C1	P	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Fig. 54.2 Recommended plate setup for cytotoxicity assay in 96-well plates. Blank indicates wells containing culture medium without cells, N indicates negative control reference compound, P indicates positive reference compound, C represents different concentration of test compounds. According to this assay design 8 concentration of test compound in six technical replicates can be tested

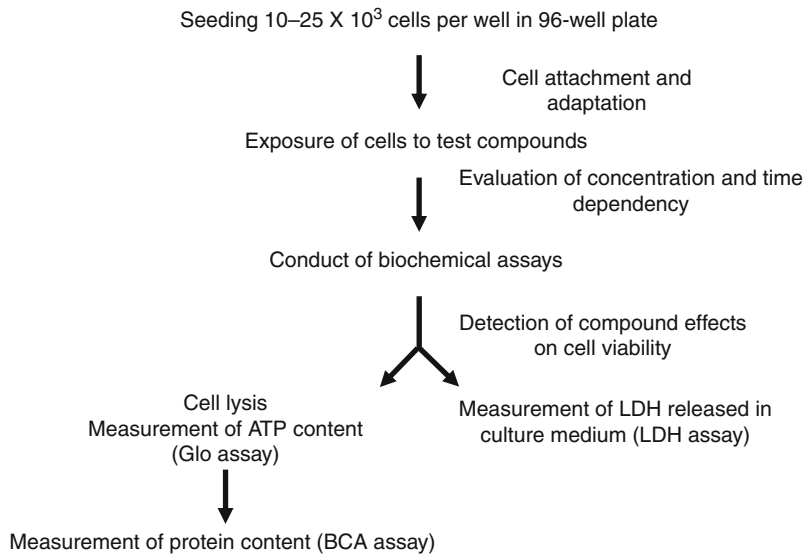

Fig. 54.3 Sequential approach for the determination of cell viability applying different physiological end points

cell numbers. In Fig. 54.4 the response of some therapeutic drugs in rat primary hepatocytes was assessed measuring the ATP content.

54.1.4.3 LDH Release, Measure of Membrane Integrity

Cell membranes protect cells from environmental insults. Therefore, their integrity is fundamental for the cell survival. Toxins can impair membrane function and integrity leading to leakage of intercellular components to the surrounding medium. Measuring leakage of components from the cytoplasm into the surrounding culture medium has been widely accepted as a valid method to estimate the number of nonviable cells. The enzyme lactate dehydrogenase (LDH-L) is expressed ubiquitously and is present in many tissues particularly in heart, liver, muscle, and kidney. This makes LDH a valuable marker for health status of cells (Fig. 54.5).

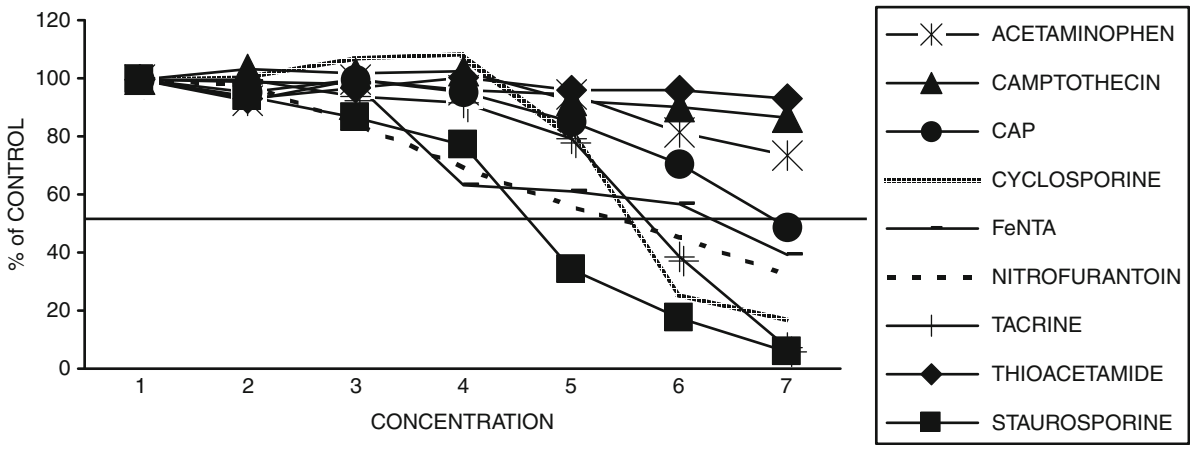

Fig. 54.4 The dose-response curve of therapeutics in rat primary hepatocytes. Rat primary hepatocytes were treated for 24 h at different concentrations and their response assessed measuring the ATP content. The toxicity potential varies among the tested compounds

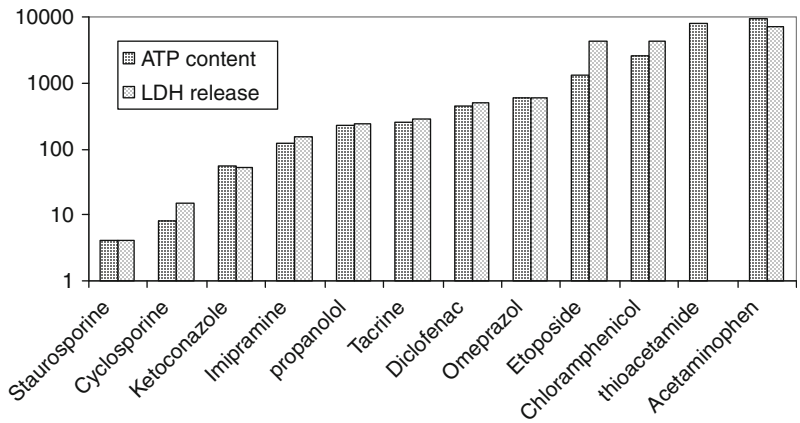

Fig. 54.5 Cytotoxicity of therapeutic drugs measured in rat primary hepatocytes. Data points are the TC50 (log scaled) of tested compounds at 24 h. Two physiological end points were tested, the ATP content of cells and the LDH release

Many colorimetric assays have been developed for the quantification of cell death and cell lysis based on the measurement of LDH activity (e.g., Roche No. 1 644 793). Principally, damaged cells leak LDH from cytosol into the culture medium, in which LDH activity ca be determined in an enzyme activity test: In the first step, NAD^+ is added to an aliquot of supernatant that is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt (yellow), which is reduced to red formazan. The amount of red color formed in the assay is proportional to the number of lysed cells. This assay, as a kit or the single components, can be purchased by many vendors.

54.1.4.4 Protein Content, Measure of Cell Viability

To maintain function and physiology, cells synthesize proteins according to their program and their differentiation status. Proteins are integral part of a cell and their content can be used as a measure of cell viability. Dead or impaired cells cannot maintain their protein stores leading to the reduction of total protein of a defined cell population. Protein assays are mainly detergent-compatible formulations based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total proteins. The BCA assay (Pierce) measure cell number based on the content of total protein. This assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an

alkaline medium (biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using the reagent BCA. The Pierce BCS assay is compatible with most ionic and nonionic detergents. To conduct the assay, 10–25 µl of cell lysate from the ATP assay is transferred into an separate well plate, 200 µl per well of the Working Reagent is added and incubated at 37°C for 30 min. The purple colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. The absorbance is measured at or near 562 nm on a plate reader.

The ATP, LDH, and BCA assays can be combined and performed in one single experiment (Figs. 54.3). Availability of data from three different physiological end points strengthens data accuracy and judgment of cytotoxicity potential of compounds under investigation.

Lysosomal Neutral Red Content (NRU), Measure of Lysosomal Integrity and Function

A measure of cell viability is the assessment of lysosomal function. In the Neutral Red Assay, the ability of intact lysosomes to store the dye neutral red is used to assess cytotoxicity. To measure neutral red retention, treated cells are incubated with a neutral red solution for 3 h (e.g., Merck No. 1.01376.0025). After incubation, the cells are washed twice and lysed subsequently. After complete lysis of the cells, the content of neutral red is determined in each well by measuring the absorption at 540 nm. Cells demonstrating less dye retention indicate lysosomal and cell damage due to treatment. The NRU assay can be adapted to high-through formats and may be used as substitute for LDH assay (Babich and Borenfreund 1992).

54.1.4.5 Detecting Reactive Oxygen Species (ROS) Indicative of Oxidative Stress

Excessive ROS production under pathological conditions results in oxidation of cellular macromolecules altering their functions. Therefore, methods are developed to detect oxidatively stressed cells. In this protocol, excessive intracellular levels of ROS are measured with the fluorescent dye marker 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Molecular Probes, Invitrogen), which is a fluorogenic indicator for reactive oxygen species in living cells. DCFDA is nonfluorescent until the acetate groups are removed by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to fluorescent dichlorofluorescein (DCF) with excitation/emission maxima of 495/529 nm.

H9c2 cells (or other cell types) are seeded into black 96-well plates containing DMEM medium at a density of 3×10^4 cells per well and incubated overnight. The overnight cultures are then stained with 20 µM H2DCFDA for 10 min in HBSS (Gibco, No.14025-092) before exposure to test compounds. Test compound dilutions are added and incubated for 0.5 h to several hours. It is wise to test several concentrations of the compound at µM range. At the end of the incubation time cells are washed two times with HBSS and reconstituted in the same buffer. The florescence signal is detected by fluorescence excitation/emission maxima of 495/529 nm in a plate reader. Quantified parameter levels are normalized to respective control conditions and mean fluorescent intensities are visualized. The graph in Fig. 54.6 demonstrates a significant increase in fluorescence intensity of DCFDA in H9c2 cells treated with the reference compound doxorubicin at two different treatment times.

54.1.5 Functional Assays

54.1.5.1 Drug-Induced Phospholipidosis

Phospholipidosis is a storage disorder induced by cationic amphiphilic drugs (CAD) and is characterized by accumulation of phospholipids and drug in acidic lysosomes. This accumulation results in unique histological effects in cells of impaired organs observed as electron-dense membranous lamellar inclusions or whorls in electron micrographs. More than 50 marketed therapeutic drugs are reported to induce phospholipidosis (Table 54.4) in a variety of organs mostly lung, liver, and macrophages. The conventional method for assessing phospholipidosis involves animal studies and histological evaluation of lamellar bodies, the hallmark feature of phospholipidosis by electron microscopy. The toxicological significance of phospholipidosis is not understood in every detail. But development of phospholipidosis is a serious issue and can result in delay or discontinuation of development compounds. Therefore, phospholipidosis assays are developed that are applied early in drug development to monitor the potential of CAD drug candidates to induce phospholipidosis. A tiered

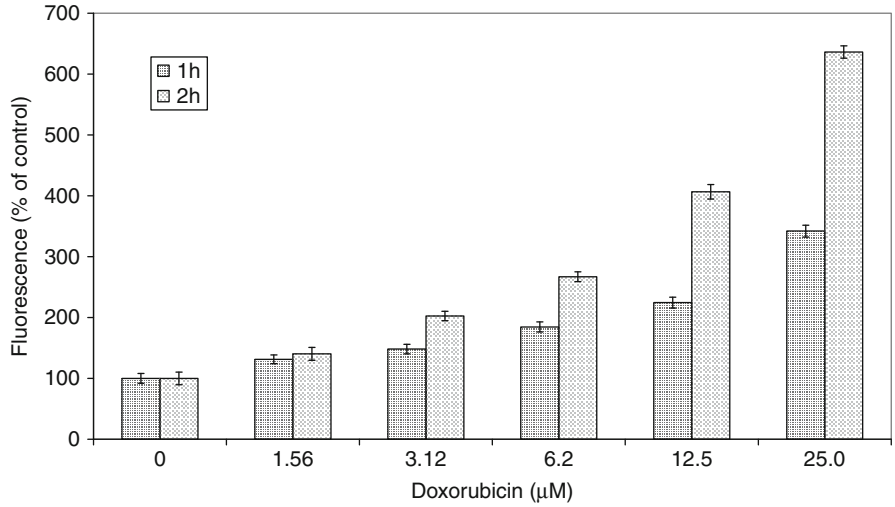

Fig. 54.6 ROS formation in H9c2 myocytes in response to doxorubicin treatment. Treatment of H9c2 cells with doxorubicin results in a significant increase in fluorescence intensity of DCFDA at both 1 h and 2 h indicating ROS production

Table 54.4 Phospholipidosis potential of reference compounds and the correlation of results from in silico, in vitro, and in vivo models

	Induction of phospholipidosis		
Reference compounds	In silico	In vitro	In vivo
Amiodarone	+	+	+
Chloroquine	+	+	+
Imipramine	+	+	+
Ketoconazole	+	+	+
Tamoxifen	+	+	+
GW532	+	+	+
Acetaminophen	–	–	–
Isoniazide	–	–	–
Ciprofibrate	–	–	–
Phenobarbital	–	–	–
Fenofibrate	–	–	–
Cyclosporin A	–	–	–
Doxorubicin	–	–	–

approach, combining in silico, in vitro, and in vivo approaches has been very successful in prediction of phospholipidosis. In general, in vitro phospholipidosis assays are carried out after in silico methods predict phospholipidosis liabilities. A positive outcome from in vitro experiments would trigger the third line experiments consisting of animal studies that provides ultimate data for a "Go or No Go" decision (Reasor and Kacew 2001; Monteith et al. 2006; Nioi et al. 2008; Nanoyama and Fukuda 2008).

For detection of drug-induced phospholipidosis several in vitro models have been described using different cell types and detection modes. But despite the diversity in assay design, all these in vitro phospholipidosis assays use mainly fluorescent dyes, for example, Nile red, or fluorescent phospholipid analogues, for example, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine phosphatidyl choline (NBD-PC) to flag lysosomal phospholipid accumulation. The method described here uses human hepatoma cell line HepG2 in combination with a fluorescent-labeled phospholipid analogue provided by LipidTOX™ phospholipidosis Kit (Invitrogen). This method is qualified for routine screening with an acceptable degree of reproducibility and easiness (Tomizawa et al. 2006; Nioi et al. 2007; Bhandari et al. 2008; Miyamoto et al. 2009).

54.1.5.2 Assay Design

HepG2 cells are seeded into collagen I-coated black 96-well micro plates at 3×10^4 cells per well for 24 h and 1×10^4 cells per well for 48 h in DMEM medium. The test compounds are dissolved in DMSO or other solvents (the solvent concentration should not exceed 0.5%) and added to culture medium with 1x LipidTox™ Red phospholipidosis detection reagents (Invitrogen). Based on cytotoxicity data, a broad range of nontoxic concentrations should be selected and tested. Six technical replicates are recommended per test concentration to fix the bias.

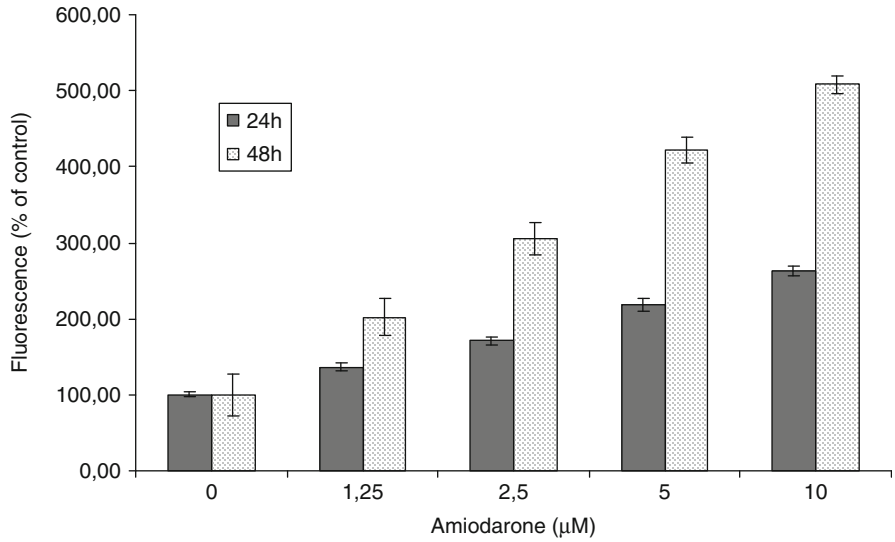

Fig. 54.7 Induction of phospholipidosis in HepG2 cells in response to amiodarone exposure and after different incubation periods. The selected concentration range was devoid of cytotoxicity

Table 54.5 Compounds that are known to induce cholestasis in vivo with their correlation to in vitro cholestasis model. Salisylic acid at concentrations up to 1 mM has no effects on Bsep transporter

	Induction of cholestasis	
Reference compounds	In vitro	In vivo
Cyclosporin A	+	+
β-estradiol-17β-D-glucoronide	+	+
Troglitazon	+	+
Erythromycin estolate	+	+
Salisylic acid	–	–

Cells are exposed to the test compound concentrations for either 24 or 48 h. After incubation, the cells are fixed with 4% formaldehyde for 30 min. Following fixation, cells are washed with HBSS (transparent, with $CaCl_2$ and $MgCl_2$) and measured in HBSS by fluorescence excitation/emission maxima 595/615 nm in a plate reader. Results can be expressed as the percent of phospholipid accumulation. Quantified parameter levels should be normalized to respective control conditions. Amiodarone in a concentration range of 1–10 µM and acetaminophen in a concentration range of 10–100 µM can be used as positive and negative reference compounds, respectively. The graph in Fig. 54.7 represents the potential of amiodarone to induce phospholipidosis. To assess the cytotoxicity aliquots of treated culture medium can be assayed for LDH release, or cytotoxicity assays with multiple end points can be conducted in parallel.

54.1.6 Drug-Induced Cholestasis

Cholestasis is one of the most manifestations of drug-induced liver injury. The drugs responsible for cholestasis (Table 54.5) are excreted by hepatocytes into bile where they interact with bile transporters and impair their function. Several forms of cholestatic liver injury can be produced by therapeutic drugs. The inhibition of bile acid efflux transporters Bsep (bile salt efflux pump) and Mrp2 (multidrug resistance–associated protein 2) seems to be an important mechanism in producing cholestatic liver injury. Inhibition of Bsep and Mrp2 may lead to reduced bile salt secretion and hence their retention within hepatocytes, which can lead to worse liver injury. The diagnosis of drug-induced cholestasis is poor, and therefore in vitro assays may help to sort out early in development the toxic agents (Pauli-Magnus et al. 2010; Padda et al. 2011; Yashikado et al. 2011). Furthermore, using hepatocytes form different species may allow cross-species comparison and at the end the relevance of findings for clinics.

The described model here is based on the sandwich cultured primary hepatocytes that allow the formation

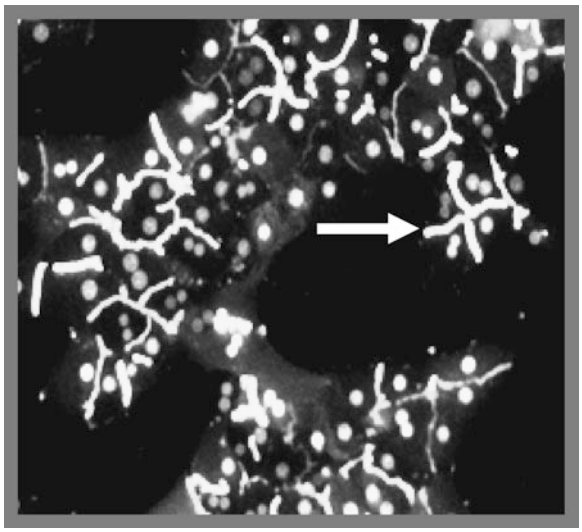

Fig. 54.8 Formation of canaliculi domains by rat hepatocytes in sandwich culture. The *arrow* indicates canaliculi domains

of canaliculi. Once the canaliculi are formed, cells are exposed to fluorescent-labeled bile salt analogue cholyl-lysyl-fluorescein (CLF; Solivas, Switzerland) that mimics cholylglycine and is therefore, a substrate for bile salt transporter Bsep. The intensity of accumulated CLF in canaliculi can then be detected in a spectralphotometer at 490 nm (Mills et al. 1991; Hopwood et al. 2006; Wolf et al. 2008; Min Dong, personal communication).

54.1.6.1 Assay Design

For this cholestasis model, freshly isolated hepatocytes in sandwich culture are used. Applying the protocol described above enables isolation of required primary cells for this protocol. Isolates from different animal models as well as human donors can be used. The monolayer cultures of hepatocytes do not form intact canaliculi. Therefore, a matrigel-based sandwich culture is used that allows the development of canaliculi domains (Fig. 54.8) capable of bile salt efflux. Cell preparations with the viability of $\geq$80% are seeded out on Collagen I coated 6-well plates in Attachment Medium with a density of 1×10^5 cells per cm^2. After hepatocytes are incubated (37°C, 5% CO_2) 1.5 h for attachment, subsequently the cells are washed with ice cold PBS to remove nonattached cells. Matrigel is diluted 1:20 v/v% in cultivation medium (ice cold) and 2.5 mL of diluted solution is put with a precooled pipette on top of the cells (6-well: 2,5 mL of 250 μg/mL). Culture plates are put back to incubator and the medium is changed every 24 h. After 2–4 days canaliculi domains are structured (examine canaliculi formation by light microscopy). When canaliculi are formed, the culture medium is discarded and cells are washed two times with 37°C HBSS. Cells are then incubated in prewarmed HBSS for 10 min at 37°C. After this short incubation, HBSS is removed and treatment medium is added (5 μM CLF and Hoechst in HBSS, plus reference or test compound at different concentrations). Treated cultures are incubated for 25 min at 37°C. At the end of the treatment period, aliquots of supernatant are sampled for LDH cytotoxicity assay. In the next step, wells are rinsed four times with HBSS to remove any remaining medium or substrate and HBSS is added. At the end, the fluorescent intensity is measured using FITC channel and signals are quantified. After the measurement, cells may be lysed and ATP, LDH, and protein assays conducted.

54.1.7 Methods to Detect Mitochondrial Dysfunction

Mitochondrial dysfunction is increasingly implicated in the etiology of drug-induced toxicities. Mitochondria are the energy powerhouses of cells and produce approximately more than 90% of the cell's energy in the form of ATP (adenosine triphosphate). Mitochondrial injury can occur through several mechanisms including disturbance of mitochondrial membrane potential, induction of mitochondrial membrane transition pore, and inhibition of respiratory chain complexes leading to uncoupling of electron transport and inhibition of ATP production. Due to these serious implications, the investigation of drug-induced mitochondrial dysfunction is receiving increasing attention (Dykens and Will 2007). Therefore, in pharmaceutical companies, a comprehensive set of assays have been developed that help recognize mitotoxic compounds early (Hynes et al. 2009). These assays also provide information on mechanisms of mitochondrial impairments late in more advanced projects. In this part, a set of developed methods that are thought to detect mitochondrial toxicities are described.

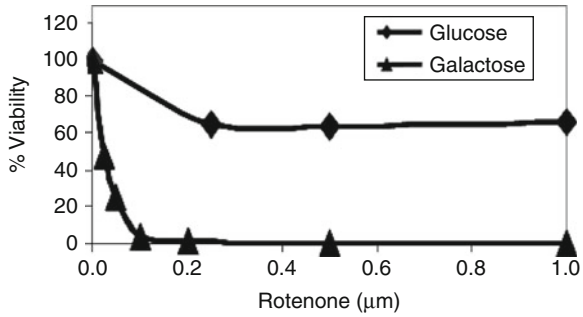

Fig. 54.9 Susceptibility of HepG2 cells grown on high glucose (25 mM) versus cells grown on galactose (10 mM). HepG2 cells grown in galactose medium increase their respiration rate to maintain their ATP level. Therefore, cells oxidizing galactose are more susceptible to mitochondrial poisoning. This is indicated in this graph in the different response of galactose-adapted cells to rotenone (TC50gal = < 0.025 μM) compared to glucose-adapted cells (TC50glu = 16.9 μM)

54.1.7.1 Galactose-Adapted HepG2 Model to Assess Mitochondrial Toxicants in Screening Mode

Many immortalized cells lines are metabolically adapted for rapid growth under hypoxic and acidic conditions, and they derive almost all of their energy from glycolysis rather than via mitochondrial oxidative phosphorylation (a circumstance known as the crabtree effect; Rodrguez-Enrquez et al. 2001). Such adapted cells are resistant to xenobiotics that impair mitochondrial function. Thus, the immortalized cell lines cannot be appropriately used in screening assays of mitochondrial toxicity. To increase detection of drug-induced mitochondrial effects in a preclinical cell-based assay, HepG2 cells are forced to rely on mitochondrial oxidative phosphorylation rather than glycolysis by substituting galactose for glucose in the growth media. Oxygen consumption doubles in galactose-grown HepG2 cells and their susceptibility to canonical mitochondrial toxicants correspondingly increases (Marroquin et al. 2007). As such, these cells are better qualified for assessments of drug-induced mitochondrial dysfunction and can be used in a high-throughput mode for a first-line screening of compound to reveal mitochondrial dysfunction.

In order to adapt HepG2 cells to the galactose supplemented culture medium, the cells are first cultured in high-glucose DMEM medium (for constituents see above). In the next step, the high glucose medium is gradually replaced first by low-glucose medium ([DMEM, Invitrogen No. 11966-025] supplemented with 5.5 mM glucose, 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate, and pen-strep as above) and then by galactose medium ([DMEM free of glucose, Invitrogen N0.11966-025] supplemented with 10 mM galactose, 2 mM glutamine [6 mM], 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate, and pen-strep as above). The adaptation period to each of the media needs to be assessed based on cell morphology and appearance.

The HepG2 cells adapted in this way can be combined either with cytotoxicity assays described above to unravel the primary cause of cytotoxicity, or they can be subjected to the oxygen consumption assay in combination with MitoXpress probe (Luxcel Biosciences) that is detailed bellow. The graph in Fig. 54.9 shows the response of galactose-adapted HepG2 cells compared to glucose-adapted cells measured in ATP cytotoxicity assay.

54.1.7.2 Real-Time Image-Based Detection of Mitochondrial Membrane Potential in Living Cells

The major function of mitochondria is concerned with ATP synthesis. The ATP production relies on the translocation of electrons across the inner mitochondrial membrane that is directed by respiratory chain complexes. The capacity of mitochondria to produce ATP can be assessed directly through measure of the rate of ATP formation and its efficiency (See ATP assay above) or indirectly by measure of the mitochondrial membrane potential.

For the measurement of mitochondrial membrane potential in living cells, the fluorescence-based specific mitochondrial dye marker tetramethyl rhodamine (TMRM, excitation/emission 550/575 nm) can be utilized combined with a confocal real-time single-cell imaging system (Nieminen et al. 1995; Scaduto and Grotyohann 1999; Heiskanen et al. 1999; Gogvadze et al. 2004). In the process, rat primary hepatocytes (also other cell types) are seeded into 96-well culture plates at 25×10^3 cells per well. The cells are allowed to grow and equilibrate for 24 h prior to being exposed to the test compounds. On the following day, test material is prepared and applied to the cells across a wide concentration range. It is recommended to include negative and positive controls. The equilibrated cells are allowed to incubate with the test compound for a defined period of time (30 min to 4 h) at 37°C. At the end of the treatment period cells are washed

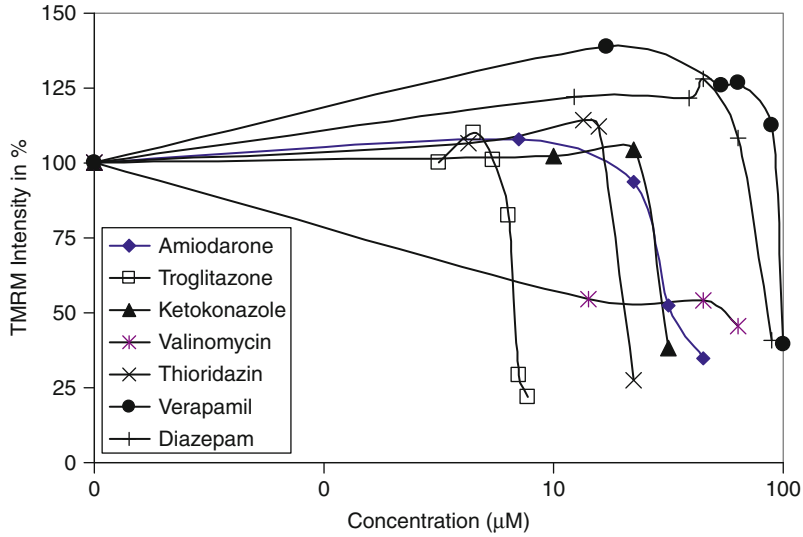

Fig. 54.10 Inhibitory effects of mitotoxic compounds amiodarone, troglitazone, ketoconazole, valinomycin, thioridazine, verapamil, and diazepam on mitochondrial membrane potential measured in rat primary hepatocytes with TMRM on an imaging system. Test compound concentrations are expressed in log scale

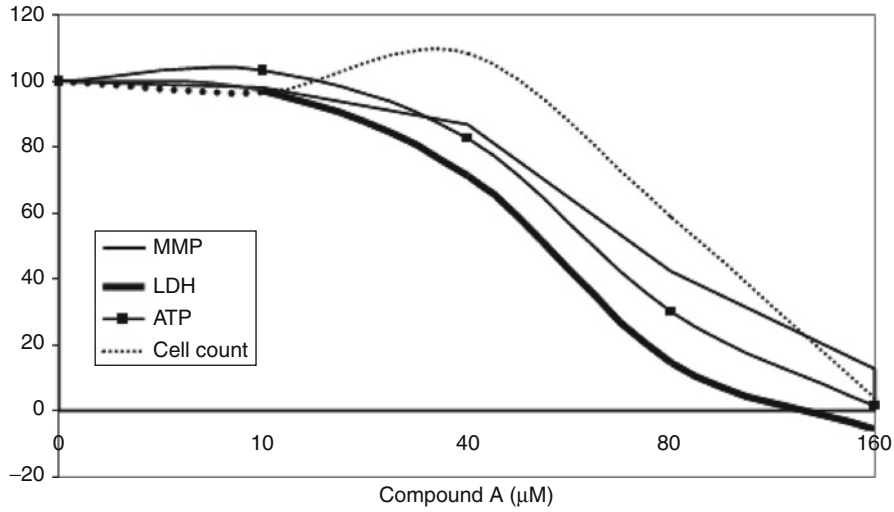

Fig. 54.11 Effects of a test compound (Compound A) on mitochondrial membrane potential (MMP) of primary rat hepatocytes exposed for 4 h. The mitochondrial membrane potential was assessed using the dye marker TMRM. The dose-dependent reduction of membrane potential is paralleled by reduction in ATP content in cells

with warmed HBSS and cultured in incubation buffer ([HBSS] supplemented with 5% heat inactivated FBS, 1% HEPES, 1% Probenecid [P36400, Invitrogen]) containing TMRM (0.5 µM) at 37°C for 15 min. Following the staining, the cells are washed and subjected to analysis either in kinetic mode or as end-point measurements. The change in mitochondrial membrane potential is assessed by quenching of the fluorescent. Signal intensities are recorded according to the manufacturer's instructions for the instrument used. Using confocal, real-time, and single-cell kinetic imaging system allows high-resolution imaging, fast and reliable quantification of effects in cells. This method can be used for quantification of mitochondrial effects in primary hepatocytes from rat, human, rabbit, and dog, as well as cell lines HepG2 and H9c2. Figure 54.10 shows the inhibitory effect of a couple of reference therapeutics that was measured on rat primary hepatocytes.

By combining this assay with membrane leakage marker LDH, cell count, and ATP content assay that are conducted in parallel to MMP analysis it is possible to evaluate the achieved data and more important get more insight into the physiological sequence of events that primarily harm the subcellular targets (Fig. 54.11). As is indicated in the diagram, LDH release occurs prior to the reduction of MMP. Furthermore, reduction in MMP is observed at concentrations where cell count

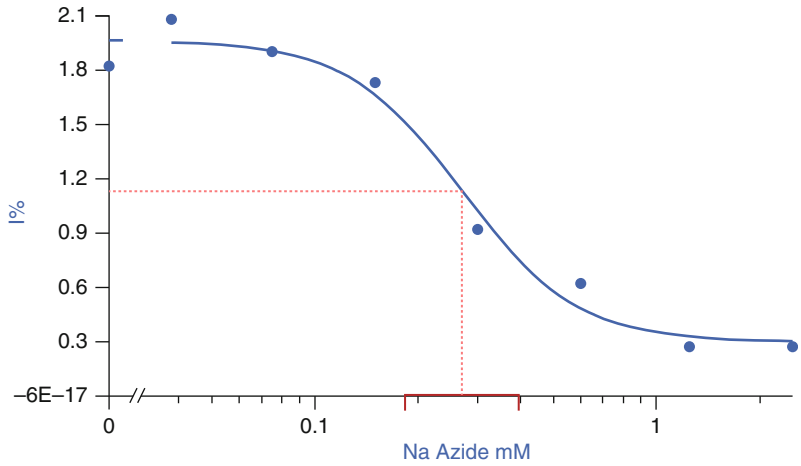

Fig. 54.12 Inhibitory effect of Na Azide on Complex IV activity. To achieve a complete dose-response curve 50 µg rat liver mitochondria were exposed to Na Azide at concentrations ranging from 0.01 to 15 mM resulting in the IC50 = 0.25 mM

goes down. These observations together indicate that reduction in MMP and ATP content may not be the primarily event that harm the cells.

54.1.7.3 Inhibition of Respiratory Chain Complexes

(Methods: Immunocapture-based respiratory chain activity assays)

The oxidative phosphorylation process in mitochondria that ends up with ATP synthesis is carried out by the respiratory chain complexes. The free energy released by oxidation of carbon hydrates is used to build NADH (nicotineamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotides), which in turn, donate electrons to Complex I and II, respectively. The translocated electrons are then passed to complex III and complex IV, and the energy that is released during the electron transport is used to build a chemiosmotic gradient across the inner mitochondrial membrane. This gradient is used by complex V for ATP synthesis.

Because of the complexity of the respiratory chain complexes, they are often the target of drugs. A set of immunocapture-based assays have been developed to identify enzyme complexes, whose inhibition is directly responsible for any observed toxicity. In three assays (for complexes I, IV, and V), a 96-well plate coated with a monoclonal antibody raised against one of the complexes is used to immunocapture a functionally active complex from small amounts of isolated mitochondria. Mitochondria are isolated either from animal tissues (e.g., rat liver or heart), or from cell cultures. Meanwhile, a global and specialized line of industry has been established that offers besides ready-to-go mitochondrial assays also high quality purified mitochondria from different sources (e.g., MitoExpress). To ensure the specificity of these complex activity assays, their respective classical inhibitors rotenone (complex I), KCN (complex IV), and oligomycin (complex V) are used as positive controls. All three activity assays allow for rapid screening of drugs and provides a low cost method of identifying mitochondrial toxicity in vitro. In Fig. 54.12, the inhibitory effect of Na Azide on complex IV was evaluated using the complex I assay.

54.1.7.4 Assays to Monitor Mitochondrial Oxygen Consumption

The uncoupling of oxidative phosphorylation modulates the rate of oxygen consumption. Oxygen consumption is also affected by the inhibition of either citric acid cycle or the β–oxidation of fatty acids. Molecular oxygen is a key substrate of all aerobic organisms and the terminal acceptor of the electron transport chain. Therefore, analyzing mitochondrial oxygen consumption is one of the most informative ways of assessing mitochondrial dysfunction. The electron transport chain (ETC), ATPase, and adenine-nucleotide translocator (ANT) inhibitors decrease ADP-activated respiration while uncouplers induce an increase in basal respiration (Hynes et al. 2009; Will et al. 2006).

The two most applied methods for measuring oxygen consumption are the polarography using the

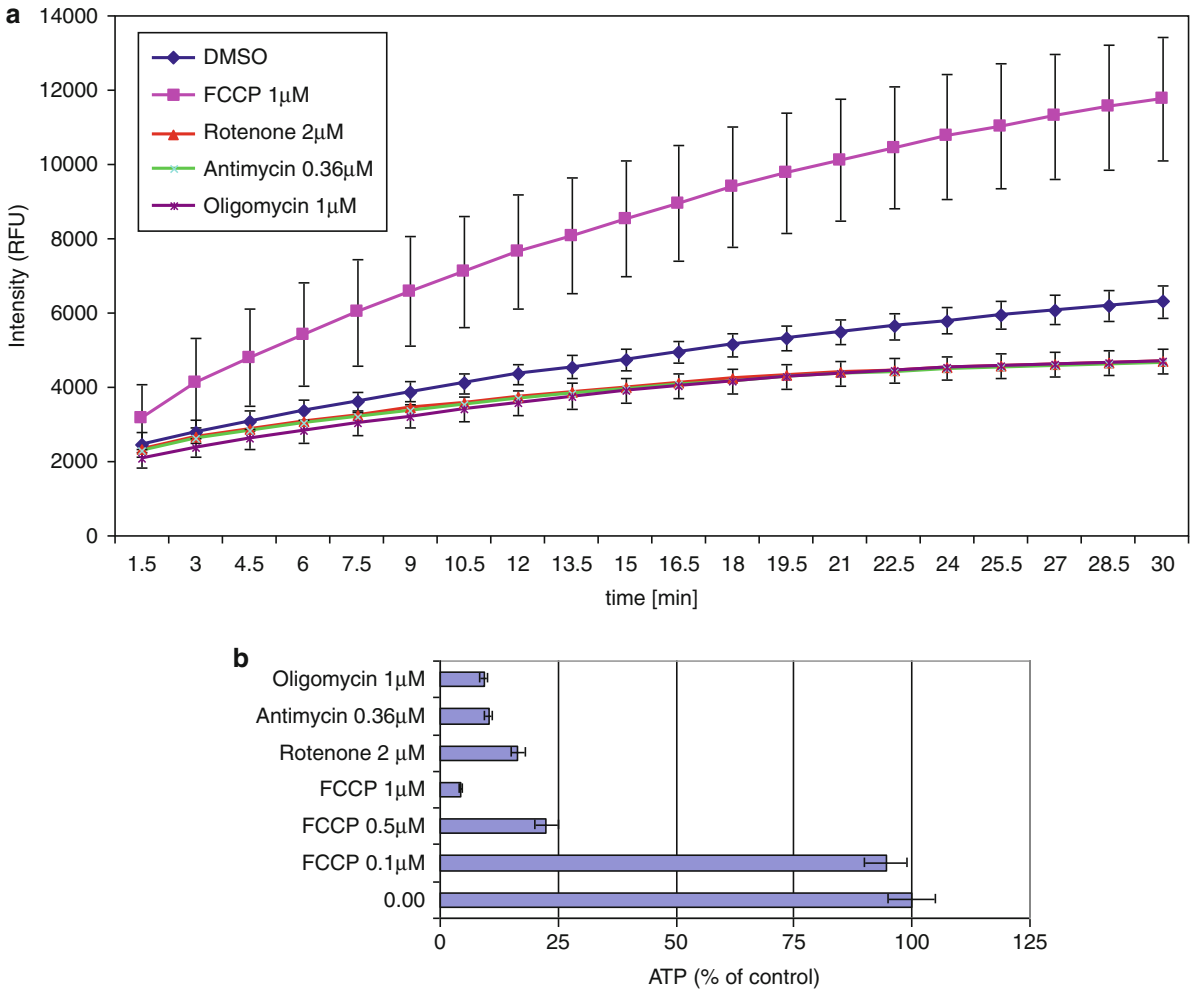

Fig. 54.13 (**a**) The effect of a panel of classical electron-transport chain inhibitors on O_2 consumption of primary rat hepatocytes using MitoXpress probe (Luxcel Biosciences), and (**b**) total ATP concentration at same indicated concentrations. Figure 54.3a illustrates treatment-related mitochondrial dysfunction with classical electron-transport inhibitors rotenone (inhibits complex I; nicotinamide adenine dinucleotide hydrogen (NADH)-ubiquinone oxidoreductase), antimycin (inhibits complex III; cytochrome c reductase), oligomycin (inhibits F1/F0 ATPase), and the uncoupler FCCP. DMSO was analyzed as control producing the base line respiration rate. While the inhibition causes a reduction in oxygen consumption (above the DMSO baseline), uncoupling increases the oxygen consumption rate (bellow the DMSO baseline). Analysis of ATP concentrations immediately posttreatment indicated dose-dependent drop in ATP production. The 0.00 bar in graph (**b**) indicates the ATP level in solvent-treated control cells

OROBOROS Oxygraph for high-resolution respirometry and the phosphorescent oxygen probe MitoXpress (Luxcel Biosciences) a 96-well-based assay.

The OROBOROS Oxygraph is used in cases where high resolution counts and the high throughput is not required, for example, measurement of oxygen consumption in living and permeabilized cells, small amounts of tissue that requires high-resolution respirometry to obtain accurate results even at high sample dilution. In contrast, the MitoXpress assay is used as a sensitive high-throughput detection assay in combination with either isolated mitochondria or cultured living cells (Luxcel Bioscience). Whereas in polarography an oxygen-sensitive electrode detects the modulation of oxygen, measurement of oxygen by MitoXpress is based on the ability of molecular oxygen to quench the excited state of the probe. As the mitochondria respire, oxygen is depleted, which is seen as an increase in probe

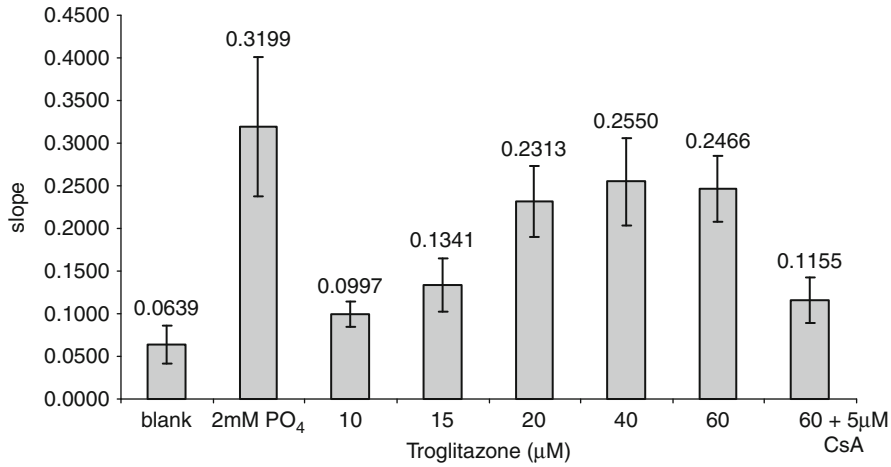

Fig. 54.14 Dose-dependent swelling of rat mitochondria. Treatment of mitochondria with troglitazone induce transition pore opening that in turn induce mitochondrial swelling. PO_4 was used as positive reference compound with maximum effect at 2 mM. Mitochondrial swelling can be inversed through incubation with cyclosporine A (CsA)

phosphorescence signal (Fig. 54.13). The protocol described here uses whole cells, but this assay can also be used with isolated mitochondria from different sources. To perform the assay, primary rat hepatocytes (or other cell types) are seeded in 96-well plates at 3×10^4 per well and incubated overnight. Next day, MitoXpress probe is reconstituted in 1 mL respiration buffer and diluted to 10.5 mL with the same buffer. Test compound dilution series are prepared in the appropriate solvent and added to the culture medium. All reagents and instruments are warmed to 30°C. The culture medium is removed and replaced by 150 µl fresh medium with or without test compound supplements. The reconstituted MitoXpress probe (10 µl) is added to each well and 100 µl of prewarmed heavy mineral oil (MP Biomedical cat. no. 150138) is quickly added to each well. At the end, the plate is inserted into the fluorescent reader preset at 1.5-min intervals over 30–60 min (Hynes et al. 2006, 2009). The recommended instrument settings for Tecan Genios reader are Gain: 65, Lag time: 100 µs, Integration: 300 µs, cycles: 20 (every 1.5 min), Filter: Exc. 370 nm, and Em.: 635 nm. Figure 54.13a is a representative of data output from oxygen consumption analysis assayed on primary rat hepatocytes.

54.1.7.5 Estimation of Mitochondrial Swelling (Opening of the Mitochondrial Permeability Transition Pore)

Mitochondrial permeability transition pore (MPTP) is a megachannel formed by the outer and inner mitochondrial membrane, composed of a number of protein complexes.

Binding of compounds to these protein complexes can lead to the opening of the channel promoting influx of solutants into the mitochondrial matrix. Consequently, mitochondria swell and lose their ability to produce ATP (Blattner et al. 2001) Therefore, swelling is one of the most reliable manifestations of mitochondrial permeability transition that can be utilized to assess the affinity of compounds to bind the mitochondrial megachannel and induce its opening (Taya et al. 2005).

Here is a 96-well-based nephelometric technique described that fits for monitoring mitochondrial swelling in screening or investigative mode. In this assay, mitochondrial swelling can be monitored continuously as changes in OD540 (Zhao et al. 2004; Taya et al. 2005). To start the assay, isolated mitochondria (0.1 mg) are added to 0.2 mL of incubation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 7.5 mM succinate, plus 3 µM Rotenone) in a 96-well plate with flat bottom. In the next step KH_2PO_4 (2 mM final, as positive reference control) or concentrations of test compounds are added and the absorption at 540 nm is measured. These data are used as null point. At the end, $CaCl_2$ from a stock solution (the final concentration is 20 µM) is added and mixed immediately. The plate is inserted into a plate reader (e.g., Tecan genius pro) preset to 37°C and changes at OD540 are measured for 30 min with 2 min intervals (shake between measures). Swelling is measured by decrease in absorbance at 540 nm. Swelling assay can

be performed on isolated mitochondria from rat heart or liver, or mitochondrial from any other resources. The quality of mitochondrial preparation is critical to this assay. Therefore, mitochondrial isolation should be in the hand of skilled labor staff that operates with special attention and according to expert protocols. Alternatively, Mitochondria can be purchased by vendors, for example, MitoScience and Mitenyi Biotec. Figure 54.14 shows the dose-dependent swelling of rat mitochondria in response to treatment with troglitazone affecting mitochondrial permeability transition pore.

54.1.7.6 Isolation of Mitochondria from Rat Liver

The mitochondrial fraction is prepared according to Schneider and Hogeboom (1950) with some modifications. The protocol is a general method for the isolation of pure mitochondria from rat liver tissue. But it is possible to scale the protocol and use heart or brain tissue. Also tissues collected from other mammalian species such as rabbits and mice can be subject to isolation taking this protocol. The mitochondria collected in this way can be utilized for all studies of mitochondrial functions.

The liver is removed from an anesthetized rat according to the protocol described above. The liver tissue is transferred immediately in ice-cold MSH buffer (5 mM HEPES, 70 mM sucrose, 210 mM mannitol, 1 mM EDTA, pH 7.35 at 4°C). The liver tissue is freed from fat and connective tissue, and minced with scissors. The minced tissue is washed with MSH buffer several times until the wash solution is free of blood and clear. Now, per gram of liver 8 mL homogenization buffer ([MSH] supplemented with aprotinin [65 μg/mL], PMSF [1 mM], and Leupeptin [1 μM]) is added and the tissue is homogenized in a glass homogenizer with a teflon pestle using six up-and-down strokes at 500 rpm. The homogenate is divided and centrifuged at 4°C for 10 min at approximately 800 × g. The supernatant is collected and immediately centrifuged at 10,000 × g for 10 min. After centrifugation, the supernatant is carefully decanted and the pellet resuspended in 80 mL of chilled mitochondrial isolation buffer (homogenization buffer without EDTA). The mitochondrial suspension is centrifuged at 3,000 × g for 5 min. The supernatant containing the mitochondria is transferred into a new tube that is centrifuged at 10,000 × g for 10 min. The supernatant is discarded and the mitochondrial pellet resuspended in mitochondrial isolation buffer. At the end, this mitochondrial suspension is centrifuged once again at 9,000 × g for 10 min and the mitochondria pellet recovered in 1 mL of mitochondrial isolation buffer. The protein concentration of the isolate is measured and the yield calculated. The isolation generally results in 20–50 mg mitochondria depending on the tissue used. These mitochondria can be used directly for planned studies or stored at −80°C for later applications.

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OMICS Technologies

55

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55.1 Introduction

Traditionally, toxicologists define the risk of a new compound to human safety using animal models supported by histopathological and biochemical approaches. However, despite decades of experience, the limitations remain:

- The extrapolation dilemma is still challenging.
- The relevance of animal data to real life continues to be a controversial issue.
- Long-term exposure in humans remains unclear.

Therefore, there is a need for the development of novel test systems that can complement or yet replace the conventional experimental approach in toxicology and thus improve the pharmaceutical drug development process and at the end the quality of new drugs.

The genomics revolution of the recent years led to development of many new and innovative technologies that can change this paradigm and address uncertainty issues in the current toxicological practice and safety assessment. The foundations of the new era build the "OMICS" triads: toxicogenomics, toxicoproteomics, and metabonomics through the identification of novel key genes, marker proteins and metabolites, or gene, protein, and metabolite profiles. These new disciplines combine toxicology with global gene, protein, and metabolite alterations in response to toxic substances and put this knowledge into a toxicological context for a better risk calculation. Global analysis of genes, proteins, and metabolites in cell or tissues has been achieved using a set of different technology platforms such as DNA and protein microarrays (chips), two-dimensional gel electrophoresis (2DGE) combined with mass spectrometer, and liquid chromatography in connection to spectral analysis. The potential of "OMICS" platforms for a better prediction, biomarker identification, and mechanistic explanations of toxicity has been demonstrated in a panel of pilot and comparative studies. Whereas the "OMICS" technologies proved superior alternative to traditional toxicological approaches for biomarker identification or mechanistic investigation, the predictive potential remains difficult and unsatisfactory. "OMICS" data are very complex in volume and content and demand the support of other sciences, for example, bioinformatics, biostatistics, and regulatory, to collect and decipher the whole information. Further variations such as strain and genetic variations, dose, and duration are challenging and demand validation. In recent years, much effort has been made to standardize study designs, experimental procedures, and data processing. Nonetheless, the replacement of conventional in vivo animal studies by "OMICS" platforms is still dreams of the future. A big step forward is the regulatory acceptance that is beginning and would be considered case-by-case. The continued development and refinement of the new methods will alleviate further regulatory appreciation.

In this chapter, Ph. Hewitt and M. Kroger describe toxicogenomics and show examples of its application in toxicological investigations. M. Kabiri deals with toxicoproteomics and outlines the established and alternative methods of global protein analysis, and provides an example of utilization. A. Amberg gives an introduction into metabonomics and presents some details of this technology.

55.2 Toxicogenomics

In toxicology, a full range of genomics technologies are now being used in efforts to uncover the cellular and biochemical mechanisms at work in response to xenobiotic/toxin exposures. The development of these new technologies represents a great opportunity to elucidate toxicological responses to pharmaceuticals, and other chemicals, at a very early stage in drug development. Toxicogenomics (or "transcriptomics") is becoming a well-accepted technology to complement traditional toxicology methods. Since molecular changes occur prior to pathological outcomes, detection of disease and organ toxicity should be possible at earlier time-points during a pathological process. In addition, these technologies are highly sensitive, so that long-term toxic effects can potentially be detected at lower doses. This has the potential to greatly impact toxicology, and to help in the risk assessment of new drug entities. Toxicogenomics represents a desire to step outside the boundaries of traditional toxicology. It is based on the measurement of thousands of genes simultaneously and has shown potential to revolutionize toxicity testing. It has been successfully used as a tool to elucidate mechanisms of toxicity as well as having the potential to predict toxicities much earlier during drug development (Schena et al. 1995; Hamadeh et al. 2002; Ganter et al. 2008).

The advanced knowledge of gene and protein expression patterns, together with modern classification algorithms, has also demonstrated practical benefits for predicting pathological events and toxic end points (Waring et al. 2001; Steiner et al. 2004). Unfortunately, these early promises are only being realized after a period of relatively expensive and deliberate test validation and generation of large reference databases, which are still essential for the future of mechanism elucidation. Without adequate study design, appropriate use of controls, and multidisciplinary development of standardized methods, acceptance has been slow.

Applications can be divided into two broad and partly overlapping classes: investigative studies and predictive toxicology. Investigative studies may help to identify new molecular targets for toxicants or provide novel and deeper insights into mechanisms of action (Man et al. 2002; Ruepp et al. 2002; Fella et al. 2005; Hewitt et al. 2005). The belief that different groups or classes of compounds will induce specific molecules or expression patterns provides the basis for predictive toxicology. Such single markers or gene/protein patterns can have a high degree of predictive power (Elcombe et al. 2002; Li et al. 2002; Petricoin et al. 2002; Ellinger-Ziegelbauer et al. 2004; Boehme et al. 2011; Hrach et al. 2011). Currently, researchers try to set up databases with expression profiles derived from known toxins. These can in the future be used to screen novel compounds in the drug discovery and preclinical evaluation processes.

The full power of toxicogenomics has yet to be realized, and there are numerous platforms available on the market. Both global expression systems (whereby all genes in a given organism are examined simultaneously) and smaller applications (hypothesis-based selection of a small number of specific genes or verification of detected genes of interest observed in microarray analysis) are widely used for many different purposes. Most people involved agree that standardization of microarray experiment procedures and of genomic signatures are keys to the broad acceptance and use of these data. Most journals now only accept papers that have used the MIAME (minimum information about a microarray experiment) guidelines. In traditional toxicology, histopathological evaluation is the gold standard to understand toxicity. Therefore, organs are fixed in paraffin and embedded in paraffin to produce tissue sections for microscopic evaluation. A huge number of formalin-fixed and paraffin-embedded (FFPE) tissues are stored in the archives of toxicology departments, providing a valuable source of molecular biological information. The enormous disadvantage is the low quality of the RNA extracted from such tissue. Thus, special technologies are needed for genomic analyses. In the meanwhile, many companies provide such platforms, either based on microarrays, qPCR, or branched DNA (bDNA) technologies. However, tissue blocks are still not commonly used for molecular profiling in the field of toxicogenomics.

This chapter will be separated into different subjects based on different levels of expression profiling: global expression arrays and multiplexed expression profiling as well as future technologies – namely next generation sequencing. All of these technologies should complement a toxicogenomics study, and their use will be dependent upon the questions being asked.

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55.2.1 Total RNA Isolation

PURPOSE AND RATIONALE

For the investigation of expression changes due to xenobiotic/toxin exposure, total RNA must be extracted, whether from body fluids, tissues (including FFPE tissues), or cells. Bolton and McCarthy first published a method for RNA isolation in 1962 (Bolton and McCarthy 1962). Since then many extraction protocols have been developed and adapted to different sample types. All protocols follow the main objective to recover high yield, high quality RNA with as little contamination by DNA and protein as possible. Sample preparation has to be performed prior to any toxicogenomics technology used in investigative studies, and predictive toxicology. For all these approaches, high-quality total RNA (or mRNA) has to be extracted first.

PROCEDURE

Prior to total RNA extraction, sample lysis procedures have to be performed. Lysis conditions are very important for the success of the RNA extraction and depend strongly upon the sample used. Due to great diversity, the biological sample can be pulverized, homogenized, or otherwise disrupted to yield a mixture that contains cells, subcellular components, and other biological debris in an aqueous buffer or suspension. Here is described the protocol for the Trizol® method of RNA extraction.

Trizol® is a mono-phasic solution of phenol and guanidine isothiocyanate, maintains the integrity of the RNA, and is an improvement on the original single-step RNA isolation method described by Chomczynski and Sacchi (1987). After addition of chloroform, vigorous shaking for several minutes, and centrifugation, the RNA exclusively remains in the aqueous phase generated. RNA is recovered by precipitation with isopropyl alcohol, after incubation, and centrifugation at 12,000 *g*. The isolated RNA is then washed with 75% ethanol, and centrifuged at a lower speed. This method facilitates isolation of a variety of RNA species, both of small and large molecular size.

The resulting cleaned RNA pellet should be briefly dried (air-dry, but not to complete dryness) before redissolving in either RNase-free water, or an appropriate buffer. To remove any remaining DNA, a DNase digestion after RNA recovery is highly recommended.

EVALUATION

After sample preparation, total RNA yield can be measured by optical density. Several methods are available. Typically OD at both 260 and 280 nm gives an indication of RNA purity and quantity; thus, the ratio of OD 260/280 should be close to 2. The integrity of RNA can be checked by gel electrophoresis. A convenient platform is the Agilent 2100 Bioanalyzer which is based on capillary gel electrophoresis (Liu et al. 2003). Strong 28 S and 18 S bands or peaks should be visible to indicate high quality RNA. This will most likely not be the case for RNA extracted from FFPE tissues. An additional parameter reflecting RNA quality is the RNA integrity (RIN) introduced by Schroeder et al. (2006). The integrity is lower with many short RNA fragments, with 18 S and 28 S peaks often not being present.

CRITICAL ASSESSMENT

Since differential expression analysis means to compare the quantities of RNA species in two samples, every step during sample preparation has to be highly reproducible. In order to maximize reproducibility, complete total RNA extraction in a one step procedure is recommended. Care must always be taken when

working with RNA, to avoid contamination with RNases, which may result in RNA degradation.

MODIFICATIONS OF THE METHOD

RNA extraction using the relatively toxic Trizol® can also be substituted by numerous other technologies available. Many vendors provide ready-to-use extraction kits including column-based extraction methods. Some kits already include the DNase digestion step, either on column or in solution.

When extracting RNA from FFPE tissues an additional proteinase K digestion step is required to release RNA (Jiang et al. 1995). Due to the "extreme" cross-linking of RNA to proteins initiated by formalin, RNA cannot easily be extracted using common methods. The quality of such FFPE RNA is dependent on several parameters, including warm ischemia time, duration of fixation, embedding process, and blockage.

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55.2.2 Global Expression Profiling

The determination of gene expression changes due to toxic insult has become the area of intense research. These changes can be monitored by comparing the level of mRNA for each gene of interest in control and treated tissues/cells/fluids, etc. More recently, it has often been shown that global expression profiling can give hints to molecular changes that lead to overt toxicity (Ellinger-Ziegelbauer et al. 2011).

Global gene expression profiling can be exploited to clarify mechanism of toxicity but also to identify marker genes for the prediction of certain toxicities (Boehme et al. 2009; Boehme et al. 2011; Hrach et al. 2011). These biomarker "fingerprints" can be utilized in later studies without running further genome-wide analyses (Fig. 55.1).

55.2.2.1 Affymetrix GeneChip

PURPOSE AND RATIONALE

There are multiple platforms available that allow one to look at the gene expression of all known genes in a given organism. The aim of this chapter is not to cover all of them, but to give an overview of one such method, the Affymetrix GeneChip®. Affymetrix can be considered to be one of the market leaders in such classical microarray technologies. Correspondingly, it is a highly reproducible, robust system and is based on single-color analysis (Shi et al. 2006). Each transcript is represented by eleven 25-mer probe pairs, with both match and mismatch probes (whereby the central nucleotide is changed). By comparing whole genome expression changes, we have an objective and hypothesis-free method to gain better understanding of the relationship between toxicity and gene expression. Currently, there are several genome-wide arrays commercially available from Affymetrix, covering more than 30 different organisms.

PROCEDURE

The following paragraph describes the sample processing using the GeneChip® 3′ IVT Express Kit generating cRNA and hybridization onto GeneChip 3′ expression arrays. In a reverse transcription reaction, cDNA is synthesized from total RNA or mRNA using reverse transcriptase and a T7 Oligo (dT) primer. Double-stranded cDNA is subsequently synthesized in a reaction mix containing DNA polymerase and RNase H. The cDNA is then used as a template for in vitro transcription using biotinylated nucleotides to produce biotin-labeled amplified cRNA (User Manual, Affymetrix).

The purified material is then assessed for yield, purity, and integrity by spectrophotometric and Agilent Bioanalyzer analyses. Fragmented (35–200 bases) in vitro transcripts (cRNAs) are generated and purified before hybridizing overnight together with

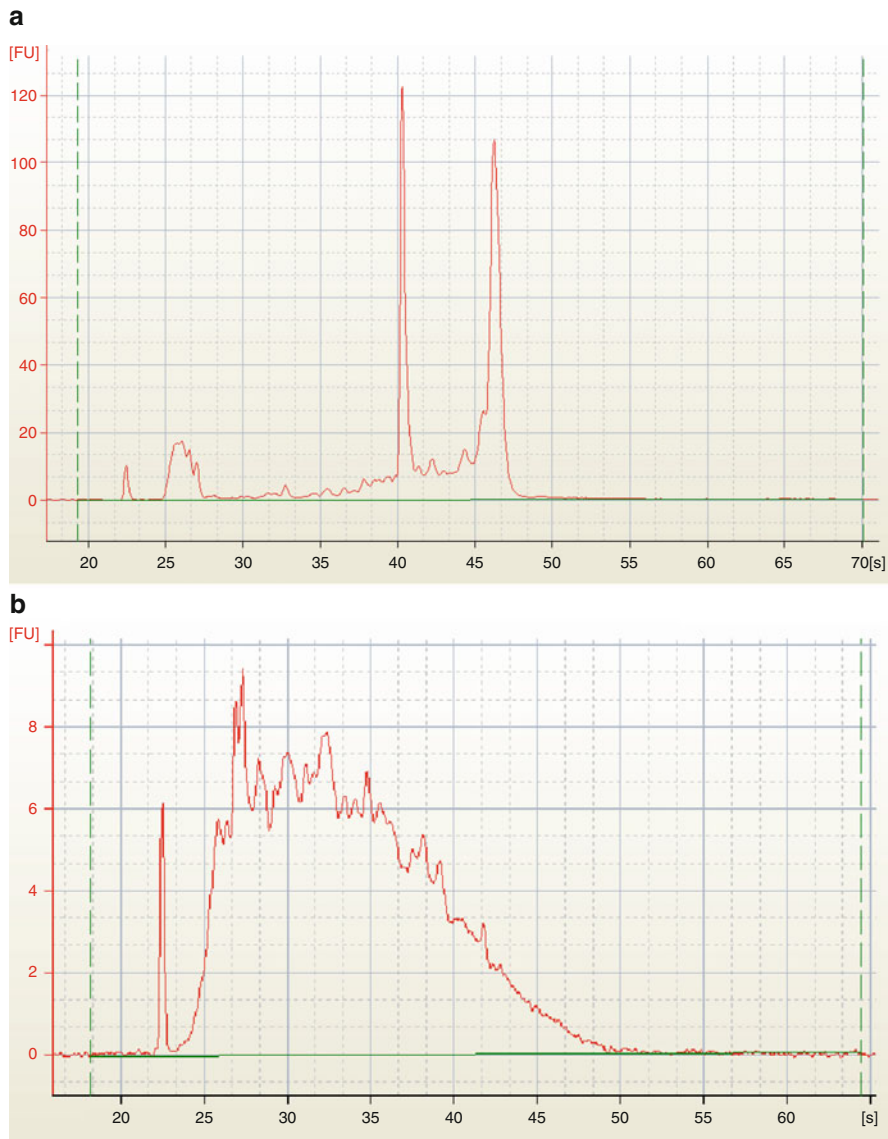

Fig. 55.1 Typical Bioanalyser spectra. Showing two distinct peaks for 18 S and 28 S RNA in high quality samples (**a**) and a trace of degraded RNA derived from FFPE tissues (**b**)

controls onto the Affymetrix GeneChip® (e.g., the Rat Expression 2.0 array contains approximately 31,000 rat-specific probe sets). The hybridized samples are stained with streptavidin-R-phycoerythrin (SAPE) and the signal is amplified using a biotinylated antibody, followed by a final staining. Washing, staining, and amplification are carried out using the manufacturer's fluidics station. The arrays are scanned using the manufacturer's fluorescent scanner (Fig. 55.2a).

EVALUATION

Normalization and scaling of the expression data across arrays can be performed based on a set of maintenance genes included on most Affymetrix arrays The raw data is firstly quality checked and transformed into expression values whereas different algorithms can be used, for example, MAS5.0 from Affymetrix or RMA. These algorithms include background correction, normalization, and summarization of the data. To identify differentially regulated genes, for example, in comparison to a control group, a threshold value of twofold is commonly applied. To aid data interpretation, it is essential that statistical analysis, false discovery rate, t-test or n-way Anova are also included. Expressionist® Refiner and Expressionist® Analyst from Genedata or GeneSpring GX

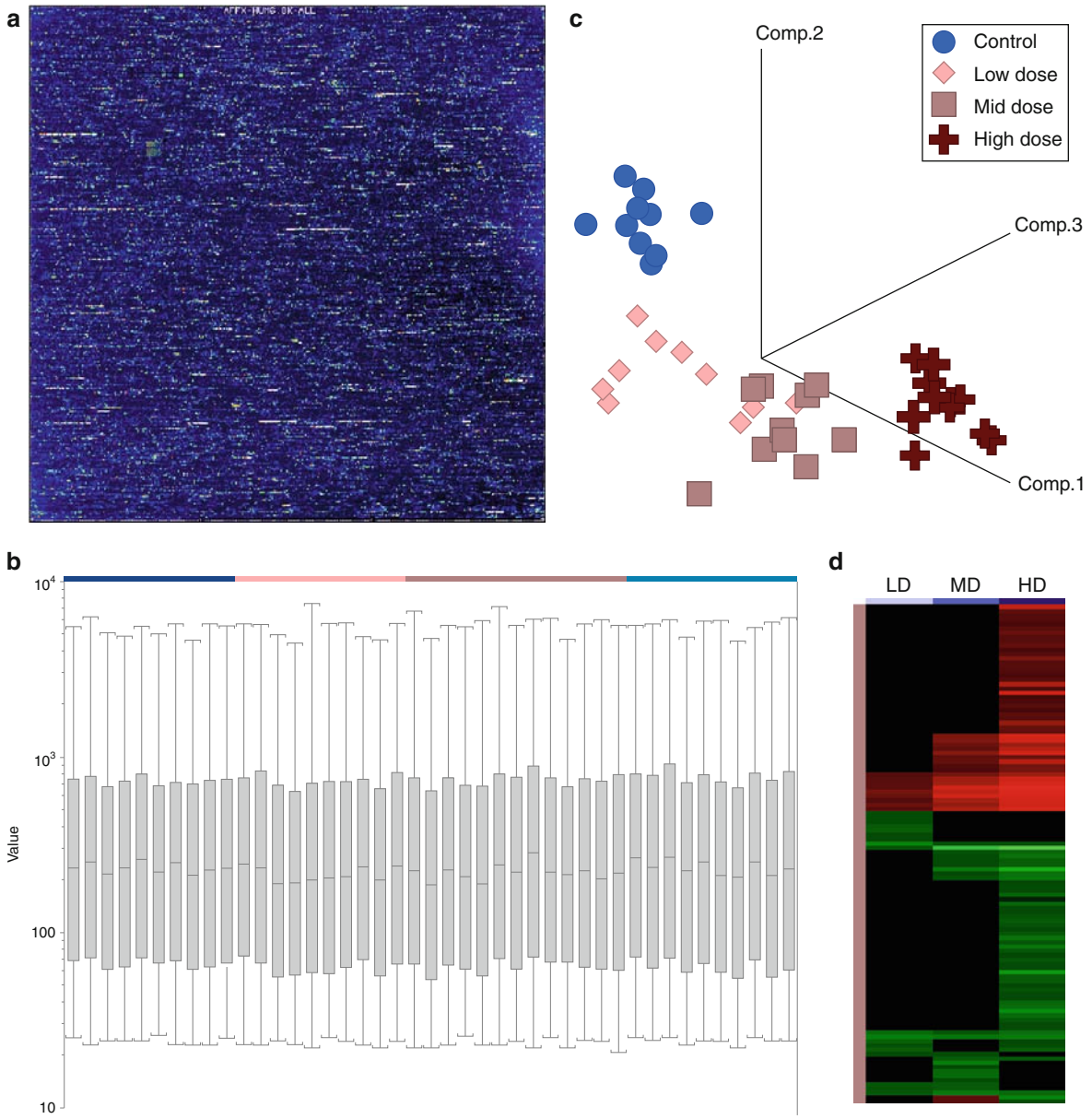

Fig. 55.2 Whole genome gene expression data of a toxicogenomic study. Computer image of a single Affymetrix GeneChip after sample hybridization (**a**). Presentation of gene expression data from rats treated with a low dose, mid dose, or high dose, respectively, of a potential drug in comparison to control animals analyzed with Expressionist® Analyst from Genedata. Non-normalized expression values of individual samples presented in separate Box Plots (**b**). Principal Component Analysis (PCA) after data normalization, showing differences between samples in their overall gene expression (**c**). The so-called heat map displays upregulated (*red*) and downregulated (*green*) genes in comparison to control animals (**d**)

from Agilent are possible software tools where such specific analysis can be performed. Dose-responses or time-effects can be evaluated by special statistical methods. An example of gene expression data from a toxicogenomic study is given in Fig. 55.2b–d.

CRITICAL ASSESSMENT

Affymetrix, as a tool, is well accepted in the scientific community and is highly reproducible (mean false change rate of triplicates $\leq 0.18\%$ and the percentage of concordant detection call is $\geq 91\%$) and sensitive

(down to 1.5 pm mRNA); therefore, chip-to-chip variation is kept to a minimum (Technical Note, Affymetrix). The major advantage of this method is that you can simultaneously monitor the expression changes of tens of thousands of individual genes. Mechanisms of toxicological response can be teased out of the data and gene expression patterns (signatures) may lead to a more predictive approach to early toxicological assessment. However, the data is cumbersome and the amount of data generated is enormous, and therefore, appropriate software tools and large databases are essential. Interpretation of such large datasets is difficult and care must be taken not to over-interpret such data. For all array technologies, a second method is recommended to confirm a small number of the gene expression changes, for example, real-time PCR (see Sect. 55.2.3).

MODIFICATIONS OF THE METHOD

The actual protocol for running Affymetrix arrays is well standardized—as recommended by the manufacturer. However, it is clear that this method is only a tool, and many researchers have reported different uses for this technology (and not only in toxicology).

There are also obviously many other companies offering similar global expression arrays. These include Agilent, Illumina, Roche NimbleGen, Applied Microarrays, to name just a few. All are based on oligonucleotides attached to an array surface where the target sequences will be captured. The most popular systems are synthesized oligonucleotides spotted onto the array (customized or standard arrays). This is in contrast to the Affymetrix array described above, where the manufacture is based on a combined chemical and photolithographic method of oligonucleotide synthesis directly on the array (Pease et al. 1994). In addition to Affymetrix, spotted cDNA arrays have been widely used for mechanistic toxicity testing (e.g., Kultima et al. 2004). Due to the availability of new genomic technologies (next-generation sequencing), the conventional microarray analysis has already lost importance, at least in other fields of research. So this new technology might also enter toxicogenomic evaluations in the near future (see Sect. 55.2.4.2).

Expression analysis using FFPE tissues is not commonly applied in toxicology but a lot is known about their use in other fields of research. Tissue blocks have been assessed for molecular toxicology processes and can successfully be used (Schmitt et al. 2009; von Landenberg et al. 2011). Due to the nature of RNA extracted from FFPE tissues, special technologies are required for successful sample processing. For example, Genisphere and NuGEN Technologies provide sample processing kits to perform expression profiling on Affymetrix and Illumina whole genome arrays. Furthermore, the DASL™ assay from Illumina was specifically designed for analyzing degraded RNA and is no available for whole genome expression analysis of FFPE tissues (Fan et al. 2004).

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EXAMPLES

Many researchers have used genome-wide expression profiling to elucidate toxic mechanisms and/or to find marker gene(s) for specific toxicity end points. Ellinger-Ziegelbauer et al. (2004) have used Affymetrix technology to characterize genotoxic carcinogens in rat liver. Ezendam et al. (2004) have used toxicogenomics for the elucidation of the mechanism of toxicity after sub-chromic hexachlorobenzene exposure in rats. Hewitt et al. (2005) have reported the use of the Affymetrix technology to elucidate the teratogenic mechanism of two drugs (retinoic acid and a novel pharmaceutical agent). In a larger in vivo study, it was demonstrated how toxicogenomic evaluations together with toxicoproteomics and metabonomics can contribute to the detection of liver toxicity, biliary injury, or hepatocellular hypertrophy, as well as kidney toxicity (Boitier et al. 2011; Ellinger-Ziegelbauer et al. 2011; Matheis et al. 2011). Yuan and coworkers successfully used toxicogenomic approaches to identify the reprotoxicity potential of specific phthalates in rat testicles (Yuan et al. 2010). Microarrays have even been applied in the fields of environmental biology and ecotoxicology (Hook 2010; Martyniuk et al. 2011). In addition, many in vitro studies have been reported, for example, in hepatocytes to evaluate liver toxicity or HepG2 cells for mutagenicity studies (Jessen et al. 2003; Kostrubsky et al. 2003; Harris et al. 2004; Boehme et al. 2011; Hrach et al. 2011).

55.2.3 Multiplexed Gene Expression Analysis

PURPOSE AND RATIONALE

The quantitative analysis of gene expression changes is important if we are to trust data generated by larger gene arrays. The real-time PCR technique (e.g., TaqMan® from Applied Biosystems) allows fast and very sensitive detection of even rare RNA molecules and is routinely used for analyzing a small set of distinct preselected genes. It can also be applied for the validation of array data. This high specificity is due to a complementarity between the primer set, the internal probe, and the target. It is widely believed to be the most sensitive and specific method with a wide dynamic range for mRNA quantification. The TaqMan® assay was used as gold standard in the MAQC project (Shi et al. 2006). Very small amounts of RNA are required, ensuring economic use of

precious samples, as well as the possibility of using micro-dissected tissue.

PROCEDURE

For the TaqMan® assay, purified RNA is first subjected to reverse transcription using random primers. The subsequent PCR reaction includes individual primers (both forward and reverse), a sequence-specific probe, and the polymerase. Ready-to-use primer/probe mixes can be purchased from the supplier or custom primer/probe mixes can be designed on their website (Primer Express® software). The software produces sequences that comply with requirements regarding the melting point, G/C content, length, and configuration. Furthermore, the amplicon should not exceed 150 bp. The characteristic TaqMan® probes are labeled with the fluorescent dye FAM (6-carboxy-fluorescein) and a nonfluorescent quencher together with a minor groove binder (MGB) at the 5′- and 3′-end, respectively. The MGB stabilizes the probe binding and enables higher melting temperatures. Probe and primers are provided in one mix and the TaqMan® Universal Master Mix comprises all remaining components necessary for a real-time PCR. The TaqMan® reaction exploits the 5′-nuclease activity of the Taq polymerase releasing the reporter dye from the 5′-end of the annealed TaqMan® probe during amplification. Reporter dye fluorescence is no longer transferred (Forester energy transfer) and suppressed by the quencher, resulting in an increasing fluorescent signal. The reaction is performed on the real-time PCR systems from Applied Biosystems (e.g., 7,500 Real-Time PCR System) and the accumulating reporter dye fluorescence is detected in real time. For amplification, the reaction starts with 2 min at 50°C and 10 min at 95°C followed by up to 40 cycles with 15 s at 95°C for denaturation and 1 min at 60°C for annealing/extension for each amplification cycle (Protocol, Applied Biosystems). Each sample is analyzed for the target gene of interest and at least one endogenous control (e.g., 18 S ribosomal RNA (rRNA)) for normalization. It is important to correctly choose an endogenous control which is consistently expressed in all samples independent of tissue source and treatment. A no template reaction can be run as the negative control. If a standard curve is required, different dilutions of a sample with known quantities are analyzed for each target, including the endogenous control. All samples and controls are run in triplicates (Fig. 55.3).

EVALUATION

In the resulting amplification curve, the baseline and the threshold within the exponential phase are set to determine the threshold cycle (C_T) (Protocol, Applied Biosystems). Two methods are usually used for the evaluation of real-time PCR data, namely, the standard curve method or the comparative C_T method. The standard curve method relies on the use of dilutions of cDNA reverse transcribed from a reference RNA, which will result in only a relative quantification. Other more specific standards can be used, for example, in vitro transcribed RNA, which gives an absolute quantitation; however, this method is very labor intensive and not commonly used (Martell et al. 1999). The standard curve is included in each PCR run, and therefore provides a correction control for the PCR efficiency, making inter-assay comparisons easier. The comparative C_T method uses algorithms to calculate relative expression levels, compared to a calibrator (e.g., a control sample). A detailed description of the mathematics is given by Livak and Schmittgen (2001). After calculation, the normalized expression of the target gene in the unknown sample relative to the normalized expression of the control (calibrator) sample is produced. It is important when using this method that the PCR efficiency of the target gene and the housekeeping gene is equal, then more samples can be run in one PCR run (i.e., no wells lost to the standard curve).

The housekeeping gene is one that is universally expressed, and does not change under the conditions of the assay employed. 18 S rRNA, β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin, mitochondrial ATP synthase 6, hypoxanthine guanine phosphoribosyl transferase 1 (HPRT), and succinate dehydrogenase complex subunit A (SDHA) have, for example, been reported (Zhong and Simons 1999; Gerard et al. 2000; Cicos et al. 2007). Assays are evaluated only when negative controls do not show any amplification products.

Statistical analyses (e.g., t-test) are performed and significant differences between treated samples compared to vehicle control are determined.

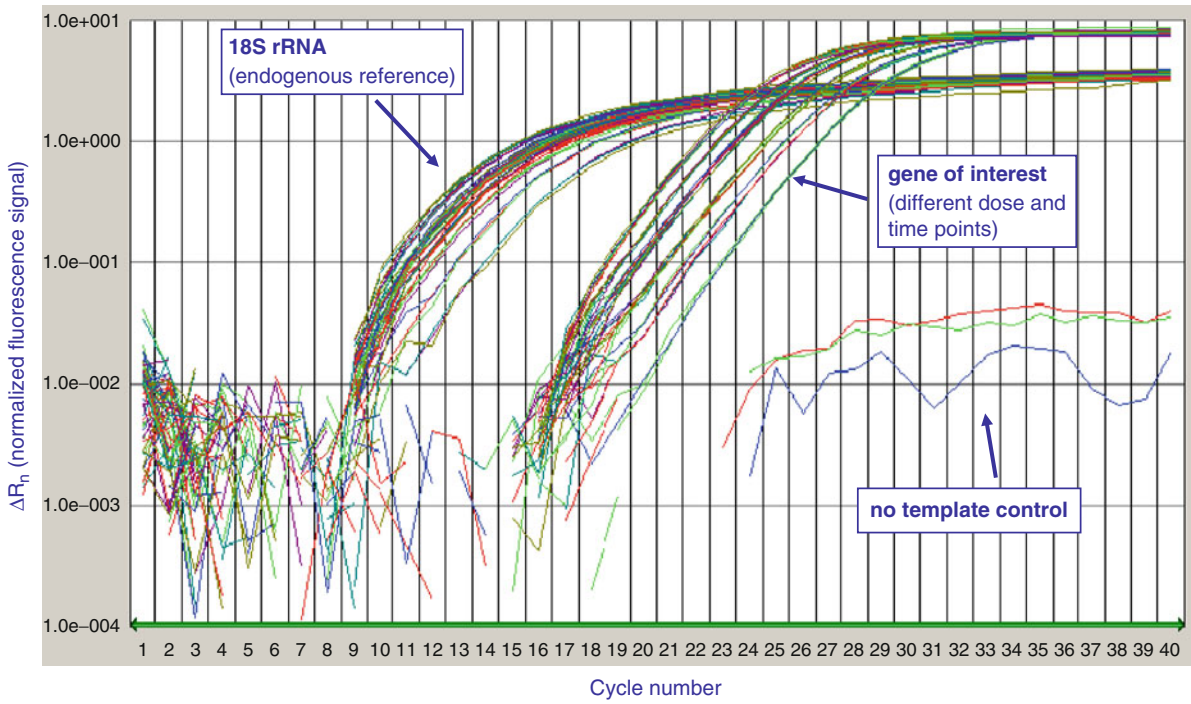

Fig. 55.3 Representative real-time PCR (TaqMan®) trace. Showing the housekeeping gene, 18 S, and the test gene of interest. Increased fluorescence intensity indicates increasing levels of cDNA and more cycles needed indicate smaller amount of starting material (RNA)

CRITICAL ASSESSMENT

Real-time PCR can be considered to be a highly sensitive, specific, and reproducible technology for quantitation of gene expression. Reliability is very high and the data generated is of the highest quality. This method would be the method of choice, when expression of a limited number of genes is required. The obvious drawback is that the number of gene expressions possible is limited. Therefore, higher-throughput gene arrays for the study of larger numbers of genes are needed.

Care must be taken when choosing housekeeping genes for normalization, as there are many citations reporting the gene expression regulation of all of these commonly used housekeepers.

Table 55.1 A selection of currently available real-time PCR systems

Company	PCR cycler
Life Technologies	7300, 7500 (Fast), 7900HT, OpenArray®, StepOne(Plus)™, ViiA™ 7 (Dx) Real-Time PCR Systems
Roche	LightCycler® 2.0, 480, 1536, Nano
Illumina	Eco® Real-Time PCR System
Eppendorf	Mastercycler® ep realplex
BioRad	MiniOpticon, MyiQ2, CFX96 Touch, CFX384 Touch
Qiagen	Rotor-Gene® Q
Cepheid	SmartCycler, GeneXpert
Agilent Technologies	Mx3000P/Mx3005P qPCR System

MODIFICATIONS OF THE METHOD

More recently intercalating double-stranded DNA-binding dyes (e.g., SYBR Green),have been introduced (Giulietti 2001) which removed the need for an expensive, specific probe to be designed. Other sophisticated tools have been developed to work in conjunction with the TaqMan® method, for example, molecular beacons, scorpions, and hybridization probes. These techniques also rely on the FRET (Fluorescence Resonance Energy Transfer) principle but the emergence of the fluorescent signal does not require the nuclease activity of the Taq polymerase. Some of the different real-time PCR providers currently available on the market are given in Table 55.1. Applied Biosystems

have introduced "microfluidics cards" or low-density gene expression arrays. These cards follow the main TaqMan® principles, but are based on a 384-well plate design. Therefore, multiple samples and genes can be monitored, quantitatively, at the same time. Maley et al. (2004) have reported the use of a multiplexed TaqMan® model for high-throughput screening applications. Instead of the nonfluorescent quencher at the 3′-end of the TaqMan® probe, a fluorescent quencher (TAMRA) is linked, especially when designing custom gene expression assays.

Other multiplexing solutions are available on the market, for example, the QuantiFast® Multiplex PCR Kit from Qiagen, which enables the analysis of up to four targets in a single tube. A flexible multiplexing PCR solution is provided by Life Technologies/Applied Biosystems. The OpenArray® technology contains several individual PCR assays on a conventional microscope slide format with 48 subarrays. The subarrays enable analysis in a mid-density manner for higher throughput applications (up to 48 samples or 224 individual assays). PCR reactions can either be TaqMan or SYBR® Green based that can be used for gene expression but also for genotyping, GWAS, and miRNA analysis.

A PCR-free system is provided by Affymetrix/Panomics. The QuantiGene® assay, based on the branched DNA technology, applies signal amplification rather than DNA amplification. Various sample types, for example, cells, tissues, FFPE tissues, blood, can be run in this assay without the need to extract pure RNA. Either a single gene or several genes can be multiplexed in one assay. After capturing the target sequence on the plate surface or on beads for single and multiplexed assays, respectively, the signal is ~400-fold increased by the use of pre-amplifiers and amplifiers which build a branch-like structure. The bead type (target) and the signal intensity are detected using the Luminex® instrument (Dunbar 2006).

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EXAMPLE

There are numerous publications where real-time PCR analysis has been used to follow the changes in expression of specific toxic relevant genes. Fox et al. (2010)

identified potential biomarkers for the identification of acetaminophen toxicity in HepG2 cells and primary rat hepatocytes by qPCR analysis. RT-qPCR analysis was applied to validate a gene marker set observed by microarray analysis describing the molecular changes in rat liver after AhR ligands exposure (Ovando et al. 2011). Testicular toxicity of emodin, a herbal medicine, was observed and analyzed by qPCR (Oshida et al. 2011). The neurotoxic effect of lead during early development was assessed by real-time PCR using zebra fish embryos (Zhang et al. 2011). Giulietti (2001) have used real-time PCR techniques to evaluate cytokine profiles in both mouse and human cells and tissues. Heregulin (a member of the neuregulin family) and its binding receptors (ErbB-2, ErbB-3, and ErbB-4) are induced by gentamicin treatment, and are therefore postulated to play an important role in hair cell regeneration following ototoxic shock (Zheng et al. 1999). Campbell et al. (2004) showed, using TaqMan®, the importance of matrix metalloproteinases in kainic acid–induced excitotoxicity in the rat brain.

55.2.4 Recent Innovations and Future Technologies

55.2.4.1 Small Noncoding RNAs in Toxicogenomics

PURPOSE AND RATIONALE

The evaluation of epigenetic factors, for example, DNA methylation, histone modifications, as well as the impact of micro RNAs (miRNAs) on mRNA regulation, has become very important in many fields of research. In the following chapter, the impact of small RNAs, with a focus on miRNA, in toxicogenomics is described. Assessing such factors has already been well established in cancer research (Munker and Calin 2011) and the importance also for toxicology has been recognized. Polymerase II promoters are often involved in the induction of toxicological effects and miRNAs are mainly transcribed by this important enzyme; thus, it has been stated that miRNAs play a crucial role in the development of certain toxicities (Taylor and Gant 2008). miRNA expression has been confirmed to be affected by extracellular signals, cellular stress, and xenobiotics (Lema and Cunningham 2010). Therefore, miRNA expression profiles could support the identification, classification, or prediction of adverse effects and serve as safety-specific biomarkers. Identifying effected miRNAs by a certain toxicant, and the posttranscriptional modification of the miRNA's target gene can be estimated which would be a crucial contribution to the clarification of a toxicity mechanism. It is also of great interest whether changes in miRNA expression affect an individual's susceptibility to xenobiotics.

miRNAs are small endogenous noncoding RNA molecules (~22 nt) with regulatory functions. The major effect of these molecules is binding to the 3′UTR region of particular mRNAs and posttranscriptionally inhibiting their translation into proteins. In the most recent release of the commonly used miRBase database (17; April 2011), 16,772 entries are recorded with 1,424 and 408 miRNA sequences for *Homo sapiens* and Rattus norvegicus, respectively (http://www.mirbase.org). In contrast to miRNAs, small interfering RNAs (siRNAs) are mainly exogenous molecules from viruses, transposons, or transgenes that achieve posttranscriptional silencing via mRNA degradation. These RNAs are used in the RNA interference (RNAi) technology to knock down genes of interest, used in gene function studies for analyzing the association of specific genes to a particular phenotype (Hannon 2002). Specifically, in toxicology, it can be studies whether the genes of interest correspond to certain toxicity.

PROCEDURE

miRNA expression can be analyzed with different technologies, including northern blotting, qPCR, microarrays, and next-generation sequencing. Since most technologies are described in other parts of this chapter, they will not be described in detail here.

Small RNAs are not extracted with common RNA extraction methods. Therefore, it has to be ensured that the extraction kit used does not lose the small RNA molecules. Products that provide small RNA enrichment (< 200 nt) in total RNA or separated small RNA extractions are available on the market. For example, the mirVana™ Kit from Ambion, Life Technologies, or the miRNeasy Mini Kit from Qiagen. Furthermore, phenol-based RNA extraction methods usually yield total RNA including small RNAs. The quality check for miRNAs can be performed using the Agilent Small RNA Assay on the 2100 Bioanalyzer.

For qPCR analysis, a special reverse transcription of miRNA is necessary. Regular oligo d(T) or random priming is not suitable due to the short nature of miRNA molecules which have no poly-A tail. Applied Biosystems describes a reverse transcription procedure where a specific stem-loop RT primer is annealed to the 3′ end of the mature miRNA (Protocol, Applied Biosystems). The primer is extended by reverse transcriptase producing a longer cDNA molecule containing the primer sequence and the first strand cDNA complementary to the mature miRNA sequence. The TaqMan® MicroRNA assay is based on the TaqMan® assay already described in Sect. 55.2.3. Here, a forward primer, as well as the TaqMan® probe, is designed specifically for the small RNA sequence, whereas the reverse primer is complementary to the RT primer sequence. Analysis of siRNA can also be performed by the same assay procedure using the TaqMan® siRNA Assay from Applied Biosystems.

EVALUATION

Quantitation of miRNA expression in the TaqMan® assay is performed based on the C_T values, similar to the evaluation described in Sect. 55.2.3. Comparable to transcriptomics data, statistically relevant differentially expressed miRNAs should be observed. Several miRNA databases are available online to obtain more information on the miRNAs of interest, for example, http://www.mirbase.org/, http://mirnamap.mbc.nctu.edu.tw/, or http://www.microRNA.org. It is often of great interest to additionally know the mRNA targets of the relevant miRNAs. Therefore, the MicroCosm Targets website is a valuable source of information (www.ebi.ac.uk/enright-srv/microcosm/).

CRITICAL ASSESSMENT

In molecular toxicology, it is often not sufficient to have information of the transcriptome. Proteins are the actual active molecules and mRNA expression alone does not mean that a protein is expressed. Due to the complexity of the proteome and the adjacent technologies, protein expression profiles are often not available. Having mRNA and the adjacent regulatory miRNA expression patterns would be a step toward protein translation which could help to postulate/predict the proteomic status of an organ/cell. An issue in interpretation of miRNA data is that many mRNAs are eligible to be targeted by one miRNA. Therefore, miRNA expression is suggested to be analyzed in conjunction with gene expression. This results in the need for additional experiments on different expression platforms. Due to the short length of miRNA primer, binding is often relatively instable and only one miRNA sequence-specific primer can be included. Others have also reported high false-positive and false-negative rates in miRNA microarray experiments (Choudhuri 2010).

MODIFICATIONS OF THE METHOD

Other qPCR technologies require a polyadenylation reaction before the reverse transcription with a universal primer. This cDNA consists of a miRNA complementary sequence at the 3′ end and a universal 5′ end. The qPCR is subsequently run with a miRNA-specific and a universal primer. The NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen) or miScript PCR System (Qiagen) are examples applying this polyadenylation reaction. Other providers use locked nucleic acid (LNA™) oligonucleotides as primers. LNA™ are chemically modified nucleic acids providing more stable binding to miRNA and therefore enabling higher melting temperatures (Exiqon).

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EXAMPLES

Several Phase I enzymes and transporters, including Cyp2E1 and MDR1, were found to be posttranscriptionally regulated by miRNAs (Nakajima and Yokoi 2011; Klaassen et al. 2011). miRNAs also regulate many transcription factors involved in xenobiotic toxicity. Thus, miRNA expression is involved in the translation of PPARα, P53, PXR, and NRF2, to name only a few. A miRNA targeting MRP1 (and MRP1 itself) was found to be a key factor in the establishment of resistance to the cytostatic Cisplatin (Pogribny et al. 2010). Lema and Cunningham reviewed how several toxicants effect miRNA expression and their targets leading to a distinct adverse effect, for example, hepatocyte proliferation induced by PPARα agonists, Tamoxifen-induced hepatocarcinogenesis or acetaminophen-induced hepatotoxicity (Lema and Cunningham 2010). Choudhuri reported that neurotoxicity, developmental toxicity, hepatotoxicity, and carcinogenesis are all affected by miRNA expression (Choudhuri 2010). Furthermore, Wang and coworkers have suggested several miRNA biomarkers for drug-induced liver injury (Wang et al. 2009).

55.2.4.2 Next-Generation Sequencing

PURPOSE AND RATIONALE

The emergence of next-generation sequencing (NGS) technologies has revolutionized the field of genomic research. Steadily decreasing cost for analysis of a genome has enabled their expansion. This is leading the displacement of traditional microarray technologies from the market. NGS platforms are able to perform massively parallel sequencing, have a higher dynamic range, and are more sensitive compared to microarrays. So far these technologies have not yet been extensively applied in the field of toxicology, but this is sure to change in future. NGS is a further development from the shotgun sequencing technology which was already an improvement of the Sanger sequencing. Numerous genomic analyses are possible, including DNA and transcriptome sequencing for genotyping and copy number variation, linkage analysis, gene regulation, and epigenetic evaluations (e.g., ChIP-Seq, methylation, small RNA discovery and analysis, histone modification) (Mardis 2007).

PROCEDURE

The most widely used NGS system is the Genome Analyzer from Illumina. It is easiest to handle with superior data quality and accuracy (Zhang et al. 2011). The Illumina NGS systems are based on the TruSeq™ technologies (Data Sheet, Illumina). For library preparation, DNA is fragmented, a blunt end generated, and an adenosine is added to the 3′end. In case of transcriptome analysis, mRNA is reverse transcribed using random primers to generate double-stranded cDNA. Adapter oligos containing universal primer sites with a 5′ overhang are ligated to both ends and fragments are purified after size selection. The fragments are hybridized with their adapter onto a flow cell and bridges built for amplification. The generated cluster can now be sequenced by synthesis. Here all four bases with different labels are added simultaneously and the hybridized base is identified by fluorescence signal (Technology Spotlight, Illumina).

EVALUATION

After read-generation "base-calling" is performed. With this information, sequences can be aligned to a reference genome or assembly can be carried out for de novo sequences.

Proper alignment or assembly is critical and many open source bioinformatic tools are available online, for example, ELAND, MAQ, and BLAST. More information about these software tools are described in the review from Zhang and coworkers (Zhang et al. 2011). High professional information technology infrastructure is not available in every lab; thus, vendors of NGS technologies also provide so-called cloud computing end-user software for data processing (e.g., the CASAVA package from Illumina). Uniquely mapped reads are then used for further analyses. To observe differentially expressed genes, for example, all reads of a specific gene transcript are counted and compared between samples. Furthermore, normalization, transformation, and statistical analyses are carried out in a similar way to microarray data analysis.

CRITICAL ASSESSMENT

NGS technologies have the potential to be of great added value to genomic research. An enormous amount of data can be obtained in a relatively short period of time from limited samples. Additionally, these technologies have the potential to pave the way toward personalized medicine. In the case of toxicogenomics, by sequencing individual genomes, interindividual differences could be potentially

Table 55.2 A selection of currently available next-generation sequencing systems

Company	Template preparation	Sequence by	System	Read length	Max output
Illumina	Bridge amplification	Synthesis	HighSeq 2000	2x100b	600 Gb
			Genome Analyzer IIx	2x150b	95 Gb
			MySeq	2x150b	~1 Gb
Roche	Emulsion PCR	Pyro-sequencing	GS-FLX+	<1,000b	700 Mb
			GS Junior	400b	~35 Mb
Applied Biosystems	Emulsion PCR	Ligation	SOLiD 4	50b	100 Gb
			5,500xl Genetic Analyzer	75b	10–15 Gb
			5,500 Genetic Analyzer	75b	7–9 Gb

detected after exposure to a toxicant/new drug entity. However, many issues exist concerning the use of NGS. Especially the handling of the huge amount of data generated is extremely challenging, specifically concerning data transfer, storage, analysis, and interpretation (Metzker 2010). Due to the short read length, reads often cannot uniquely be aligned and therefore cannot be included in the analyses, repetitive sequences alignment is especially difficult. Improvement of software tools is thus essential to be able to properly adopt this technology. These technologies still need to be validated for data quality, reliability, reproducibility, and biological relevance. In 2008, a consortium has been founded coordinated by the FDA to deal with these challenges, namely, the MAQC-III project, called Sequencing Quality Control (SEQC) (www.fda.gov/MicroArrayQC).

Furthermore, sequencing is still relatively expensive—when compared to the costs of whole genome microarrays. This is an especially sensitive issue for toxicogenomics where usually large numbers of animals are need to be analyzed/sequenced; therefore, costs would be too high to use sequencing in routine studies. To reduce costs, it can be considered to sequence only preselected regions rather than analyzing the whole genome. As the technologies are continually and rapidly being improved, there is hope that in the near future they will become affordable soon.

MODIFICATIONS OF THE METHOD

Three major NGS technologies are currently available on the market. One is the technology from Illumina described above, with different sequencers available (HighSeq, HiScanSQ, Genome AnalyzerIIx and the MySeq) and the two others are from Roche and Applied Biosystems. The major differences of all technologies are in template preparation or in sequencing. Templates can be prepared by emulsion PCR or the amplification is performed on a solid phase. The 454 Sequencing Systems (Roche) are pyrosequencer and performs emulsion PCR for template preparation. Sequencing by ligation is required on the SOLiD platform (Applied Biosystems). Here the template is amplified on a solid phase. In addition, all platforms differ in the obtained number and length of reads (Table 55.2). All sequencing technologies are described and compared by Voelkerding et al. (2009) and Metzker (2010).

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EXAMPLES

The first toxicogenomics study applying NGS was described by Su and coworker in 2011. They compared

the RNA-Seq data to data from an Affymetrix microarray analysis, which was based on a real-life toxicological study where rats were treated with aristolochic acid. Differentially expressed genes compared to control animal were assessed. NGS was observed to be more sensitive in identifying a great number of deregulated genes. Although they found only 40–50% common genes, gene expression patterns obtained from both technologies were similar, as was the biological outcome. For example, typical nephrotoxic and carcinogenicity GO processes were observed with both technologies (Su et al. 2011). NGS has been applied in several phases of drug discovery and development process, and has been extensively reviewed by Woolland et al. (2011). NGS can support target identification, but can also be used in drug resistance studies, biomarker discovery, and RNA-protein identification studies. For personalized medicine, the identification of interindividual variations is important for therapy which might improve drug efficacy and safety.

55.3 Application of Toxicoproteomics in Profiling Drug Effects

PURPOSE AND RATIONALE

Traditionally, toxicologists define the preliminary risk of a new compound to human safety using animal models supported by histopathological and biochemical approaches. However, despite decades of experience, the extrapolation dilemma and the relevance of animal data to real-life, long-term exposure in humans remained unclear. The genomics revolution of the recent years led to development of many new and innovative technologies that can change this paradigm and address uncertainty issues in the current toxicological practice and safety assessment through the identification of novel key genes, marker proteins, or protein profiles. Thus, these technologies provide a superior alternative to traditional rodent and canine bioassays to identify and accurately assess the safety of chemicals and drug candidates for human safety.

Toxicogenomics, the use of DNA microarray for comprehensive RNA expression analysis, has recently caused a great deal of interest (Pennie et al. 2000; Nuwaysir et al. 1999). This technology has been used to monitor changes in gene expression in response to drug treatment. However, analysis of the information produced by toxicogenomics has proven to be unsatisfactory (Anderson and Seilhamer 1997; Mann 1999; Srinivas et al. 2001). Fundamental studies have illustrated the usefulness and potential of the toxicoproteomics, the proteomic approach, to complement RNA microarray data. Proteomic technology helps identify corresponding changes in the level of protein, which is critical because the protein is the basic component of a cell. Additionally, toxicoproteomics helps resolve issues involving differential protein modifications. These are critical for the function of many proteins, in that they may lead to changes in the activity of gene products. Primarily, the manifestation of protein modifications is the reason for undesired, compound-related effects. Toxicoproteomics helps to determine such changes, and to gain insight into the mode of action of drugs (Kumagai et al. 2006; Gao et al. 2009). Furthermore, toxicoproteomics technologies can also be applied in identification, characterization, and evaluation of new prognostic and diagnostic biomarkers (Merrick 2006; Bjørnstad et al. 2006; Provan et al. 2006; Glückmann et al. 2007).

Toxicoproteomics studies, as they have been conducted so far, focused mainly on investigation of toxic effects of known toxicants and reference chemicals. These studies were of paramount importance to show the potential of proteomics technologies and their application in toxicological research. In recent publications, however, researchers report investigations with new chemical entities within pharmaceutical and chemical industry, and scientific institutions. These studies herald the next period of practicing toxicoproteomics with more focus on issue resolution or biomarker identification. To achieve this goal, the knowledge from proteomics approach needs to be combined with the information from classical disciplines such as toxicology and pathology.

PROCEDURE

55.3.1 Available Technology Platforms

The most common implementation of proteomic analysis involves protein separation 2-D gel electrophoresis (2DGE), quantification of proteins with analytical methods for their identification in mass

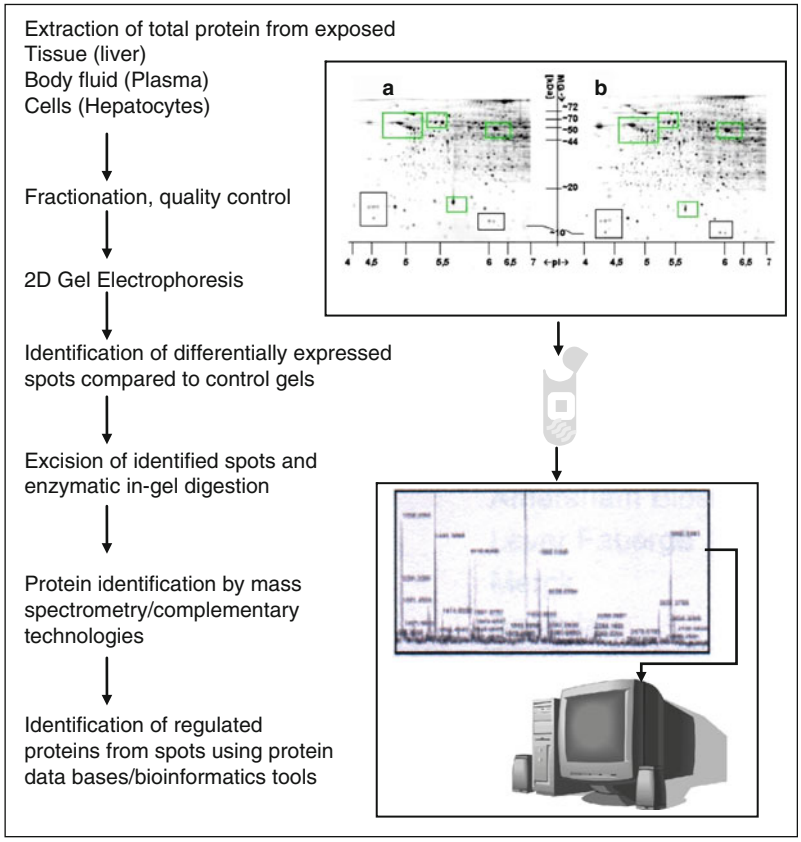

Fig. 55.4 A general flow chart of sequential steps for conducting standard proteomics studies

spectrometer (MS), and at the very least data integration and analysis using bioinformatic tools. Figure 55.4 represents the sequential steps for conducting standard proteomics studies.

55.3.1.1 Protein Extraction and Fractionation

The first step in the protein identification and characterization process by different proteomics technologies is the extraction of proteins from cell cultures, body fluids, or tissue samples. As total protein extracts harbor many abundant proteins such as immunoglobulins or albumin, the removal of abundant proteins could be considered for the purpose of increasing number of detectable and identifiable proteins (Merrick 2006).

The first step in the preparation of protein extracts is to mix samples (cell or tissue pieces) with a lysis buffer, for example, 20 mM Tris-HCL, pH 7.4, 1% CelLytic-M (Sigma). To avoid the enzymatic degradation of proteins by proteases, protease inhibitors are added. When organ samples are used for protein extraction, as soon as these constituents are added, tissue are homogenized using a Polytron type homogenizer. During the homogenization process, the tube is kept submersed in a water ice bath to maintain the sample at 4°C. After homogenization, samples are centrifuged to separate the solubilized proteins from cell debris and insoluble membrane components. The supernatant is aspirated and can be divided into aliquots. At the end, the total protein concentration can be determined using a BCA protein assay with BSA as the standard. Depending on the purpose and experimental design, a protein fractionation can be followed, or the protein extracts are analyzed without further processing. If the protein extracts are not used immediately, they can be frozen at < -70°C until tested.

55.3.1.2 2DGE

Initially, proteins in a sample are separated according to their isoelectric point in a pH gradient. Next, the proteins are separated according to size on a SDS-polyacrylamide gel. A dye marker such as coomassie blue, silver, or fluorescent dyes then detects the resolved proteins. In order to analyze differentially expressed protein spots in an experimental set of gels,

a computerized detection and matching system is required. Finally, MS identifies selected protein spots. The 2-D gel electrophoresis allows separation of around 3,000 proteins. Even though the 2DGE is an effective method for the separation and quantitation of proteins from different sources, it has some limitations. Working with 2DGE technique takes time and demands intensive skilled labor, and hence is not practical for high throughput. Other limitations like lack of reproducibility narrow the application of 2DGE. Liquid chromatography is an alternative and straightforward method with the advantage of direct connection to mass fingerprinting technologies (Link et al. 1999).

55.3.1.3 MS

Mass fingerprinting of excised and trypsin-digested gel spots is the method of choice to identify proteins. The masses of the tryptic fragments in a sample are accurately and quickly measured using a matrix assisted laser-desorption/ionization time-of-flight (MALDI-TOF) instrument. In this technique, purified or partially purified proteins are mixed with a crystal-forming matrix, placed on an inert metal target, and subjected to a pulsed laser beam to produce the phase ions that traverse a field-free flight tube. They are then separated according to their mass-dependent velocities. The mass peak list obtained is searched by means of in silico digest of sequence databases for comparison and identification of proteins.

EVALUATION

55.3.2 Performed Studies to Figure Out the Mechanism of Organ Toxicity by Proteome Analysis

The proteomic investigation has been applied to a series of compounds to examine the response of in vitro and in vivo models after exposure to toxicants. The main focus of these studies has been to understand the mechanism of their toxicity. In an attempt to develop a rodent liver proteomic toxicity database, Anderson and colleagues characterized the effect of a range of xenobiotics on protein expression in the liver (Anderson et al. 1996a). Using this database, it was possible to detect, classify, and characterize a broad range of hepatotoxins. Toxicoproteomics was the key tool used to gain new insights into the molecular mechanism involved in cyclosporine A (CsA) nephrotoxicity. The initial study reported by Steiner et al. (1996) investigated changes in the kidney protein pattern of CsA treated rats in order to determine the nephrotoxic mechanism of this drug. Using this proteomic approach, the investigators discovered an association between decreased calcium binding protein, calbindin-D 28 kDa, and CsA-mediated medullar nephrotoxicity (Aicher et al. 1998). Since these early studies, the potential of toxicoproteomics to identify protein changes associated with nephrotoxicity has

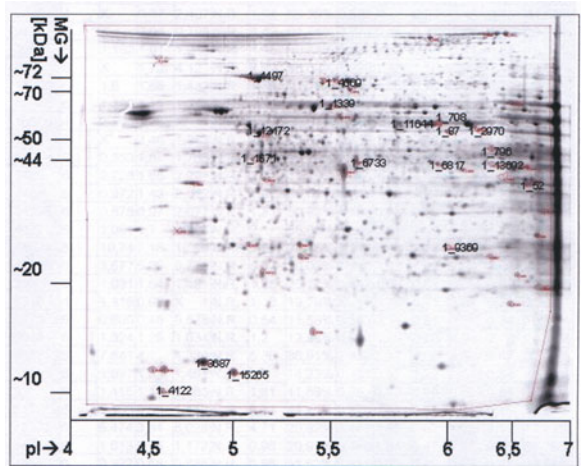

Fig. 55.5 Protein pattern of treated cells compared to control obtained after separation of 350 µg protein sample. Cells were exposed to 0.5 mM fenofibrate for 24 h. Spot IDs marks differentially regulated proteins

Table. 55.3 Identified proteins from rat primary hepatocytes involved in energy metabolism and electron transfer

Protein identification[a]	Molecular mass (kDa)[b]	pI[b]	Regulation
ATPase chain B, liver	51.35	4.95	Down
ATP synthase δ-chain	15.79	4.74	Down
NADH ubiquinone oxidoreductase	26.52	6	High
malate dehydrogenase	36.48	6.16	Down
NAD-specific isocitrate dehydrogenase	39.6	6.47	High
Enoyl-CoA hydratase	31.5	8.4	High
Aldehyde dehydrogenase	48.27	6.06	High
Guanidino acetate N-methyl transferase	26.4	5.7	High

[a]Peptide masses were identified in the positive ion reflector mode (MALDI-TOF) and protein identification was performed using the program MS-Fit
[b]Theoretical calculated pI and molecular masses

been shown in further investigative animal and clinical studies (Witzmann and Li 2004; Janech et al. 2007; Mischak et al. 2009; Klawitter et al. 2010). Data on H1 receptor antagonist, pyrilamine, and the non-genotoxic carcinogenic analogue, methapyrilene, showed differing proteomic profiles despite a similar chemical structure. Widespread changes in hepatic protein composition were observed for methapyrilene but not for pyrilamine (Cunningham et al. 1995). The proteomic approach further assisted numerous mechanistic investigations followed by explanation of regulatory changes implemented at the protein level. This included testing of pharmaceuticals for carcinogenic potential, hepatocellular hypertrophy, and peroxisome proliferation (Arce et al. 1998; Anderson et al. 1996b). The proteome profiling in these studies was partially used for lead prioritization, emphasizing the potential role for toxicoproteomics in lead candidate selection.

To investigate the significance of the 2-DE technology in determining changes in protein expression, Kabiri et al. (unpublished data) exposed rat primary hepatocytes to the peroxisome proliferator fenofibrate. Several important aspects of this study are discussed here. To investigate the proteome profile, we isolated and cultured primary hepatocytes in the presence of various concentrations of fenofibrate. To reduce the complexity, protein extracts were narrowed by prefractionation procedure and cytosolic fraction was collected for further analysis. After 2-DE separation, protein spots with an alteration in their abundance were excised and subjected to the MS. Figure 55.5 shows the 2-D pattern obtained after separation of 350 μg protein sample from treated cells as an example. When primary cells were treated with 0.5 mM fenofibrate over 24 h, a total of 30 protein spots were strongly affected. Some of these are summarized in Table 55.3. Many of the identified proteins are involved in cell proliferation, protein metabolism, and energy. In addition, proteins associated with defense reactions to cellular stress are expressed at high levels in response to fenofibrate exposure. These results are consistent with mRNA abundance as indicated in microarray experiments (data not shown). However, we detected expressed changes in protein, for which no changes at RNA level were measured. We have investigated the comparative effects of additional compounds on the rat primary hepatocytes and the results show the potential of toxicoproteomics to serve as a complementary technology to microarray-based approach.

CRITICAL ASSESSMENT OF THE METHOD

55.3.3 Alternative Proteomic Technologies and Options

Although 2DGE is unchallenged in its ability to resolve thousand of proteins, it has several limiting factors. Firstly, it is labor intensive, requires large quantities of proteins, and may not be suitable to serve as an effected diagnostic tool. Secondly, the application of 2DGE is limited because it fails to detect proteins at the extremes of separation either by size or by isoelectric point, and because it is insufficiently sensitive for low-abundant proteins (Moseley 2001). Therefore, additional innovative methods are needed to measure broad protein abundances and activity.

55.3.3.1 SELDI-TOF/Protein Biochips

SELDI-TOF is beginning to offer an alternative to 2DGE. Surface-enhanced laser-desorption/ionization (SELDI) is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip (Weinbergera et al. 2002, Ciphergen Protein Chip® Arrays).

This system has already been used to identify markers of prostate cancer and changes in renal cell carcinoma. It also has been applied to the discovery of new toxicity markers (Grizzle et al. 2004; Jr et al. 1999; Paweletz et al. 2001; Vlahou et al. 2001). Taking advantage of the recent development in SELDI and the protein chip technology, it will be possible to simultaneously analyze protein profiles of body fluids such as serum and urine samples very rapidly. The SELDI mass spectrometry in conjunction with bioinformatics tools could greatly facilitate the discovery of new and improved toxicologic biomarkers (Collins et al. 2010).

Protein biochips (gel-based microarrays) represent a further development of proteomics technologies with the potential for screening of protein subsets. In this technology, proteins (mostly antibodies) are immobilized and trapped in gel-based matrices and thus are made accessible for target proteins. The combination with dye marker like fluorescence and chemiluminescence and detection of their signal intensities allows detection of protein modifications or deregulations. The gel-based protein chips can also be

combined with MALDI-TOF MS to achieve a specific analysis of protein subclasses (Rubina et al. 2003; Rubina et al. 2008).

55.3.3.2 ICAT

A recent and exciting development by Aebersold and colleagues is the isotope-codes affinity tag (ICAT) method, which can be used to label proteins before separation (Gygi et al. 1999). By using two different isotopes for labeling, it is possible to perform a binary comparison in a single step. After labeling, test and control samples are pooled and digested with proteases to produce peptide fragments. ICAT-labeled peptides are separated and analyzed by tandem MS. Due to the fact that the ICAT method is designed to combine labeling, separation, and the analysis of peptides into a single automated procedure, it is possible to scan several thousand peptide pairs a day.

55.3.4 Phosphoproteomics and Detection of Posttranslational Modifications

Proteomics is complicated by the fact that the absolute quantification does not always reflect the molecular function of proteins, because protein activities are highly regulated posttranslationally. Posttranslational modifications modulate the function of proteins and thus directly impact their capacity to participate in cellular regulatory events (Cravatt and Sorensen 2000). Due to posttranslational modifications, the numbers of proteins in human are estimated to be at least three times the amount of genes. Therefore, the elucidation of protein posttranslational modifications is the most important justification for biochemical and structural relationships. Hence, these modifications need to be evaluated.

However, establishing a proteomics platform initially requires implementation and combination of a series of systems to allow a flexible and reliable approach for analysis and identification of differences observed on 2-D gels. Proteomics in this sense is more interdisciplinary, combining aspects of biology, chemistry, toxicology and pharmacology, bioinformatic and information sciences. Use of bioinformatics is essential for analyzing the massive amount of data generated by proteomics.

The throughput of proteomics is currently much lower than that of RNA microarrays, largely due to the requirement of MS analysis, or similar technologies. However, the microarray-based approaches of protein detection may overcome this limitation.

While the combination of 2DGE with protein analytic techniques has been established for toxicoproteomics, the integration of bioinformatic and appropriate software is yet to be implemented.

For toxicoproteomics, the ideal proteomics platform would be one that is:

- Sensitive enough to detect high- and low-abundance proteins
- Easily implemented and performed quickly
- Able to detect modifications and alternative splice forms in addition to abundance
- Able to deliver sophisticated data for protein-protein networking

There are many obstacles to overcome in regard to current limitations of toxicoproteomic technologies. Thus, in the near future, proteomics will play an important role in the research toxicology.

MODIFICATIONS OF THE METHOD

55.3.5 Subcellular Proteomics

One-step characterization of a eukaryotic cell proteome is difficult if not impossible to achieve. There is a growing trend in eukaryotes proteomics toward characterization of subcellular and organellar structures. The reason for this shift from global proteomics to subcellular proteomics is the complexity of eukaryotic cells and subcellular organelles. Therefore, the proteomic analysis of subcellular organelles will be an important aspect of toxicoproteomics (Lee et al. 2010; Gatto et al. 2010).

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55.4 Metabonomics

Metabonomics, or "metabolomics" or "metabolic profiling" as it is often mentioned in the literature, is closely related to the other "-omics" technologies toxicogenomics and toxicoproteomics, linking genotype to phenotype. This is also reflected by the most cited definition of metabonomics that was published in 1999 as "the quantitative measurement of the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999). Therefore, metabonomics means the investigation of endogenous metabolites in biofluids or tissues and the changes on this system caused by different factors such as drug treatment, environmental influences, nutrition, lifestyle, genetic effects, or diseases. Metabonomics has become increasingly popular in drug development, molecular medicine, and other biotechnology fields, since it profiles directly the phenotype and changes therefore in contrast to other "-omics" technologies (Dieterle et al. 2011). It is also often described as the analysis of the "metabolic profile" or "metabolome," which is defined as all small, nonprotein metabolites with molecular weight not more than 1,500–2,000 Da, estimated up to several thousand different molecules. Therefore, analytical technologies for metabonomics are faced with the challenge to analyze all these chemically diverse high and low molecular weight molecules in a wide variety of different concentrations simultaneously. There are various technologies capable to analyze these metabonomics samples, including nuclear magnetic resonance Spectroscopy (NMR) and mass spectrometry coupled with liquid chromatography (LC-MS) or gas chromatography (GC-MS), which will be described later in this chapter. Metabonomics investigations can be performed by the principal approaches of global or targeted metabonomics analysis. In global metabonomics analysis, all the changes on the metabolic profile, measured by the different analytical technologies, are analyzed by statistical methods followed by an identification of the changed analytical signal with the help of databases with known endogenous metabolites. In targeted metabonomics analysis, the changes of a predefined subset of known endogenous metabolites of interest are quantified in the analyzed samples for which no statistical analysis and good databases are needed which is often crucial in metabonomics analysis.

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55.4.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

PURPOSE AND RATIONALE

The principle of nuclear magnetic resonance (NMR) analysis is described in many publications (Ernst et al. 1990; Goldman 1991). For the acquisition of a NMR spectrum, a liquid sample is placed in a static magnetic field. After irradiation with high-frequency pulses (pulse-sequences), the response of the NMR sample is detected by an induced current. The highest field strength available today in NMR spectrometers is 21 T corresponding to 900 MHz ^{1}H (proton) frequency. Most applications in metabolic profiling are using 600 MHz (14.1 T) instruments.

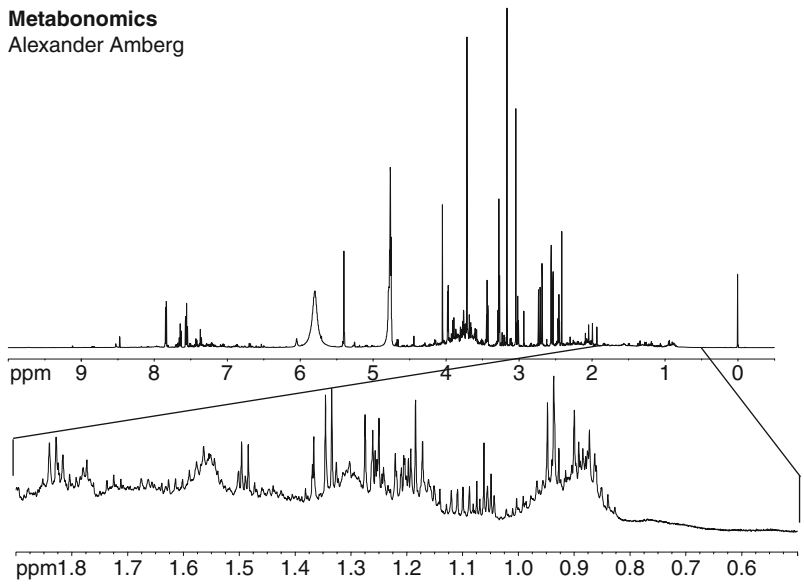

Fig. 55.6 Typical 600 MHz ^{1}H-NMR spectrum of rat urine after dilution with 10% D_2O buffer

The amplitude response of a NMR spectrometer is perfectly linear to the concentration of the sample, which allows easy quantification of compound concentrations for metabolic profiling in the µM to mM range. All steps involved in the acquisition and processing of NMR data, including preparation and exchange of samples, can be performed fully automated for hundreds of samples without the need for manual interaction (Dieterle et al. 2011).

PROCEDURE

With NMR it is possible to analyze all different kinds of liquid samples, including the noninvasively biofluids urine and blood (plasma or serum) but also many other biofluids like seminal fluid, amniotic fluids, cerebrospinal fluid, synovial fluid, etc. Organs, tissue, or cells can be analyzed after extraction of the endogenous metabolites using standard extraction methods (Lindon et al. 2006).

NMR analysis of biofluids needs no sophisticated sample preparation, details for the different biofluids are described by Lindon et al. 2000. In most cases, adding H_2O/D_2O (90:10) or buffer to account for pH variation or to reduce viscosity is sufficient as sample preparation before the NMR measurement (Keun et al. 2002). This means that one potential source of variance due to sample extraction procedures is absent (Dieterle et al. 2011).

The most used analytical technology in NMR metabonomics is ^{1}H proton spectroscopy of biofluids. For the analysis of these samples, water suppression techniques have to be applied, since endogenous metabolites can be present in low µM concentrations in an aqueous environment. Different water suppression techniques are available and described by Prince (1999). Additionally, blood serum/plasma samples or tissue extracts have high concentrations of macromolecules like proteins and lipoproteins, this means the NMR signals of these macromolecules have to be removed in the spectra by employing diffusion editing methods to avoid overlapping with NMR signals from the other endogenous metabolites (Dieterle et al. 2011).

After recording of the NMR raw data, the NMR signals (FID) are processed by application of the Fourier transformation (FT) followed by a phase correction and baseline correction to obtain the final NMR spectrum. A typical example of a 600 MHz ^{1}H spectrum of rat urine is shown in Fig. 55.6.

EVALUATION

The next step applied to the NMR spectra is the exclusion of spectral regions without interest in metabonomics. This includes the exclusion of the water region between 4.6 and 5.0 ppm, as this region does not contain any information due to the water suppression. In urine samples also, the urea is excluded because the amplitude of the strong urea signal is falsified due to proton exchange with water.

Small changes in the chemical shift of NMR signals can be caused by variations of the pH, salt

concentrations, overall dilution, etc. To reduce this effect for data analysis and interpretation, different mathematical methods can be applied. Normally the equidistant binning method is used for this, which is the integration of the signals in small spectral regions called "bins" or "buckets" of 0.04 ppm, for example (Dieterle et al. 2011).

To better identify the changes of a NMR signal in samples with varying concentration like urine, normalization methods of the quantified concentration have to be performed. Mostly, creatinine normalization is used for which each peak is divided by the creatinine signals at 3.05 and 4.05 ppm. The assumption behind this method is that the excretion of creatinine into urine is constant over time. Additionally, integral normalization is used very often for which each peak is divided by the total integral of all peaks. Alternatively also quotient normalization can be used for which each peak is divided by the corresponding peak of a reference spectrum (Dieterle et al. 2011).

The last and most important, but also often very time intensive step then is the assignment of the changed NMR signals of interest. This can be performed by reference spectra, many reference spectra are also available in different public and commercial databases (Dieterle et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

The inter-laboratory comparability of NMR data was tested for a set of samples shipped to different laboratories. Data were acquired with NMR spectrometers operated at different field strengths. Compared to any other analytical technology, NMR shows an impressive analytical reproducibility and repeatability reflecting itself in a coefficient of variation of 2% for a study invoking a large set of spectra (Goldman 1991). Thus, the observed variances in NMR spectra of a biological study are highly dominated by biological effects (Dieterle et al. 2011). This is also supported by other authors who see the main advantages of NMR metabonomics in the nonselectivity, lack of sample bias, and cross-laboratory/cross-platform reproducibility and in the good reference databases that are available for NMR. Some of the limitations are the lower sensitivity compared to mass spectrometry and the issue with the resolving of many different metabolites in the same region of a NMR spectrum (Keun et al. 2002; Robertson 2005; Lindon et al. 2006).

MODIFICATION OF THE METHOD

With the use of two-dimensional NMR techniques, more structural information can be extracted from the NMR spectrum, which can be used especially for the elucidation of an unknown structure of a newly found biomarker in a biological sample. But the use of 2D NMR in metabonomics is limited to small sample arrays as the measuring time is up to several hours per sample (Dieterle et al. 2011).

Intact tissue samples can directly be analyzed only by magic angle spinning (MAS) NMR without sample extraction. For this a small sample of intact tissue is placed into the spectrometer and is analyzed directly. But this technology requires specialized equipment and expertise for the conduction and is therefore used less often like the other technologies (Robertson 2005).

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55.4.2 Liquid Chromatography: Mass Spectrometry (LC-MS)

PURPOSE AND RATIONALE

Other metabonomics technologies which are capable to analyze a complex mixture of molecules simultaneously include the separation of the different compounds by chromatographic methods, like liquid chromatography (LC) in combination with mass spectrometry (MS) used as detector. In LC analysis, liquid samples flow with a mobile phase at high pressure through a column that is packed with a stationary phase of different particles. In most metabonomics applications, a reversed-phase LC is used. The separation here is based on adsorption of the compounds on a nonpolar stationary phase followed by elution of the compounds as a result of the hydrophobic interaction with the mobile phase by using a gradient from a polar to nonpolar solvent. As a consequence compounds are eluted over time from the column based on their polarity with different retention times. These analyses are known as HPLC (high performance liquid chromatography) or UPLC (ultra performance liquid chromatography), which are using higher pressures that results in better separations. The opposite principle using a polar stationary phase and a gradient from a nonpolar to polar solvent is called normal phase LC. These approaches are known as HILIC (hydrophilic interaction liquid chromatography).

After LC separation, the compounds are analyzed and quantified by mass spectrometry (MS). For this the solvent that is coming from the column is vaporized, afterward the remaining compounds are ionized, and the charged molecules are accelerated and separated in electromagnetic fields according to their mass-to-charge (m/z) ratio. In most metabonomics investigations, electrospray ionization mass spectrometry (ESI-MS) is used as ionization method in negative mode (ESI−) or positive mode (ESI+).

PROCEDURE

The samples which can be used for metabonomics LC-MS analysis are the same already described for NMR analysis. The analysis of the noninvasively biofluid urine can be challenging due to the high salt content, the complex composition, and the varying dilution. To overcome these problems, urine samples can be desalted before LC-MS analysis by solid phase extraction (Wagner et al. 2007) or column switching procedures (Waybright et al. 2006).

For the analysis of blood plasma or serum, it is of key importance to selectively remove proteins before analysis without affecting the low molecular weight metabolome. This is mandatory to reduce signal suppression of low-abundance compounds and to avoid protein precipitation under reversed-phase (RP) liquid chromatography conditions. Several procedures for deproteinization exist, such as extraction of low molecular weight compound by organic solvents, acids, or denaturation of proteins by heat (Want et al. 2006; Boernsen et al. 2005; Trygg et al. 2005). With regard to reproducibility, number of metabolic features detected, and robustness, extraction by methanol followed by evaporation and resuspension in the mobile LC phase proved to be the best method (Dieterle et al. 2011).

The most common method for metabonomics analysis is a reversed-phase LC (e.g., LC column packed with C_{18} particles) by using a water/acetonitrile (both with 0.1% formic acid) gradient as mobile phase (e.g., starting from 5% to 90% acetonitrile). As mass analyzers, mainly quadrupole- and time-of-flight (TOF)-based analyzers and hybrid forms of them are used (Dunn and Ellis 2005; Ackermann et al. 2006). Quadrupoles are robust, flexible, have a high linear dynamic range, but are limited in full-scan data acquisition due to long duty cycles. TOF instruments on the other hand have fast scanning capabilities, wide mass range, and high resolution. Quadrupole TOF (Q-TOF) hybrid instruments combine the stability and robustness of the quadrupole analyzer with TOF features and allow for MS-MS experiments (Dieterle et al. 2011).

Nontargeted metabolite profiling approaches require a sensitive full-scan mode and exact masses. Therefore, Q-TOF instruments or linear ion trap FT-MS instruments are advantageous. In contrast, for targeted analysis of selected metabolites, triple quadrupole instruments and Q TRAP instruments with their capability for multiple reaction monitoring are frequently used (Dieterle et al. 2011).

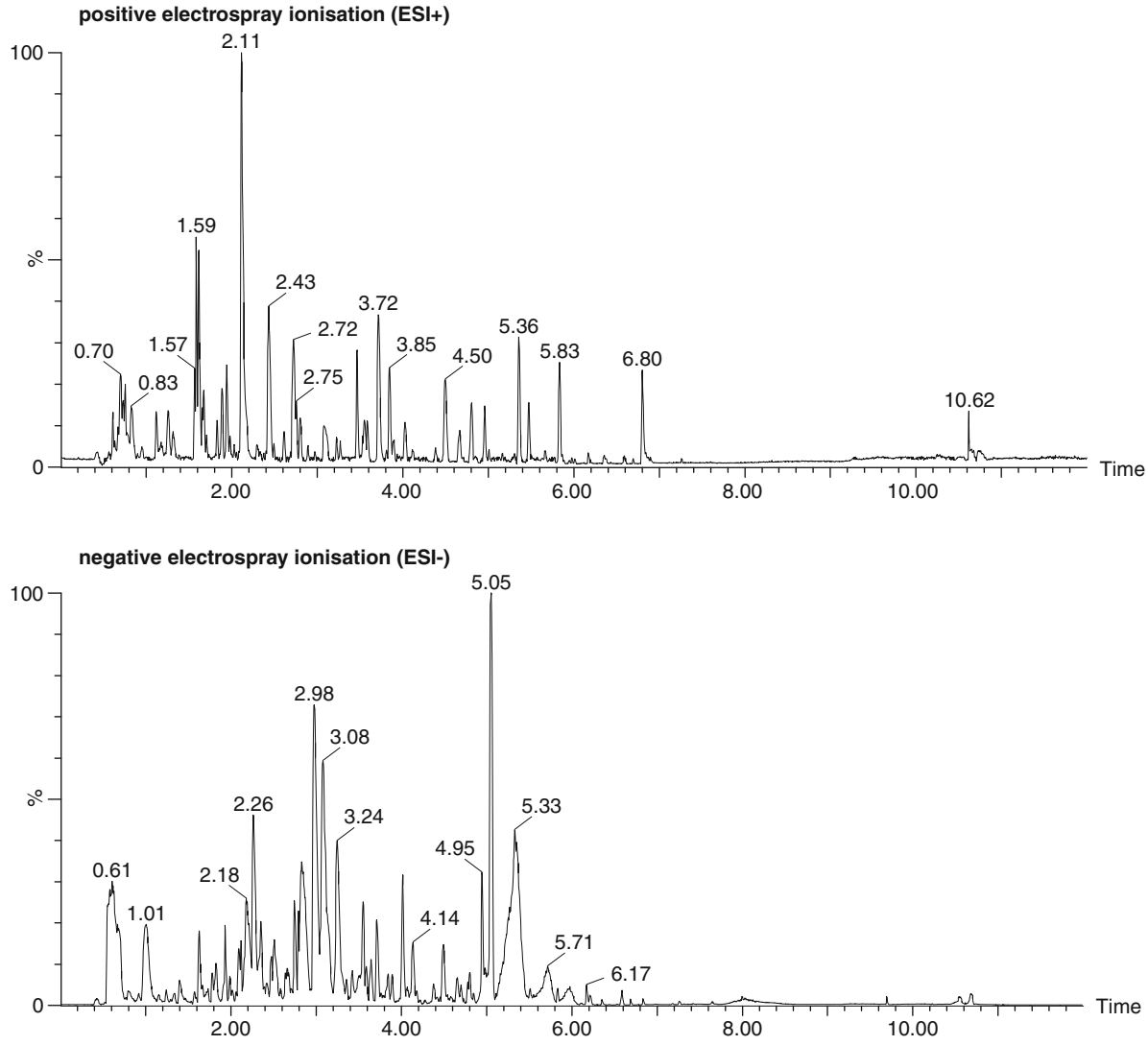

Fig. 55.7 Typical UPLC-TOF-MS chromatogram of rat urine with positive electrospray ionization (ESI+) and negative electrospray ionization (ESI−) after dilution of urine with water (1:3)

A typical example of an UPLC-TOF-MS chromatogram of rat urine is shown in Fig. 55.7.

EVALUATION

Full-scan LC-MS chromatograms contain many data (full mass spectra at the different retention times) from all the analyzed compounds in complex samples like urine, serum, or plasma. Therefore, an extraction of the relevant information (most expressed as m/z to retention time pairs) and de-noising from full-scan metabolic profiles is an important step in processing and statistical analysis of such LC-MS data. But a prerequisite for statistical analysis is to apply data alignment procedures and to reduce variance between samples that is not attributed to true differences. The major sources of such variances are nonlinear shifts in retention time, peak overlap, and m/z shifts. In general, three preprocessing strategies are used for LC-MS data sets. First, the spectra are aligned along the chromatographic and spectral axis. Then the dimensionality is reduced by binning or bucketing procedures. With the last step, significant peaks are automatically detected and quantified. Several commercial and open source routines for automatic

alignment, de-noising, deconvolution, and extraction of peak have been published and are reviewed by Katajamaa et al. (2007).

For samples with varying concentrations like urine, analogue normalization methods which are already described for NMR metabonomics analysis can be used.

Also in LC-MS metabonomics analysis, the last and most important step is the assignment of the changed LC-MS signals (m/z to retention time pairs) of interest with reference LC-MS spectra/chromatograms, which are also available in different public and commercial databases (Weckwerth and Morgenthal 2005; Dieterle et al. 2011).

CRITICAL ASSESSMENT OF THE METHOD

The main advantage of LC-MS metabonomics analysis is the better sensitivity with lower detection limits compared to NMR. This is very important in metabonomics analysis especially if novel biomarkers want to be identified. Other advantages are better resolutions by chromatographic separation of complex samples mainly for analysis of higher molecular compounds. As already described, some limitations of LC-MS metabonomics analysis are the potential for sample bias and the lower cross-laboratory/cross-platform reproducibility compared to NMR (Robertson 2005). But in summary, NMR and LC-MS approaches are highly complementary and use of both is often necessary for molecular characterization (Lindon et al. 2006).

MODIFICATION OF THE METHOD

HILIC (hydrophilic interaction liquid chromatography) as an alternative approach to reversed-phase LC was recently applied in metabonomics studies as a complementary tool to study polar metabolites (Idborg et al. 2005). However, until now, HILIC has not reached the level of reliability, stability, and reproducibility of HPLC or UPLC methods (Dieterle et al. 2011).

Also alternative mass spectrometer like ion traps, Fourier transform and Orbitrap instruments can be used in metabonomics analysis. Benefits of ion trap instruments are their capability to perform progressive fragmentation steps (MS^n), compact size, and fast full scanning but with low resolution. Linear ion traps quadrupole hybrid instruments (Q TRAP or QqLIT) combine the MS^n capabilities of ion trap instruments with the neutral loss and precursor ion scan capabilities of triple quadrupole instruments. Therefore, the shortcomings of both approaches are overcome.

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55.4.3 Gas Chromatography: Mass Spectrometry (GC-MS)

PURPOSE AND RATIONALE

An alternative chromatographic method which can be applied for metabonomics analysis is the use of gas chromatography (GC) in combination with mass spectrometry (MS) as detector. In GC analysis, gaseous compounds from the samples are analyzed or compounds which are vaporized before analysis. The compounds to be analyzed flow with an inert carrier gas, such as helium or nitrogen, as mobile phase through a column. The stationary phase in the column is a microscopic layer of a liquid film of a polymer coated on the surface of the column, normally a nonpolar but sometimes also a more polar stationary phase. The separation of a complex mixture is based on the interaction of the compounds from the sample between the liquid stationary phase and the gaseous mobile phase by using a temperature gradient of the gas and column which is located in an oven. As a consequence compounds are eluted over time from the column with different retention times which is based primarily on their boiling point or vapor pressure but also on the interaction with the stationary phase.

After GC separation, the compounds are analyzed and quantified by mass spectrometry (MS). This is very similar to the previously described LC-MS analysis, including ionization of the compounds and acceleration and separation in vacuum of the charged molecules in electromagnetic fields according to their mass-to-charge (m/z) ratio. As the ionization method in GC-MS metabonomics investigations, typically electron ionization (EI) is used or chemical ionization (CI) as a softer alternative method with the help of a reagent gas like methane.

PROCEDURE

The samples which can be used for metabonomics GC-MS analysis are the same already described for NMR and LC-MS analysis. But since these are primarily very polar samples and the prerequisite for GC-MS is the analysis of volatile compounds, sample preparations methods have to be performed before analysis. This includes normally an extraction procedure that maximizes the number and amounts of the endogenous metabolites combined with a derivatization that converts polar compounds (e.g., sugars, amino acids, organic acids, etc.) into volatile compounds (Dieterle et al. 2011). Most extraction procedures are based on methods from Bligh and Dyer's (Bligh and Dyer 1959; Peña-Alvarez et al. 2004) with little variations and optimization combined with 2-stage derivatization methods (Gullberg et al. 2004; Schröder et al. 2003). In the first step, a methoxymation converts aldehyde and keto groups into oximes using hydroxylamines or alkoxyamines to reduce the number of tautomeric forms (due to the limited rotation along the C = N bond). The second step of silylation then derivatizes polar functional groups (e.g., −OH, −SH, −NH) into trimethylsilyl groups (TMS ethers, TMS sulfides, TMS amines) resulting in more volatile compounds (Dieterle et al. 2011).

After this sample preparation, GC analysis is normally performed with a nonpolar column (e.g., DB5-MS with 0.18 μm film column) by using a temperature gradient typically from 70°C to 320°C with an increase of 15°C/min and a flow of 1 ml/min of the carrier gas helium. The mass analyzers in GC-MS are analogous to the previously described LC-MS mass analyzers, this means mainly quadrupole- and time-of-flight (TOF)-based analyzers are used.

As ionization methods in GC-MS, an EI or CI can be used depending on the results that are favored. With EI ionization, the molecules break down into different fragments that give some structural information of the molecules. On the other hand, CI ionization is a less energetic process and often results in less fragmentations and the formation of the molecular ion species to access the mass of the molecules (Dieterle et al. 2011).

A typical example of a GC-TOF-MS chromatogram of rat urine is shown in Fig. 55.8.

EVALUATION

Full-scan GC-MS chromatograms have the same format and are in principle the same like LC-MS chromatograms, they differ only in the chromatographic method that was used. Therefore, the same processing, normalization, and assignment methods previously described for the evaluation of LC-MS chromatograms are valid and are used for GC-MS chromatograms.

For GC-MS, reference spectra/chromatograms are also available in different public and commercial databases (Dieterle et al. 2011).

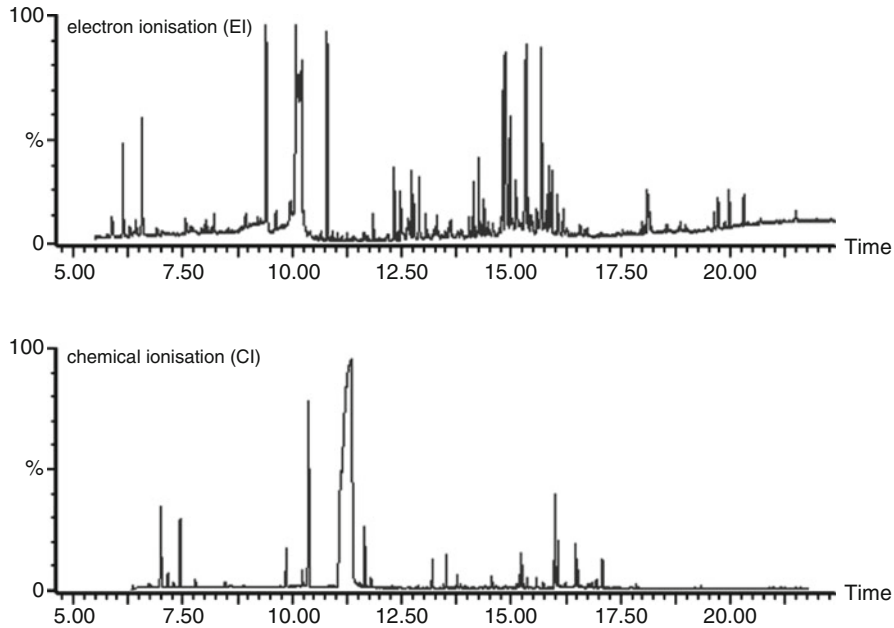

Fig. 55.8 Typical GC-TOF-MS chromatogram of rat urine with electron ionization (EI) and chemical ionization (CI) after extraction and silylation derivatization of the urine

CRITICAL ASSESSMENT OF THE METHOD

All advantages and limitations which were already described for the LC-MS analysis are also valid for metabonomics analysis with GC-MS compared to NMR. Further advantages are that GC-MS analyses show no problems with ion suppression of co-eluting compounds as observed in LC-MS analysis. Also, the assignment of the identity of peaks via a database of mass spectra is straightforward, due to the extensive and reproducible fragmentation patterns obtained in full-scan mode. Further limitations of GC-MS analyses exist for thermally labile compounds at the temperatures required for their separation or for compounds that are not volatile at all. Also since most endogenous metabolites in metabonomics analysis contain polar functional groups and are therefore less volatile, additional sample preparation steps of derivatization prior to GC-MS analysis are needed in most analysis to extend the application range of GC-based methods (Koek et al. 2011). But in summary, also GC-MS approaches are highly complementary to NMR and LC-MS approaches and the use of all the different technologies is often necessary for molecular characterization (Lindon et al. 2006).

MODIFICATION OF THE METHOD

Besides normal GC-MS also two-dimensional GC×GC-MS techniques can be used to increase the resolution of peaks in complex mixtures (Shellie et al. 2005; Van Mispelaar et al. 2003). Many different columns are available for GC analysis with different stationary phases, common GC columns are packed, for example, with different polysiloxane, polyethylene glycol polymers, resulting in columns with different polarities and other different characteristics.

Modifications in the mass analyzers which were already described for LC-MS analysis are also valid for metabonomics analysis with GC-MS (Weckwerth and Morgenthal 2005; Dieterle et al. 2011).

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