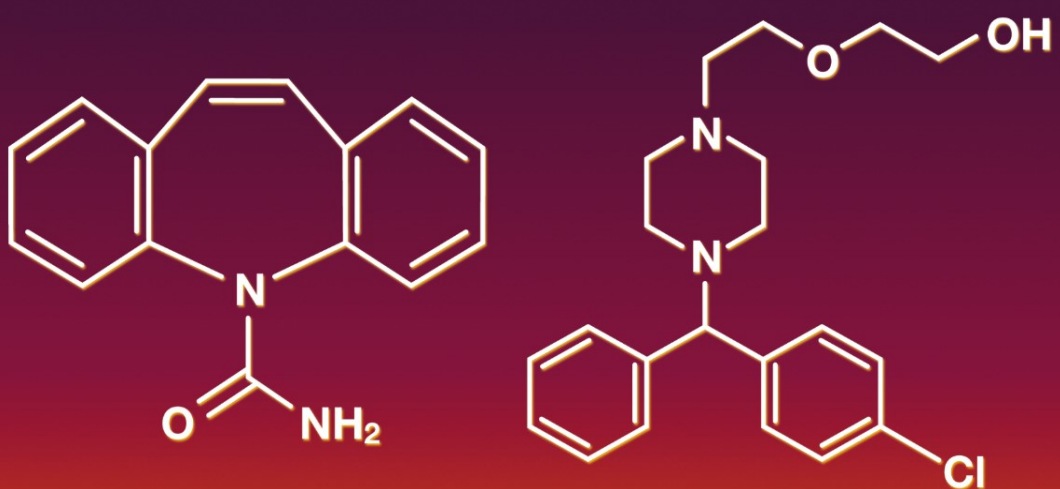
# HANDBOOK OF DRUG MONITORING METHODS

## *Therapeutics and Drugs of Abuse*

Edited by

**Amitava Dasgupta**, PhD, DABCC, FACB

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*To my wife Alice*


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# Preface

Starting from the 1970s, therapeutic drug monitoring has evolved from monitoring concentrations of a few antiepileptic drugs to a major discipline in today's laboratory practice. For a drug with a narrow therapeutic range, therapeutic drug monitoring becomes an essential part of patient management, especially because of the development of immunoassays for measuring concentrations of drugs in a biological matrix. In current practice, 15–20 therapeutic drugs are routinely monitored even in medium-size clinical laboratories, and a list of well over 50 therapeutic drugs can be found in the laboratory test guides of major medical centers in the United States, academic medical centers, and reference laboratories. These centers not only employ immunoassays but also take advantage of sophisticated analytical techniques such as gas chromatography/mass spectrometry and high-performance liquid chromatography coupled with mass spectrometry for therapeutic drug-monitoring services.

Similarly, in the past two decades, drugs of abuse testing became a routine part of emergency room testing and clinical laboratory service. Federal and state governments, as well as the private sector, now recognize the necessity of a drug-free work environment. Moreover, drug testing is a routine part of law enforcement activity in crime and forensic laboratories. Strict laws are also enforced throughout the United States against driving under the influence of alcohol. Therefore, alcohol and drug testing is an important component of most toxicological laboratory services.

*Hand book of Drug Monitoring* attempts to bridge different analytical techniques used in today's practice of therapeutic drug monitoring and drugs of abuse as well as alcohol testing with relevant theory, mechanism, and in-depth scientific discussion on each topic. As a handbook at the bench of a clinical laboratory the book serves as a quick reference to find the potential source of a false-positive or a false-negative result. At the same time, this book is a reference for medical technologists, supervisors, laboratory directors, clinical chemists, toxicologists, and pathologists looking in-depth for the cause of a potential interference, as well as a guide to the tests that can be ordered to circumvent such problem.

The book has 22 chapters, 13 focusing on various issues of therapeutic drug monitoring, one on analysis of heavy metals, one on alcohol testing, and seven on issues of drugs of abuse testing. Chapters are written by experts in their relative subspecialties and also by the editor. I am grateful to this outstanding group of contributors because without their generosity and dedication this book would never have been written.

The chapters on therapeutic drug monitoring cover a wide range of topics from clinical utility of free drug monitoring to interferences in digoxin assay, and include issues in monitoring anticonvulsant drugs, immunosuppressants, tricyclic antidepressants, and antiretrovirals used in treating AIDS patients. One chapter is focussed on common interferences from endogenous substances such as bilirubin, hemoglobin, and lipids in immunoassays for therapeutic drugs. Another chapter is dedicated to

interferences from heterophilic antibodies in therapeutic drug monitoring. Pharmacogenomics and personalized medicine is the future frontier of therapeutic drug monitoring. Chapter 11 is dedicated to this subject.

Use of complementary and alternative medicines by the general population is increasing steadily not only in the United States but also worldwide. Unexpected concentrations of therapeutic drugs because of use of complementary and alternative medicine have been well documented in the literature; for example, low concentrations of many therapeutic drugs because of self-medication with St. John's Wort, an herbal antidepressant. Chapter 13 is dedicated to important issues of drug–herb and drug–food interactions and their impact on therapeutic drug monitoring.

People try to beat drug tests to avoid consequences of a failed test. Chapter 17 discusses common household adulterants, as well as other adulterants such as nitrite, pyridinium chlorochromate, and glutaraldehyde, which people add in vitro to their urine to cheat on drug tests. Routine specimen integrity testing may not detect some adulterants, and practical tips are given in Chapter 17 to identify such adulterated specimens. There is much interference with drugs of abuse testing of the amphetamine class. Therefore, Chapter 20 addresses this important issue. Designer drugs and rave party drugs may escape detection in routine laboratory procedures for drugs of abuse testing. This topic and how to avoid such pitfalls are addressed in Chapter 19. Alternative specimens for drugs of abuse testing such as hair, saliva, sweat, and meconium are the topics of Chapter 18.

An analytic true positive may be a clinically false positive, for example, positive opiate test because of ingestion of poppy seed product. This important issue is addressed in Chapter 21, which will be helpful to medical review officers as well as to any toxicologist. Another chapter is dedicated to the topic of expert witness testimony by technologists performing alcohol and drugs of abuse testing and toxicologists supervising such tests, when often called as factual or expert witnesses in a court of law.

I express my sincere thanks to Robert L. Hunter, MD, PhD, Professor and Chairman, Department of Pathology and Laboratory Medicine, University of Texas-Houston Medical School, for his continued support for the last one-and-half years as I worked on this project. In addition, he critically read all chapters I wrote and made excellent recommendations for improvement. Alice Wells, MT(ASCP), read the entire manuscript and checked whether references were put in correct order and number and also helped me in editing this book. John Mohr, PharmD, assistant professor of internal medicine at our institution, reviewed therapeutic ranges and helped me with important suggestions. I also thank him. I also thank two of our pathology residents, Michelle Rodriguez, MD, and Anna Richmond, MD, for critically reading several chapters and making helpful suggestions. Last but not least, I express my thanks to my wife Alice for tolerating the long hours spent on this project and her continued support. Finally, readers will be the judge of the final success of this project. If they find this book helpful, we will feel our effort is well rewarded.

*Amitava Dasgupta*

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# 1 Introduction to Therapeutic Drug Monitoring

*Amitava Dasgupta, PhD*

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**Summary**

Therapeutic drug monitoring is defined as measuring serum concentrations of a drug in a single or multiple time points in a biological matrix after a dosage. The purpose of therapeutic drug monitoring is to individualize the dosage to achieve maximum efficacy of a drug and at the same time minimize adverse drug reactions. Therapeutic drug monitoring has clinical importance for drugs with a narrow therapeutic window, such as various anticonvulsants, cardioactive drugs, theophylline, immunosuppressants, tricyclic antidepressants, antiretroviral drugs, certain antibiotics, and neoplastic drugs. Altered pharmacokinetic parameters are observed for many drugs in disease states including hepatic and renal impairment, cardiovascular disease, thyroid dysfunction, and cystic fibrosis. Altered drug disposition also occurs in pregnant women. Therapeutic drug monitoring helps to identify such altered drug disposition, and dosage adjustment can be made for proper management of the patient to avoid adverse reactions. Therefore, therapeutic drug monitoring is cost effective in heath care.

**Key Words:** Anticonvulsants; antineoplastic drugs; cardioactive drugs; immunosuppressants; pharmacokinetics; therapeutic drug monitoring; tricyclic antidepressants.

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## 1. INTRODUCTION

Pharmacological response of a drug given in a selected dosing regimen depends on several factors, including compliance of the patient, bioavailability of the drug, rate of drug metabolism (depending on the genetic make up of the patient) as well as the protein-binding ability of the drug. It is well established that only unbound (free) free drug can bind with the receptor and produce the desired effect. For certain drugs, a good correlation exists between serum drug concentrations and pharmacological response. Therefore, monitoring serum concentrations of these drugs is beneficial for patient management especially if the drugs have very narrow therapeutic ranges. Moreover, for these drugs, adjustment of dosing may be more useful based on serum drug concentration rather than routine assessment of a patient. For example, adjusting phenytoin dosing in patients based on their serum phenytoin concentrations rather than seizure frequencies not only decrease the morbidity but also prevent unnecessary toxicity of phenytoin in these patients. Peterson et al. *(1)* reported that in their study with 114 patients, total phenytoin concentrations provided as good an indication of the clinical response as the free phenytoin concentrations in most patients, but in 14.2% patients, free phenytoin concentrations were better correlated with clinical picture than total phenytoin concentrations. Another report indicated that quality of life improved in a group of patients with congestive heart failure where digoxin dosing was based on target therapeutic concentrations *(2)*.

Therapeutic drug monitoring has been used in clinical practice to individualize drug therapy since the beginning of the 1970s. The goal of therapeutic drug monitoring is to optimize pharmacological responses of a drug while avoiding adverse effects. Usually for drugs that are routinely monitored in clinical laboratories, serum concentrations are a better predictor of desired pharmacological effects than the dose. Moreover, therapeutic drug monitoring is also utilized to monitor a patient's compliance with a drug regimen and to identify potential drug–drug or food–drug interactions.

Therapeutic drug monitoring not only consists of measuring the concentration of a drug in a biological matrix but it also involves the proper interpretation of the value using pharmacokinetic parameters, drawing appropriate conclusion regarding the drug concentration and dose adjustment. The International Association for Therapeutic Drug Monitoring and Clinical Toxicology adopted the following definition, "Therapeutic drug monitoring is defined as the measurement made in the laboratory of a parameter that, with appropriate interpretation, will directly influence prescribing procedures. Commonly, the measurement is in a biological matrix of a prescribed xenobiotic, but it may also be of an endogenous compound prescribed as a replacement therapy in an individual who is physiologically or pathologically deficient in that compound" *(3)*.

Only a fraction of drugs currently used worldwide require routine monitoring. For a drug in which the gap between therapeutic and toxic concentration is wide, therapeutic drug monitoring is not indicated unless in the case of intentional overdose, for example with salicylate or acetaminophen. In an Italian collaborative study on the utilization of therapeutic drug monitoring, it was noted that only 16.3% of the population was given drugs for which therapeutic drug monitoring was available in the hospital. Digoxin was the most frequently ordered drug in their study population *(4)*.

## 2. IMPLICATIONS OF THERAPEUTIC DRUG MONITORING

When appropriate, patients gain both medically and economically from therapeutic drug monitoring. Many reports in the literature indicate that therapeutic drug monitoring can decrease hospital stay and have important implications on the cost of medical care. Reduced drug-related toxicities are beneficial for patients and also diminish the liability of physicians. Ried et al. *(5)* evaluated the effectiveness of therapeutic drug monitoring in reducing toxic drug reactions by meta-analysis of 14 studies. The authors concluded that patients monitored for appropriate drugs suffered fewer toxic drug reactions than patients for whom therapeutic drug monitoring was not undertaken. Another study reported that determination of serum drug concentrations and evaluation of such results by clinical pharmacists resulted in significant cost savings *(6)*. Crist et al. evaluated the impact of therapeutic drug monitoring of aminoglycoside (gentamicin or tobramycin) using 221 patients on the length of hospital stay, cost effectiveness, and related factors. The mean length of hospital stay was 8.4 days in the patient group that received individualized aminoglycoside doses (study group) versus 11.8 days in the control group. In addition, the hospital cost was lower by $725 per patient in the study group which would produce a savings of $640,000 at the author's institution *(7)*.

## 3. CHARACTERISTICS OF DRUGS REQUIRE MONITORING

Drugs that are candidates for therapeutic drug monitoring have several characteristics. A list of commonly monitored and less frequently monitored therapeutic drugs is given in Table 1. The following are the characteristics of a drug where monitoring is beneficial:

1. Narrow therapeutic range where the dose of a drug that produces the desired therapeutic concentrations is near the dose that may also produce toxic serum concentration.
2. There is no clearly defined clinical parameter that allows dose adjustments.
3. There is an unpredictable relationship between dose and clinical outcome. For example, a certain dose may produce a desirable pharmacological response in one patient but the same dose may cause toxicity in another patient.
4. Toxicity of a drug may lead to hospitalization, irreversible organ damage, and even death.
5. There is a correlation between serum concentration of the drug and its efficacy as well as toxicity. For strongly protein-bound drugs (protein binding >80%), a better correlation may be observed between unbound (free) drug concentration and clinical outcome rather than between traditionally monitored total drug concentration (free + protein bound) and clinical outcome. This is particularly applicable to a special patient population with hepatic or renal impairment. Moreover, elderly patients and critically ill patients may also demonstrate elevated concentrations of free drugs. Therefore, for these patients, monitoring free drug concentrations (free phenytoin, free valproic acid, free carbamazepine etc) is strongly recommended instead of monitoring total drug concentrations. Clinical utility of free drug monitoring is discussed in detail in Chapter 2.

**Table 1**
**Commonly and Less Frequently Monitored Therapeutic Drugs in Clinical Laboratories**

| *Class of Drug* | *Commonly Monitored* | *Less Frequently Monitored* |
|---|---|---|
| Anticonvulsants | Phenytoin[a], carbamazepine[a] Valproic acid[a], phenobarbital[a] Primidone[a], ethosuximide[a] Lamotrigine | Diazepam, clonazepam Felbamate, methsuximide Gabapentin, zonisamide |
| Cardioactive | Digoxin[a], quinidine[a] Disopyramide[a], lidocaine[a] Procainamide[a], NAPA[a] | Flecainide, verapamil Mexiletine, tocainide Propanol, amiodarone |
| Antiasthmatic | Theophylline[a], caffeine[a] | |
| Immunosuppressants | Cyclosporine[a], tacrolimus[a] Mycophenolic acid[a] | Sirolimus, Everolimus |
| Antidepressants | Amitriptyline, nortriptyline Doxepin, imipramine Desipramine, clomipramine Trimipramine, lithium[b] | Fluoxetine/norfluoxetine Paroxetine, sertraline Haloperidol |
| Antibiotic | Amikacin[a], gentamicin[a] Tobramycin[a], vancomycin[a] | Ciprofloxacin, cefazolin Chloramphenicol, nafcillin |
| Antiviral | | Indinavir, nelfinavir Ritonavir, saquinavir Delavirdine, nevirapine |
| Antineoplastic | Methotrexate[a] cisplatin | Doxorubicin, tamoxifen Cyclophosphamide, 5-fluorouracil |
| Analgesic | Acetaminophen[a], salicylate[a] | Ibuprofen, pentobarbital |

[a] Immunoassay commercially available.
[b] Automated assay commercially available.

## 4. FACTORS AFFECTING SERUM DRUG CONCENTRATIONS

The serum concentration of a particular drug is determined by absorption, distribution, metabolism, and excretion of a drug. Major characteristics that affect serum drug concentrations include genetic make up of a patient as well as age, gender, weight, habits (such as smoking), and diet. Elderly and newborns may metabolize a particular drug more slowly than others. Some drugs, for example theophylline, distributes to lean weight only where other drugs, such as phenytoin, distributes to total weight. Diseases may alter serum drug concentrations dramatically. Hepatic disease may alter metabolism of a drug where a patient with renal failure may clear a drug in urine more slowly than a patient with normal renal function. Pregnancy alters metabolism of several drugs while drug–drug interactions may also significantly alter serum drug concentrations.

### *4.1. Pharmacokinetics and Serum Drug Concentrations*

When a drug is given orally, it undergoes several steps in the body and its concentration in serum or whole blood is affected by certain steps.

1. *Liberation:* The release of a drug from the dosage form (tablet, capsule, extended release formulation)
2. *Absorption:* Movement of drug from site of administration (for drugs taken orally) to blood circulation
3. *Distribution:* Movement of a drug from the blood circulation to tissues. This distribution in most cases is reversible. Certain drugs also cross the blood brain barrier.
4. *Metabolism:* Chemical transformation of a drug to the active and inactive metabolites. Cytochrome P450 enzyme system is the major drug-metabolizing agent of body.
5. *Excretion:* Elimination of the drug from the body through renal, biliary, or pulmonary mechanism.

Liberation of a drug after oral administration depends on the formulation of the dosage. Immediate release formulation releases the drugs at once from the dosage form when administered. On the contrary, the same drug may also be available in sustained release formulation. The rationales for specialized oral formulations of drugs include prolongation of the effect for increased patient convenience and reduction of adverse effects through lower peak plasma concentrations. Local and systematic adverse effects of a drug can also be reduced by use of controlled release delivery systems *(8)*. Over the past decade, there has been a significant growth in the introduction of these new formulations of existing drugs designed to improve patient management *(9)*. Controlled release dosage formulations include osmotic pumps and zero-order kinetics system to control the release rate of a drug, bio-adhesive systems and gastric retention devices to control gastrointestinal transit of a drug, bio-erodible hydrogels; molecular carrier system such as cyclodextrin-encapsuled drugs; externally activated system; and colloidal systems such as liposomes and microspheres *(8)*. The effect of food intake on bioavailability of a drug is more apparent on a single unit non-disintegrating dosage form, although controlled release formulations are not completely immune from the food intake. Polymers occupy a major portion of materials used for controlled release formulations and drug-targeting systems because this class of substances presents seemingly endless diversity in chemistry and topology *(10)*. Microparticles are small solid particulate carriers containing dispersed drug particles either in solution or in crystalline form. The importance of microparticles is growing because of their utilization as carriers for drugs and other therapeutic agents. Microparticles are made from natural or synthetic polymers. Different materials have been used for microparticles systems, such as albumin, gelatin, starch, ethyl cellulose, and synthetic polymers, such as poly lactic acid, poly cyanoacrylates, and poly hydroxybutyrate *(11)*. Enteric coded formulations resist gastric acid degradation and deliver drugs into the distal small intestine and proximal colon. Budesonide, a synthetic glucocorticoid with high topical anti-inflammatory activity and little or no systemic effect, has been administered through inhalation for the treatment of inflammatory airways infection. Budesonide is also manufactured into two commercially available oral control release formulations, and both the formulations are enteric coded *(12)*. Recently, enteric coded formulation of mycophenolic acid mofetil, a prodrug of immunosuppressant mycophenolic acid is commercially available *(13)*. Solid nanoparticles were introduced in the 1990s as an alternative to microemulsions, polymeric nanoparticles, and liposomes. These nanoparticles have several advantages such as biocompatibility and their capability of controlled and targeted drug release *(14)*.

Oral controlled release drug delivery systems can be further classified into two broad categories; single-unit dosage forms (SUDFs) such as tablets or capsules and multiple-unit dosage forms (MUDFs) such as granules, pellets, or mini-tablets. Mini-tablets are tablets with a diameter equal to or smaller than 2–3 mm *(15)*. Several mini-tablets can be either filled into hard capsules or compacted to a bigger tablet that after disintegration releases these subunits as multiple dosage form. Many drugs are available in sustained release formulations. For example, the immediate release venlafaxine, an antidepressant formulation, requires twice-daily administration whereas the extended release formulation is designed for once-daily administration. Another antidepressant fluoxetine is available in a sustained release dosage form, which requires once-weekly administration for continuation of therapy for depression *(16)*. Calcium channel antagonists are a heterogenous group of drugs with different cardiovascular effects and are effective in the treatment of hypertension and angina pectoris. A number of these agents are commercially available in sustained release formulations *(17)*. Anticonvulsants, such as carbamazepine and valproic acid, are also available in sustained release formulations *(18,19)*. Theophylline is available in prolonged release form *(20)*. Procainamide, a class IA antiarrhythmic drug, is also administered as sustained release formulation *(21)*. McCormack and Keating *(22)* recently reviewed the use of prolonged release nicotinic acid in treating lipid abnormality.

Absorption of a drug depends on the route of administration as well as drug formulation. Generally, an oral administration is the route of choice in the practice of pharmacotherapy, but under certain circumstances (nausea, vomiting, convulsions etc), rectal route may present a practical alternative for drug administration. Rectal administration is now well accepted for delivering anticonvulsants, non-narcotic and narcotic analgesics, theophylline, and antibacterial and antiemetic agents. This route can also be used for inducing anesthesia in children. The rate and extent of rectal drug absorption are often lower compared with oral absorption possibly because of small surface area available for drug absorption. The composition of rectal formulation (solid vs. liquid, nature of suppository) also plays an important role in the absorption of a drug. However, for certain drugs, rectal absorption is higher compared with absorption of the same drug given orally. This phenomenon may be due to avoidance of the hepatic first-pass metabolism after rectal delivery. These drugs include lidocaine, morphine, metoclopramide, ergotamine, and propranolol. Local irritation is a possible complication of rectal drug delivery *(23)*.

When a drug is administered by direct injection, it enters the blood circulation immediately. Sometimes, a drug may be administered by the intravenous or intramuscular route as a prodrug if the parent drug has potential for adverse drug reactions at the injection site. Fosphenytoin is a phosphate ester prodrug of phenytoin developed as an alternative to intravenous phenytoin for acute treatment of seizure. However, the bioavailability of derived phenytoin from fosphenytoin relative to intravenous phenytoin administration is almost 100% *(24)*.

There is considerable interest to deliver a drug through the transdermal route. However, the skin, particularly the stratum corneum, poses a formidable barrier to drug penetration, thus limiting topical and transdermal bioavailability of a drug *(25)*. As early as in 1967, it was demonstrated that the bioavailability of topically applied hydrocortisone alcohol was only 1.7% *(26)*. For a drug to be delivered passively

through the skin, it should have adequate lipophilicity and a molecular weight <500 D *(27)*. Penetration enhancement techniques are usually used to improve bioavailability following transdermal delivery of a drug. This enhancement technique is based on drug/vehicle optimization such as drug selection, prodrug and ion pairs, supersaturated drug solutions, eutectic systems, complexation, liposome vesicles, and particles. Enhancement through modification of stratum corneum by hydration, and chemical enhancers acting on the lipids and keratin of stratum corneum are also utilized for transdermal drug delivery *(25)*. Major routes of administration of drugs in a patient and its advantages and disadvantages are summarized in Table 2.

When a drug enters the blood circulation, it is distributed throughout the body to various tissues. The pharmacokinetic term most often used to describe distribution is called volume of distribution ($V_d$). This is the hypothetical volume to account for all drugs in the body and is also termed as the apparent $V_d$

$$V_d = \text{Dose/plasma concentration of drug}$$

The amount of a drug at a specific site, where it exerts its pharmacological activity or toxicity, is usually a very small fraction of the total amount of the drug in the body because of its distribution in tissue and blood. Even in a target tissue, only a fraction of the drug binds with the receptors and exerts its pharmacological activity. Protein binding of a drug also limits its movement into tissues. Muscle and fat tissues may serve

**Table 2**
**Routes of Administration of Drugs and Their Advantages as well as Disadvantages**

| *Route* | *Advantages* | *Disadvantages* |
|---|---|---|
| Oral | Route of choice because of ease of administration | Longer time to peak level; Food, alcohol may affect levels |
| | Sustained release formulation prolonged effect | Gastric-emptying time, First-pass metabolism affect levels |
| Rectal | Can be used if patient has nausea, vomiting, convulsion<br>Inducing anesthesia in children<br>Few drugs show higher absorption compared with oral route because of avoidance of first-pass metabolism such as lidocaine | Absorption may be low; Local irritation |
| Intravenous | Rapid peak concentration and action | Need a intravascular access for administration/discomfort |
| Intramuscular | No first-pass metabolism<br>100% Bioavailability | |
| Transdermal | Ease of application | Poor systematic absorption |
| Sublingual | Rapid absorption and action<br>Ease of application | First-pass metabolism |

as a reservoir for lipophilic drugs. For central nervous system drugs (neurotherapeutics), penetration of blood brain barrier is essential. Usually, moderately lipophilic drugs can cross the blood brain barrier by passive diffusion, and hydrogen-bonding capacity of a drug can significantly influence the central nervous system uptake. However, drugs may also cross the blood brain barrier by active transport *(28)*. When a CNS drug is given as a prodrug, a delay may be observed in the accumulation of the drug in the brain because of the time required for conversion of the prodrug to the original drug. Walton et al. *(29)* reported that when fosphenytoin, the prodrug of phenytoin, was administered in rats, lower brain levels of phenytoin were typically observed compared with brain phenytoin levels when phenytoin was directly administered in rats. Many drugs do not effectively penetrate the blood brain barrier. Ningaraj et al. *(30)* recently commented on challenges in delivering new anticancer drugs to brain tumors because most new anticancer drugs that are effective outside the brain have failed in clinical trials in treating brain tumors, in part because of poor penetration across the blood brain barrier and the blood brain tumor barrier. However, there are also advantages when a drug does not effectively penetrate the blood brain barrier. Second generation antihistamines have a low tendency to cross the blood brain barrier and thus reduce sedation and impairment in patients *(31)*.

Drugs usually undergo chemical transformation before elimination, and the process is termed as metabolism. Drug metabolism may occur in any tissue including the blood. For example, plasma cholinesterase, a glycoprotein synthesized in the liver metabolizes drugs such as cocaine and succinylcholine. Hoffman et al. *(32)* reported that decreased plasma cholinesterase activity is associated with the increasing risk of life-threatening cocaine toxicity. However, the liver is the main site for drug metabolism. The role of metabolism is to convert lipophilic non-polar molecules to more polar water-soluble compounds for effective excretion in urine. The drug molecule can be modified structurally (oxidation, reduction, or hydrolysis), or the drug may undergo conjugation (glucuronidation, sulfation) that increases its polarity. The rate of enzymatic process that metabolizes most drugs is usually characterized by the Michaelis–Menten equation and follows first-order kinetics (rate of elimination is proportional to drug concentration). However, for certain drugs for example, phenytoin, the metabolism is capacity-limited.

The half-life of a drug is the time required for the serum concentration to be reduced by 50%. The fraction of a drug that remains in the body after five half-lives is approximately 0.03 (Fig. 1). However, after multiple doses, usually a drug reaches a steady state after five to seven half-lives. Half-life of a drug can be calculated from elimination rate constant (K) of a drug.

$$\text{Half-life} = 0.693/\text{K}$$

Elimination rate constant can be easily calculated from the serum concentrations of a drug at two different time points using the formula where $Ct_1$ is the concentration of drug at a time point $t_1$ and $Ct_2$ is the concentration of the same drug at a later time point $t_2$:

$$K = \frac{\ln Ct_1 - \ln Ct_2}{t_2 - t_1}$$

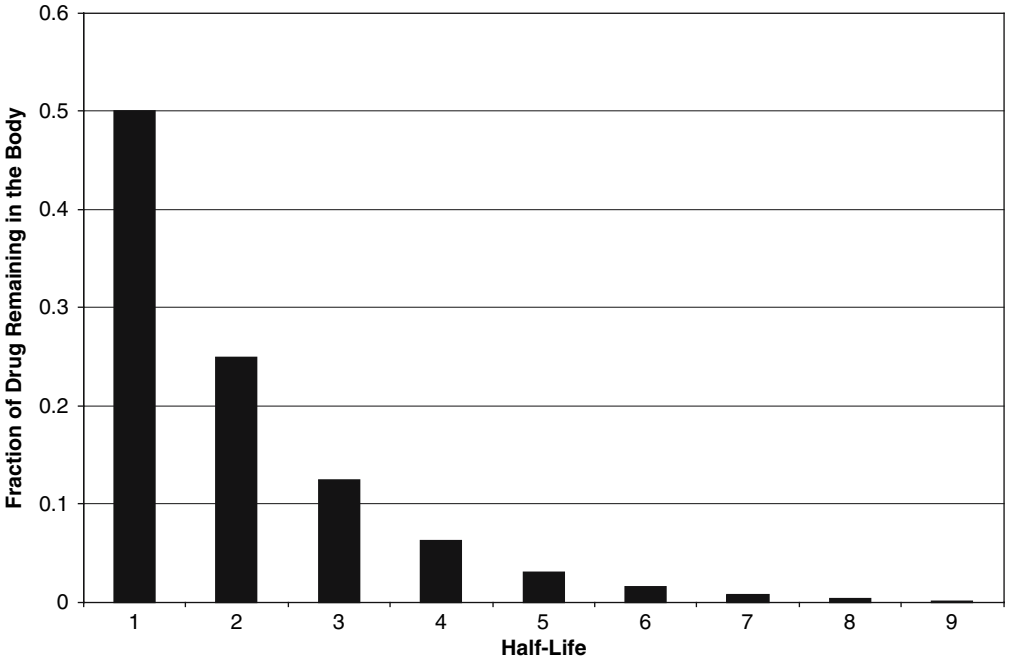


**Fig. 1.** Fraction of drug (given in a single dose) remaining in the body after different time (half-life) periods.

A drug may also undergo extensive metabolism before fully entering the blood circulation. This process is called first-pass metabolism. The drugs that are eliminated by conjugation (estrogen, progesterone, morphine, etc.) undergo significant first-pass metabolism because the gut is rich in conjugating enzymes. Factors such as gender, disease state, enzyme induction and inhibition, genetic polymorphism, and food may cause significant variability in pharmacokinetics of a drug undergoing first-pass metabolism. Drug concentrations obtained from individuals given the same dose may vary even sevenfold *(33)*.

Renal excretion is a major pathway for the elimination of drugs and their metabolites. Therefore, impaired renal function may cause accumulation of drugs and metabolites in serum, thus increasing the risk of adverse drug effect. This may be particularly important for drugs that have active metabolites, such as procainamide and carbamazepine. Moreover, other pathological conditions such as liver disease, congestive heart failure, and hypothyroidism may also decrease clearance of drugs. Drugs may also be excreted through other routes, such as biliary excretion. The factors that determine elimination of a drug through the biliary track include chemical structure, polarity, and molecular weight as well as active transport sites within the liver cell membranes for that particular drug. A drug excreted in bile may be reabsorbed from the gastrointestinal track or a drug conjugate may be hydrolyzed by the bacteria of the gut, liberating the original drug, which can return into the blood circulation. Enterohepatic circulation may prolong the effects of a drug. Cholestatic disease states, in which flow of normal bile flow is reduced, will reduce bile clearance of a drug and may cause drug toxicity *(34)*. Moreover, drug–drug interaction may involve bile clearance pathway of

a drug. For example, quinidine not only reduces renal clearance of digoxin but also causes an average reduction of 42% in bile clearance of digoxin *(35)*.

### *4.2. Genetic Factors Affecting Serum Drug Concentrations*

There are wide variations in a patient's response to drug therapy. One patient may demonstrate desirable pharmacological effect after administration of a particular dose of a drug whereas another patient may show only subtle effects. Although such variability may be related to renal disease or liver disease or due to drug–drug interactions, alteration of drug-metabolizing capacity caused by hereditary enzymatic deficiency or over-expression may also lead to an altered response of a patient to a drug. As early as in 1964, Kurt et al. *(36)* reported that phenytoin toxicity in a patient receiving the usual dose of phenytoin was probably related to a rare genetic deficiency in phenytoin hydroxylation.

Although over 15 different enzymes have been identified in the liver, in practice the cytochrome P450 isoenzymes that mediate the oxidative metabolism of many drugs include CYP1A2, CYP2C9, CYP2D6, CYP2E1, and CYP3A4. These enzymes show marked variations in different people. Some of these enzymes also exhibit genetic polymorphism (CYP2C19, CYP2D6), and a subset of the population may be deficient in enzyme activity (poor metabolizer). Therefore, if a drug is administered to a patient who is a poor metabolizer, drug toxicity may be observed even with a standard dose of the drug. Phenotyping procedures commonly involve administration of a probe drug and calculation of the urine or plasma metabolic ratio. CYP3A4 is the most abundant hepatic oxidative enzyme in the liver, and it accounts for almost 30% of the cytochrome P450 enzyme system *(37)*. This isoenzyme is also present in significant amounts in the epithelium of the gut, and orally administered drugs, which are substrate of CYP3A4, may undergo significant metabolism before entering circulation. CYP3A4 exhibits significant inter-individual variation that may be as high as 20-fold. The knowledge that a drug is metabolized by a certain cytochrome P450 enzyme is indicative that this drug can competitively inhibit the metabolism of other drugs, which are also substrates of this enzyme. Often the cytochrome P450 can be induced by another drug or a herbal supplement, such a St. John's wort resulting in a lower plasma concentration of a drug because of increased metabolism of the drug. Drug interactions with St. John's wort are discussed in detail in Chapter 13.

Pharmacogenomics approach to personalized medicine is based on the utilization of genetic information data in pharmacotherapy and drug delivery thus ensuring better drug efficacy and safety in patient management. Currently, the concept of personalized medicine and pharmacogenetics are likely to improve the areas of pharmacokinetics and pharmacodynamics because genetic polymorphisms have already been detected and analyzed in genes coding drug-metabolizing enzymes, transporters as well as well as target receptors. The potential of applying genotyping and haplotype analysis in future medical care could eventually lead to pharmacotyping referring to individualized drug delivery profiling based on genetic information *(38)*. The United States Federal Drug Administration (FDA) has granted market approval for the first pharmacogenetic testing using a DNA microarray, the AmpliChip CYP450, which genotypes cytochrome P450 (CYP2D6 and CYP2C19). The test uses software to predict phenotypes and tests for 27 CYP2D6 alleles *(39)*. Pharmacogenomics issues are discussed in Chapter 11.

### *4.3. Gender Differences and Serum Drug Concentrations*

Biological differences between men and women result in differences in response to drug therapy. Both pharmacokinetic and pharmacodynamic differences exist between men and women. In general, men have a larger body size than women, which results in larger distribution volumes and faster total clearance of many drugs in men compared with that in women. Moreover, greater body fat in women may lead to increases in distribution of lipophilic drugs in females *(40)*. Slower gastric emptying of women can significantly delay the onset of effectiveness of enteric-coated dosage form as well as differences in gastric pH between men and women may also affect dissolution of a drug between genders *(41)*. Hepatic metabolism of drugs by Phase I (oxidation, reduction, and hydrolysis through cytochrome P450's 1A, 2D6, and 2E1) and Phase II (conjugative metabolism by glucuronidation, glucuronyl transferase, methyltransferases, and dehydrogenases) mechanism and by combined oxidation/conjugative mechanism may result in faster drug clearance in men compared with that in women. However, metabolism of drugs by CYP2C9, CYP2C19, and *N*-acetyltransferase appears to be similar in both males and females. In contrast, metabolism of certain drugs that are substrates for CYP3A4 appeared to be mildly or moderately faster in women compared with that in men. Clearance of drugs that are substrates to P-glycoprotein appears to be comparable in both genders *(40)*. Additional gender-related factors, such as intake of hormonal contraceptives, may also have further modulating effects on CYP2D6, CYP2C19 as well as phase II metabolism of drugs *(42)*.

Women experience more adverse reactions to treatment with drugs than men. A Bayesian statistical analysis of sex difference in adverse drug reactions indicated that although about same numbers of adverse events were reported for both men and women, those reported for women were more serious. One example of a sex difference in toxicity of drugs is the drug-induced cardiac arrhythmia, torsades de pointe *(43)*. The efficacy of antiretroviral therapy in HIV-infected patients appears to be similar between men and women, but women may experience higher toxicity profiles *(44)*. Fleisch et al. recently commented that because of gender differences in pharmacokinetic and pharmacodynamic responses in drugs, more women should be recruited in clinical trials for new drugs. Traditionally, women are underrepresented as participants in clinical drug trials *(45)*.

Theophylline is metabolized by CYP1A2. In one study involving 24 subjects, it was observed that theophylline metabolism is faster in women than in men (6 h in female non-smoker vs. 9.3 h in male non-smokers) *(46)*. Phenytoin and naproxen are mainly metabolized by CYP2C9. Rugstad et al. *(47)* reported that there was an increase in plasma naproxen concentrations with age and that females also had higher plasma concentrations of naproxen compared with males. Although women showed slightly lower concentration of phenytoin compared with men when corrected for body weight and height, the difference was not statistically significant *(48)*. The activity of CYP2C19 may be higher in males than in females. The metabolism of mephobarbital was significantly faster in males than in females when compared after a single oral dose of 400 mg of the drug. The sex difference was more significant with the R-enantiomer *(49)*. Clomipramine, which is metabolized by CYP2D6 and CYP2C19, has a higher clearance rate in males compared with that in females *(50)*.

Propranolol metabolism is also faster in males than in females *(51)*. The metabolism of methylprednisolone is mediated by CYP3A4, and in one report, metabolism was higher in women than in men *(52)*. Many other drugs are also metabolized by CYP3A4, the major isoenzyme of human cytochrome P450 enzyme system. Higher clearance of a drug that is a substrate of CYP3A4 in women has been reported. Wolbold et al. found twofold higher CYP3A4 levels in women compared with that in men based on their analysis of 94 well-characterized surgical liver samples. Higher expression in women was also observed in CYP3A4 messenger RNA (mRNA) transcripts, suggesting a pre-translational mechanism. Expression of pregnane X receptor (PXR), which plays a major role in induction of CYP3A4, was also correlated with CYP3A4 in mRNA level, but no sex difference was observed in the expression of PXR mRNA. No sex difference was also observed in P-glycoprotein expression *(53)*. In contrast, Bebia et al. observed no sex difference in phenotype of CYP2C19, CYP3A4, and CYP2D6 based on a study of 161 normal subjects. CYP2E1 showed an age-associated increase, which developed earlier in male subjects than in female subjects *(54)*. Many drugs are metabolized by conjugation. In one study, acetaminophen (paracetamol) clearance was 22% greater in young males compared with that in age-adjusted young females. This difference was entirely because of increase in activity of the glucuronidation pathway in males, and no sex difference was observed in other pathways of paracetamol metabolism *(55)*.

### *4.4. Food Intake and Serum Drug Concentrations*

It has long been recognized that food alters absorption and metabolism of many drugs. Grapefruit juice, Seville orange juice, orange juice, and cranberry juice alter pharmacokinetics of many drugs. Food–drug interactions are discussed in detail in Chapter 13.

### *4.5. Alcohol and Drug Interactions*

Fatal toxicity may occur from alcohol and drug overdoses. In many instances, in the presence of alcohol, a lower concentration of drug may cause fatality because of drug–alcohol interactions. In a Finnish study, it was found that median amitriptyline and propoxyphene concentrations were lower in alcohol-related fatal cases compared with cases where no alcohol was involved. The authors concluded that when alcohol is present, a relatively small overdose of a drug may cause fatality *(56)*. Although alcohol is mostly metabolized in the liver by hepatic alcohol dehydrogenase, long-term intake of large amount of alcohol induces other pathways of metabolism, in particular, the microsomal alcohol-oxidizing system involving CYP2E1. In contrast, acute ingestion of alcohol is likely to cause inhibition of this enzyme *(57)*. CYP2E1 also metabolizes and activates many toxicological substrates to more active products, and induction of CYP2E1 plays an important role in oxidative stress and toxicity in ethanol-induced liver injury *(58)*.

There are two types of interactions between alcohol and a drug: pharmacokinetic and pharmacodynamic. Pharmacokinetic interactions occur when alcohol interferes with the hepatic metabolism of a drug. Pharmacodynamic interactions occur when alcohol enhances the effect of a drug, particularly in the central nervous system. In this type of interaction, alcohol alters the effect of a drug without changing its concentration in the

blood *(59)*. The package insert of many antibiotics states that the medication should not be taken with alcohol although only a few antibiotics have reported interactions with alcohol. Erythromycin may increase blood concentration of alcohol by accelerating gastric emptying *(59)*. Histamine $H_2$ receptor antagonists, such as cimetidine, ranitidine, nizatidine, and famotidine, reduce the activity of alcohol dehydrogenase *(60)*. DiPadova et al. studied the interactions between alcohol and cimetidine, ranitidine as well as famotidine using human subjects. Relative to baseline, ranitidine increased the mean peak concentration and area under the curve (AUC) of blood alcohol by 34 and 41%, respectively. First-pass metabolism of ethanol was also decreased significantly with an increase in bioavailability from 79.6 to 92.6%. Cimetidine showed a greater effect on blood alcohol levels compared with ranitidine, but famotidine showed no significant effect. The authors concluded that patients taking cimetidine or ranitidine should be warned of possible impairments after consumption of alcohol in quantities usually considered as safe in the absence of therapy with these medications *(61)*. However, another study contradicted these findings and concluded that under real life conditions, the concomitant administration of alcohol and cimetidine, ranitidine, or omeprazole is unlikely to have significant physical, social, or forensic implications because no significant difference was found between percentage of first-pass metabolism, peak blood alcohol concentration, or AUC following administration of cimetidine, ranitidine, or omeprazole *(62)*. Another report also found no significant interaction between alcohol and lansoprazole or omeprazole *(63)*.

The effect of alcohol, even low-dose alcohol, on the enhanced antithrombotic effect of warfarin is of clinical significance. A 58-year-old Caucasian man was receiving long-term anticoagulation therapy with warfarin and had a stable International Normalization Ratio (INR). His INR increased when he started taking low-dose beer for cardiovascular protection. After he stopped the alcohol, his INR returned to normal *(64)*. This excessive warfarin activity from low alcohol consumption may be related to the inhibition of warfarin metabolism by cytochrome P450. Conversely, in people who chronically drink alcohol, long-term alcohol consumption activates cytochrome P450 and may increase warfarin metabolism *(59)*.

Alcohol increases sedative effect of tricyclic antidepressants (TCAs) through pharmacodynamic interactions. In addition, alcohol can also cause pharmacokinetic interactions. Alcohol appears to interfere with first-pass metabolism of amitriptyline, thus increasing serum levels of this drug. Alcohol has pharmacodynamic effects with antihistamines, increasing the sedative effects of these over the counter and prescription drugs. Alcohol also increases the sedative effect of phenobarbital and may also increase its serum concentration through pharmacokinetic interactions. Interactions between benzodiazepines and alcohol have also been reported. Alcohol consumption may result in accumulation of toxic breakdown products of acetaminophen *(59)*.

### *4.6. Smoking and Serum Drug Concentrations*

Approximately 4800 compounds are found in tobacco smoke including nicotine and carcinogenic compound, for example polycyclic aromatic hydrocarbons (PAHs) and *N*-nitroso amines. Compounds in tobacco smoke can induce certain cytochrome P450 enzymes responsible for metabolism of many drugs. PAHs induce CYP1A1, CYP1A2, and possibly CYP2E1. Smoking may also induce other drug metabolism

pathways such as conjugation *(65)*. Cigarette smoke is responsible for pharmacokinetic drug interactions, not nicotine. Therefore, nicotine replacement therapy does not cause hepatic enzyme induction *(66)*.

Theophylline is metabolized by CYP1A2. In one study, the half-life of theophylline was reduced by almost twofolds in smokers compared with that in non-smokers *(65)*. Lee et al. *(67)* reported that theophylline clearance was increased by 51.1% and that steady state serum concentrations were reduced by 24.5% in children who were exposed to passive smoking. Clinically significant drug interactions with smoking have also been reported for caffeine, chlorpromazine, clozapine, flecainide, fluvoxamine, haloperidol, mexiletine, olanzapine, proprandol, and tacrine. With all medications, serum concentrations of drugs are significantly reduced in smokers because of increased metabolism of drugs. Smokers may therefore require higher doses than non-smokers to achieve pharmacological responses *(66)*. Warfarin disposition in smokers is also different compared with that in non-smokers. One case report described an increase in INR to 3.7 from a baseline of 2.7–2.8 in an 80-year-old man when he stopped smoking. Subsequently, his warfarin dose was reduced by 14% *(68)*. Another report also demonstrated an increase in INR in a 58-year-old male after cessation of smoking. His warfarin dose was lowered by 23% *(69)*.

Pharmacodynamic drug interactions in smokers may be due to nicotine, which may counteract the pharmacological effects of a drug. The half-life of nicotine is approximately 2 h, and the pharmacological effects of nicotine, such as heart rate increases, blood pressure, diminishes rapidly after cessation of smoking. On the contrary, if nicotine replacement therapy is initiated in a hospitalized patient, heart rate may increase by 10–15 beats/min and blood pressure may increase by 5–10 mmHg *(70)*. The transdermal nicotine patch may have a lesser effect on blood pressure and heart rate *(71)*. Smokers taking benzodiazepines, such as diazepam and chlordiazepoxide, experience less drowsiness than non-smokers, and this interaction appears to be pharmacodynamic in nature because several studies did not find any significant difference between metabolism of benzodiazepines between smokers and non-smokers. Therefore, larger doses may be needed to sedate a smoker *(66)*. Smokers may also need higher doses of opioids (codeine, propoxyphene, and pentazocine) for pain relief *(65)*. In one study, to determine whether smokers require more opioid analgesic, it was found that 20 smokers (10 cigarettes a day or more for at least 1 year) required 23% more (when adjusted for body weight) and 33% more (when adjusted for body mass index) opioid analgesics compared with 69 non-smoking patients *(72)*.

## 5. EFFECT OF DISEASE ON SERUM DRUG CONCENTRATIONS

Several pathophysiological conditions affect metabolism and excretion of drugs. Altered drug metabolism and excretion have been reported in patients with hepatic disease, renal impairment, thyroid disorder, cardiovascular disease, and pregnancy. Moreover, critically ill patients often metabolize or excrete drugs differently compared with ambulatory patients.

### *5.1. Effect of Hepatic Disease on Drug Metabolism*

Severe hepatic disease alters the metabolism of many drugs. Mild to moderate hepatic disease causes an unpredictable effect on drug clearance. Hepatic cytochrome

P450 enzyme activities and gene expression can be profoundly altered in disease states. In general, the levels of affected cytochrome P450 enzymes are depressed by diseases causing potential and documented impairment of drug clearance causing drug toxicity *(73)*. In one study, it was reported that hepatocellular carcinoma decreased expression of CYP2E1 *(74)*. Trotter et al. reported that total mean tacrolimus dose in year one after transplant was lower by 39% in patients with hepatitis C compared with that in patients with no hepatitis C infection. The most likely explanation for these findings is decreased hepatic clearance of tacrolimus caused by mild hepatic injury from recurrent hepatitis C virus *(75)*. Zimmermann et al. reported that oral dose clearance of sirolimus (rapamycin) was significantly decreased in subjects with mild to moderate hepatic impairment compared with that in controls, and authors stressed the need for careful monitoring of trough whole blood sirolimus concentrations in renal transplant recipients exhibiting mild to moderate hepatic impairment *(76)*.

The liver is responsible for producing albumin and other proteins, and hepatic impairment diminishes this process by decreasing concentrations of serum albumin and other proteins. Many drugs are bound to serum protein, and elevated concentration of strongly protein-bound drugs such as phenytoin and valproic acid in patients with hepatic impairment is well documented in the literature. Because free fraction of a drug is responsible for pharmacological action as well as toxicity monitoring, free drug concentrations and dose adjustment based on free drug levels is required in patients with liver disease. This issue is discussed in detail in Chapter 2.

### *5.2. Renal Impairment and Drug Clearance*

Renal disease causes impairment in the clearance of many drugs by the kidney. Correlations have been established between creatinine clearance and clearance of digoxin, lithium, procainamide, aminoglycoside, and many other drugs. The clearance of a drug is closely related to glomerular filtration rate (GFR), and creatinine clearance is a valid way to determine GFR. Serum cystatin C is another marker of GFR. In clinical practice, the degree of renal impairment is widely assessed by using the serum creatinine concentration and creatinine clearance predicted using Cockcroft–Gault formula *(77)*. However, creatinine clearance may be a poor predictor of GFR under certain pathological conditions. Caution should be exercised when medications are prescribed to elderly patients because they may have unrecognized renal impairment. Serum creatinine remains normal until GFR has fallen by at least 50%. Nearly half of the older patients have normal serum creatinine but reduced creatinine clearance. Dose adjustments based on renal function is recommended for many medications in elderly patients even with medications that exhibit large therapeutic windows *(78)*. Dosage adjustments are made for amikacin, gentamicin, tobramycin, and vancomycin based on GFR. Schuck et al. *(79)*, based on a study with 126 patients, concluded that no significant differences exist between serum concentrations of creatinine or its predicted creatinine clearance by Cockcroft–Gault formula, cystatin C, and predicted GFR with regard to dose adjustments. O'Riordan et al. *(80)*, using 22 healthy volunteers who received a single dose of intravenous digoxin, concluded that serum cystatin C is no better than serum creatinine concentration in predicting renal clearance of digoxin. In contrast, Hoppe et al. *(81)* reported that serum cystatin C is a better predictor of drug clearance than serum creatinine concentrations.

Renal disease also causes impairment of drug protein binding because uremic toxins compete with drugs for binding to albumin. Such interaction leads to increases in concentration of pharmacologically active free drug concentration, which is clinically more important for strongly protein-bound drugs. This topic is addressed in Chapter 2.

### *5.3. Thyroid Disorder and Drug Metabolism*

Patients with thyroid disease may have an altered response to drugs. Thyroxin is a potent activator of the cytochrome P450 enzyme system, and hypothyroidism is associated with inhibition of hepatic oxidative metabolism of many drugs. Croxson et al. *(82)* measured serum digoxin concentration using a radioimmunoassay in 17 hyperthyroid and 16 hypothyroid patients and observed significantly lower levels of digoxin in patients with hyperthyroidism and significantly higher levels of digoxin in patients with hypothyroidism. Although there is a general conception that serum phenytoin clearance is not affected by thyroid function state, Sarich and Wright *(83)* reported a case where a 63-year-old female, who developed decreased serum level of free $T_4$, showed phenytoin toxicity that may be related to decreased cytochrome P450-mediated hydroxylation of phenytoin. Another case report also indicated phenytoin intoxication induced by hypothyroidism. A 42-year-old woman with a 29-year history of hypothyroidism and 18-year history of epilepsy was treated with phenytoin, mephobarbital, valproic acid, and thyroid replacement therapy. However, 1 month after sudden withdrawal of the thyroid powder, she was sick and was admitted to the hospital. Her serum phenytoin and phenobarbital levels were significantly elevated over the therapeutic range (26.4 μg/mL for phenytoin and 36.4 μg/mL for phenobarbital), but her valproic acid concentration was low. The endocrinological examination revealed hypothyroidism. Thyroxine administration was started and her phenytoin concentration was decreased to a sub therapeutic level even with the same dose of phenytoin *(84)*.

Hypothyroidism also affects the metabolism of immunosuppressants. A 25-year-old man with a renal transplant had a therapeutic trough whole blood cyclosporine concentration (108–197 ng/mL) after transplant. On the 105th day, his trough cyclosporine concentration was elevated to 1060 ng/mL. His cholesterol was also elevated from 254 to 422 mg/dL, and the patient has an onset of hypothyroidism after transplantation. The authors concluded that elevated cyclosporine concentration may be due to a decrease in cyclosporine clearance resulting from decreased cytochrome P450 activity in hypothyroidism. Moreover, decreased thyroid hormone level and increased plasma lipoprotein level may have affected the distribution of cyclosporine *(85)*. Haas et al. reported a case where a patient developed hypothyroidism 6 months after single lung transplantation and was admitted to the hospital for anuric renal failure. The patient showed a toxic blood level of tacrolimus, which was resolved with the initiation of thyroxine replacement therapy and dose reduction of tacrolimus *(86)*.

The iodine-rich amiodarone affects the thyroid gland causing thyroid disorder, which may affect warfarin sensitivity. Kurnik et al. *(87)* described three cases where patients developed amiodarone-induced thyrotoxicosis, resulting in a significant decrease in

warfarin requirement. Mechanism of interaction of thyroid hormone with warfarin is complex. One proposed mechanism is the alteration of kinetics of the clotting factors with an increase in catabolism of vitamin K-dependent factors in patients with hyperthyroidism. This interaction increases sensitivity to warfarin in patients with hyperthyroidism but decreases sensitivity of warfarin in patients with hypothyroidism *(88)*.

### *5.4. Cardiovascular Disease and Serum Drug Concentration*

Cardiac failure is often associated with disturbances in cardiac output, influencing the extent and pattern of tissue perfusion, sodium and water metabolism as well as gastrointestinal motility. These factors affect absorption and disposition of many drugs requiring dosage adjustment. $V_d$ and clearance of lidocaine are decreased in cardiac failure. For drugs that are metabolized by the liver, decreased blood flow in the liver accounts for reduced clearance, but impaired hepatic metabolism in these patients also plays a role. Accumulation of active metabolites of lidocaine and procainamide in these patients are clinically significant. Theophylline metabolism, which is largely independent of hepatic blood flow, is reduced in patients with severe cardiac failure and dose reduction is needed. Digoxin clearance is also decreased. Quinidine plasma level may also be high in these patients because of lower $V_d$ *(89)*. Elimination half-life is directly related to the $V_d$ and inversely related to clearance. Pharmacokinetic changes are not always predictable in patients with congestive heart failure, but it appears that the net effect of reduction in $V_d$ and impairment in metabolism usually results in higher plasma concentrations of a drug in a patient with congestive heart failure compared with that in healthy subjects. Therefore, therapeutic drug monitoring is crucial in avoiding drug toxicity in these patients *(90)*. Recently, Kotake et al. *(91)* reported that heart failure elevates the serum level of the drug cibenzoline, which is used in the treatment of arrhythmia.

Physiological changes in critically ill patients can significantly affect the pharmacokinetics of many drugs. These changes include absorption, distribution, metabolism, and excretion of drugs in critically ill patients. Understanding these changes in pharmacokinetic parameters are essential for optimizing drug therapy in critically ill patients *(92)*. Moreover, usually free fractions of strongly protein-bound drugs are elevated in the critically ill patients because of low serum albumin concentrations. This issue is discussed in Chapter 2.

### *5.5. Drug Metabolism and Clearance in Pregnancy*

Epidemiologic surveys have indicated that between one-third and two-thirds of all pregnant women will take at least one medication during pregnancy. Drug therapy in pregnant women usually focuses on safety of the drug on the fetus. However, pharmacokinetics of many drugs is altered during pregnancy. Therapeutic drug monitoring during pregnancy aims to improve individual dosage improvement, taking into account pregnancy-related changes in drug disposition *(93)*. Physiological changes that occur during pregnancy alter absorption, distribution, metabolism, and elimination of drugs thus affecting efficacy and safety of the drugs toward pregnant women unless careful dosage adjustments are made. During third trimester, gastrointestinal function may be prolonged. Moreover, the amount of total body water and fat increase

throughout pregnancy and are accompanied by increases in cardiac output, ventilation, and renal and hepatic blood flow. In addition, plasma protein concentrations are reduced, increasing the unbound fraction of a drug. Therefore, careful therapeutic drug monitoring of the free (unbound) concentration of strongly protein-bound drugs, such as phenytoin, is recommended in pregnant women. Moreover, changes occur in the drug-metabolizing capacity of the hepatic enzymes in pregnancy. Renal absorption of sodium is increased. Placental transport of a drug, compartmentalization of a drug in the embryo/placenta, and metabolism of a drug by the placenta and the fetus also play important roles in the pharmacokinetics of a drug during pregnancy *(94)*.

The increased secretion of estrogen and progesterone in normal pregnancy affects hepatic drug metabolism differently depending on the specific drug. A higher rate of hepatic metabolism of certain drugs, for example phenytoin, can be observed because of the induction of the hepatic drug-metabolizing enzymes by progesterone. On the contrary, the hepatic metabolism of theophylline and caffeine is reduced secondary to the competition of these drugs with progesterone and estradiol for enzymatic metabolism by the liver. Cholestatic effect of estrogen may interfere with the clearance of drugs, for example, rifampin *(93)*. By the end of pregnancy, total and unbound phenobarbital concentrations are reduced up to 50% of the original concentration, but primidone concentrations are altered marginally. Total phenytoin concentrations may fall by 40% compared with serum phenytoin levels before pregnancy. Total and free carbamazepine values may also alter because of pregnancy, but reports are conflicting *(95)*. Significant increases in clearance of lamotrigine have been reported in pregnancy. Apparent clearance seems to increase steadily during pregnancy until it peaks approximately at 32nd week when 330% increases in clearance from baseline values can be observed *(96)*. Another study involving 11 pregnant women also demonstrated significant decreases in the ratio of plasma lamotrigine concentration to dose (65.1% during second trimester and 65.8% in third trimester) compared with pre-pregnancy values. Five patients experienced seizure deterioration during pregnancy, and there were significant inter-patient variations in the pharmacokinetics of lamotrigine *(97)*.

Lower serum concentrations of lithium have been reported in pregnancy, and this may be related to an increase in the GFR in pregnancy. Altered pharmacokinetics of ampicillin can be observed in pregnancy where serum concentrations may be lower by 50% in pregnant women compared with that in non-pregnant women because of altered pharmacokinetics. Faster elimination of phenoxymethylpenicillin (Penicillin V) in pregnant women has also been demonstrated *(93)*.

Combined antiretroviral therapy can reduce transmission of the human immunodeficiency virus (HIV) from mother to fetus significantly. However, pregnancy may alter the pharmacokinetics of the antiretroviral drugs. Available data indicate that pharmacokinetics of zidovudine, lamivudine, didanosine, and stavudine are not altered significantly during pregnancy. However, nevirapine half-life is significantly prolonged in pregnancy. For protease inhibitors, reduction of maximum plasma concentration of indinavir was observed in pregnancy. This may be due to induction of cytochrome P450. Standard adult doses of nelfinavir and saquinavir produced lower drug concentration in HIV-infected pregnant women compared with that in non-pregnant women *(94)*.

During pregnancy, the thyroid is hyper-stimulated resulting in changes in thyroid hormone concentrations. Gestational age-specific reference intervals are now available for thyroid function tests. Knowledge of expected normal changes in thyroid hormone concentrations during pregnancy allows individual supplementation when needed *(98)*. Hypothyroidism is common in pregnancy, and therapeutic drug monitoring of antithyroid drugs is important. Consistently lower serum concentrations of propylthiouracil were observed in pregnant women compared with that in non-pregnant women *(99)*.

## 6. DRUG METABOLISM AND CLEARANCE IN NEONATES, CHILDREN, AND ELDERLY

In the fetus, CYP3A7 is the major hepatic cytochrome responsible for steroid metabolism. Variably expressed in the fetus, CYP3A5 is also present in significant level in half of the children. However, in adults, CYP3A4 is the major functional hepatic enzyme responsible for metabolism of many drugs. CYP1A1 is also present during organogenesis whereas CYP2E1 may be present in some second trimester fetuses. After birth, hepatic CYP2D6, CYP2C8/9, and CYP2C18/19 are activated. CYP1A2 becomes active during the fourth to fifth months after birth *(100)*.

In general, age is not considered to have a major influence on the absorption of drugs from the gut except for the first few weeks of life when absorption steps may be less efficient. Neonates and infants demonstrate increased total body water to body fat ratio compared with adults whereas the reverse is observed in the elderly. These factors may affect $V_d$ of drugs depending on their lipophilic character in infants and elderly compared with that in adult population. Moreover, altered plasma binding of drugs may be observed in both neonates and some elderly because of low albumin, thus increasing the fraction of free drug. Moreover, drug-metabolizing capacity by the liver enzymes is reduced in newborns particularly in premature babies but increases rapidly during the first few weeks and months of life to reach values which are generally higher than adult-metabolizing rates. In contrast, efficiency of cytochrome P450 enzymes declines with old age. Renal function at the time of birth is reduced by more than 50% of adult value but then increases rapidly in the first 2–3 years of life. Renal function then starts declining with old age. Oral clearance of lamotrigine, topiramate, levetiracetam, oxcarbazepine, gabapentin, tiagabine, zonisamide, vigabatrin, and felbamate is significantly higher (20–120%) in children compared with that in adults depending on the drug and the age distribution of the population. On the contrary, clearance of these drugs is reduced (10–50%) in the elderly population compared with that in the middle-aged adults *(101)*.

Clearance of aminoglycoside is dependent on the GFR, which is markedly decreased in neonates, especially in premature newborns. These drugs appear to be less nephrotoxic and ototoxic in neonates compared with that in the adult population. The $V_d$ of aminoglycoside increases in neonates, which may also contribute to a longer half-life of aminoglycoside in neonates. Decreased renal clearance in neonates is responsible for decreased clearance of most beta-lactam antibiotics *(102)*. Higher $V_d$ and lower clearance of gentamicin was also observed in neonates *(103)*. Conversion of theophylline to caffeine in human fetuses has been reported *(104)*. Kraus et al. studied maturational changes in theophylline disposition in 52 infants and observed

**Table 3**
**Factors and Diseases Affecting Disposition of Drugs**

| *Factor or disease* | *Comments* |
|---|---|
| Gender difference | Men may have faster clearance of drugs than women except for drugs cleared by CYP3A4<br>Women may be more susceptible to drug toxicity |
| Alcohol intake | Pharmacodynamic interactions with many drugs causing significant toxicity with lower amounts of drugs when alcohol is present<br>Cimetidine and ranitidine may increase blood alcohol level<br>Alcohol may increase International Normalization Ratio (INR) in patients taking warfarin<br>Alcohol increases serum levels of amitriptyline |
| Smoking | Theophylline serum concentrations reduced in smokers<br>Reduced serum concentrations of many other drugs<br>Interaction with warfarin |
| Hepatic impairment | Decreased clearance of tacrolimus and sirolimus requiring dosage reduction<br>Elevated free concentrations of strongly protein-bound drugs |
| Renal impairment | Decreased clearance of drugs where renal excretion is the major pathway<br>Elevated free concentrations of strongly protein-bound drugs |
| Thyroid disease | Elevated concentration of certain drugs (cyclosporine, phenobarbital etc.) in hypothyroidism<br>Thyrotoxicosis may reduce warfarin requirement |
| Cardiovascular disease | Reduced metabolism of many drugs because of decreases in hepatic blood flow. Reduced clearance of digoxin, theophylline, and other drugs |
| Pregnancy | Elevated free concentrations of drugs because of reduced plasma proteins<br>Increased metabolism of certain drugs (phenytoin, indinavir), but clearance of some drugs (theophylline) may also be reduced<br>Lower serum concentration of lithium |
| Children | Increase oral clearances of many antiepileptic drugs<br>Conversion of theophylline to caffeine in children |

that postconceptional age was an import factor in describing theophylline metabolism in neonates. Disappearance of serum caffeine concentrations and maturation of theophylline clearance were primarily related to the demethylation pathway that produced 3-methylxanthine. Theophylline clearance and urine metabolite pattern reached adult values in infants 55 weeks after postconceptional age *(105)*. Major factors affecting drug distribution and metabolism are summarized in Table 3.

## 7. THERAPEUTIC DRUG MONITORING OF INDIVIDUAL DRUGS

Usually, concentration of a therapeutic drug is measured in the serum or plasma. However, whole blood concentration of immunosuppressant drugs such as cyclosporine and tacrolimus is usually measured for therapeutic drug monitoring. Obtaining blood for measurement of a drug during the absorption or the distribution phase may lead to misleading information. Moreover, to measure the peak concentration of a drug, timing of the sample will depend on the route of administration. After intravenous administration, the peak concentration of a drug may be achieved in a few minutes. On the contrary, for a sustained release tablet, the mean time to reach the peak plasma concentration of theophylline was 7.9 h in one study *(106)*. The trough concentration is clinically defined as the serum drug concentration just before the next dose. Usually, trough concentrations are monitored for most drugs, but for aminoglycosides and vancomycin, both peak and trough concentrations are monitored. For a meaningful interpretation of a serum drug concentration, time of specimen collection should be noted along with the time and date of the last dose and route of administration of the drug. This is particularly important for aminoglycoside because without knowing the time of specimen collection, the serum drug concentration cannot be interpreted. Information needed for proper interpretation of drug level for the purpose of therapeutic drug monitoring is listed in Table 4.

### *7.1. Therapeutic Drug Monitoring of Anticonvulsants*

Phenytoin, phenobarbital, ethosuximide, valproic acid, and carbamazepine are considered as conventional anticonvulsant drugs. Many people with epilepsy suffer from side effects of anticonvulsants as well as suboptimum seizure control, which can be minimized by regular medication review and dosage adjustments based on serum drug levels *(107)*. All these antiepileptic drugs have a narrow therapeutic range. Phenytoin, carbamazepine, and valproic acid are strongly bound to serum proteins.

**Table 4**
**Essential Information Required for Interpretation of Serum Concentrations of a Drug**

| *Patient Information* | *Other Information* |
|---|---|
| Name of the patient | Dosage regimen |
| Hospital identification number | Time of taking dosage |
| Age | Type of specimen (serum, urine, saliva, other body fluid). Number of specimens |
| Height and weight | (if more than one) and type of drug concentration requested (total vs. free) |
| | Time of specimen collection (peak vs. trough) |
| Gender (if female pregnant?[a]) | Time of last dose |
| Ethnicity | Concentration of the drug |
| Albumin level, creatinine clearance[a] | Pharmacokinetic parameters of the drug |

[a] Optional information.

Although free drug concentration can be predicted from traditionally measured total drug concentration (free + protein-bound drug), under certain disease conditions, such as uremia and hepatic impairment, free drug concentration may not be predicted from total drug concentration because of impairment of the protein-binding ability of serum for these anticonvulsants. Moreover, monitoring free drug concentration is also recommended for these drugs in elderly patients, critically ill patients, pregnant women, and patients with a low serum albumin concentrations. This topic is discussed in Chapter 2.

Minimally effective serum total phenytoin concentration is considered as 10 μg/mL, whereas the upper end of the therapeutic range is 20 μg/mL. Carbamazepine is an iminostilbene derivative structurally similar to the TCA imipramine. It was approved in the USA in 1974 as an antiepileptic for many seizure disorders and in 1979 for use in children over 6 years of age. The current uses of carbamazepine include partial seizures with complex symptomatology, generalized tonic-clonic seizures, and mixed seizures. Carbamazepine and phenytoin are considered as the drugs of choice for treating these seizure disorders *(108)*. Carbamazepine is also frequently added to the existing TCA therapy *(109)*. Carbamazepine, like lithium, may help some individuals with episodic behavioral dysfunction, such as loss of control and aggression, even in the absence of epileptic, affective, or organic features *(110,111)*. TCA and anticonvulsants are also used in the treatment of pain in polyneuropathy *(112)*. The minimally effective serum concentration of carbamazepine is 4 μg/mL, and the toxicity may be encountered at serum level over 12 μg/mL. Carbamazepine is metabolized to an active metabolite carbamazepine 10, 11-epoxide. Carbamazepine 10, 11-epoxide is present in 15–20% of the total carbamazepine concentration at steady state. The concentration of metabolite may be significantly higher in carbamazepine overdose and in patients with renal failure. Moreover, concentration of carbamazepine 10, 11-epoxide may be further elevated in patients also receiving valproic acid and lamotrigine. For these patients, measuring both carbamazepine and carbamazepine 10, 11-epoxide is clinically useful *(113)*. Another issue is the cross-reactivity of epoxide with carbamazepine immunoassays. The cross-reactivity of carbamazepine 10, 11-epoxide with different immunoassays may vary between 0% (Vitros Ortho Diagnosics Rariton, NJ) and 94% (Dade Dimension Deerfield, IL) *(114)*. Parant et al. *(115)* also reported high cross-reactivity of PETINIA (Dade Behring Deerfield, IL) carbamazepine assay with carbamazepine 10, 11-epoxide and negligible cross-reactivity with the EMIT 2000 assay. Ideally, a high-performance liquid chromatography (HPLC) method should be used as a reference method for measuring carbamazepine and carbamazepine 10, 11-epoxide concentrations, but immunoassays are widely used for routine monitoring of serum carbamazepine concentrations in many clinical laboratories. Therefore, caution should be exercised in the interpretation of serum carbamazepine concentrations in light of cross-reactivity of the specific immunoassay with carbamazepine 10, 11-epoxide.

Valproic acid is an antiepileptic drug, which is structurally unrelated to phenytoin, phenobarbital, or carbamazepine. The chemical name of valproic acid is 2-propylpentanoic acid, which was synthesized in 1881, but the antiepileptic property of valproic acid was not discovered until 1963. Other than epilepsy, valproic acid in the form of divalproex sodium is used as a prophylaxis for migraine *(116)*. Valproic acid is also used in treating a variety of psychiatric disorders *(117)*. Therapeutic responses to valproic acid are usually observed at serum concentrations equal to or greater than

40 μg/mL, and toxicity is encountered at serum levels exceeding 100 μg/mL. Seizure controls of phenobarbital start with a serum concentration of 15 μg/mL, and concentrations greater than 40 μg/mL may cause toxicity.

In the past decade, 10 new antiepileptic drugs have been approved for use. These drugs include felbamate, gabapentin, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, tiagabine, topiramate, vigabatrin, and zonisamide. In general, these antiepileptic drugs (except felbamate) have better pharmacokinetic profiles, improved tolerability in patients, and are less involved in drug interactions compared with traditional anticonvulsants: phenytoin, carbamazepine, phenobarbital, and valproic acid. Gabapentin, levetiracetam, and vigabatrin are mainly eliminated by the renal route with a fraction of unchanged drug in the urine of 65, 66, and 100%, respectively. These anticonvulsants are not involved in drug interactions. Other new anticonvulsants are metabolized by cytochrome P450 and uridine glucuronosyltransferase enzyme and may be involved in pharmacokinetic drug interactions with conventional anticonvulsants or other drugs *(118)*. Clinical utility of therapeutic drug monitoring as well as guidelines of serum drug concentrations have not been clearly established for these new anticonvulsants. However, careful monitoring of liver function tests and blood cell counts is strongly recommended for felbamate because of its known toxicity *(119)*. The drug is only 20–25% bound to serum protein, and currently, there is no indication for monitoring free felbamate level. There is no systematic study to establish a therapeutic range for gabapentin. A tentative target range of 70–120 μmol/L has been suggested. There are more indications for therapeutic monitoring of lamotrigine. The therapeutic range suggested is 12–55 μmol/L. Tiagabine is strongly protein bound and is a candidate for free drug monitoring. However, more studies are needed to establish a therapeutic range. The traditional approach to therapeutic drug monitoring does not apply to vigabatrin *(120)*. Reference ranges and costs of therapeutic drug monitoring of anticonvulsants are given in Table 5.

### *7.2. Therapeutic Drug Monitoring of Cardioactive Drug*

Therapeutic drug monitoring of several cardioactive drugs, including digoxin, disopyramide, lidocaine, procainamide, mexiletine, tocainide, and quinidine are routinely performed in clinical laboratories because of the established correlation between serum drug concentrations and pharmacological response of these drugs. Moreover, drug toxicity can be mostly avoided by therapeutic drug monitoring. Digoxin is one of the most frequently ordered drugs among all cardioactive drugs in clinical laboratory. This drug has a narrow therapeutic window, and immunoassays employed in monitoring serum digoxin concentration are subjected to interference from both exogenous and endogenous compounds. This topic is discussed in detail in Chapter 6.

Disopyramide (4-diisopropylamino-2-phenyl-2-pyridyl) butyramide was synthesized in 1954, and its antiarrhythmic properties were discovered in 1964. The plasma protein binding of disopyramide is extremely variable in patients because of fluctuation of $\alpha_1$-acid glycoprotein concentrations in the serum. Moreover, binding of disopyramide to the serum proteins is stereoselective with R(–)-isomer approximately 66% protein bound and S(+)-isomer 79% protein bound *(121)*. The therapeutic range of disopyramide is considered as 1.5–5.0 μg/mL. Echizen et al. *(122)* recommended monitoring free fraction of disopyramide. Lidocaine is another cardioactive drug bound to $\alpha_1$-acid

**Table 5**
**Therapeutic Drug Monitoring of Anticonvulsants**

| *Drug* | *Specimen Requirement* | *Therapeutic Range[b] Trough (μg/mL)* | *Cost[a]* |
|---|---|---|---|
| Carbamazepine | Serum | 4–12 | $ |
| Carbamazepine, 10, 11-epoxide | Serum or plasma | 0.4–4 | $$ |
| Clonazepam | Serum or plasma | 10–50 | $$ |
| Diazepam and | Serum | 0.1–1.0 | |
| Nordiazepam | | 0.1–0.8 | $$$ |
| Felbamate | Serum | Range not well established (very toxic) | $$ |
| Ethosuximide | Serum or plasma | 40–75 | $$ |
| Gabapentin | Serum or plasma | 2–12 | $$$ |
| Lamotrigine | Serum or plasma | 1–4 | $$$ |
| Methsuximide | Serum or plasma | 10–40 | $$$ |
| Phenytoin | Serum or plasma | 10–20 | $ |
| Phenobarbital | Serum or plasma | 15–40 | $ |
| Primidone | Serum or plasma | 5–12 | $ |
| Valproic acid | Serum or plasma | 50–100 | $$ |
| Zonisamide | Serum or plasma | 10–40 | $$$ |

$, < $75; $$, < $100; $$$, > $100; $$$$, > $150.

[a] The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges based on ranges used in our hospital laboratory as well as published ranges in textbooks and test catalogues of reference laboratories. Reference ranges may vary significantly depending on patient population, disease states, and others.

glycoprotein. Free fraction of lidocaine may vary considerably in disease state, and it is discussed in Chapter 2. Mexiletine was synthetically developed, and this drug is mainly metabolized by the liver to parahydroxy mexiletine, hydroxymethyl mexiletine, and their corresponding alcohols, and metabolites are not considered active. The therapeutic range is considered to be 0.5–2.0 μg/mL, although many patients experience toxicity at a serum level just exceeding the upper limit of the therapeutic range *(123)*. Koch-Weser *(124)* established that the therapeutic range of procainamide is 4–10 μg/mL. Procainamide is metabolized to an active metabolite; *N*-acetyl procainamide (NAPA). Lima et al. *(125)* reported that the combined concentrations of procainamide and NAPA over 25–30 μg/mL increase the risk of toxicity. Decreased renal function may cause significant accumulation of procainamide and NAPA resulting in severe intoxication *(126)*. Quinidine is a natural alkaloid found in cinchona bark. Since 1918, quinidine has been used as an antiarrhythmic drug. Wide variations in quinidine serum levels coupled with a narrow therapeutic range make therapeutic drug monitoring of quinidine essential. This drug is strongly bound to $\alpha_1$-acid glycoprotein, and the variations of pharmacologically free fractions have been reported in altered pathological conditions. Monitoring free quinidine concentration is discussed in Chapter 2.

Tocainide was developed as an oral analogue of lidocaine because lidocaine cannot be administered orally because of high first-pass metabolism. Tocainide and lidocaine have similar electrophysiological properties. Tocainide is cleared by hepatic metabolism and urinary excretion of unchanged drug. Unbound drug concentration is likely to correlate with total drug concentration, and there is no indication for monitoring free tocainide concentration because tocainide is only poorly bound to serum proteins (5–20%). Flecainide is a strong sodium channel blocker used in the treatment of various supraventricular tachyarrhythmias. Flecainide is mainly metabolized by cytochrome P450 (CYP2D6) and CYP2D6. The poor metabolizers of this drug showed a 42% reduction in flecainide clearance. This population represents 5–10% of Caucasians and less than 1% of Asians *(127)*. The reported therapeutic range of trough flecainide concentration is 200–1000 ng/mL (0.2–1.0 μg/mL), although severe adverse effect such as ventricular arrhythmia has occurred occasionally in patients whose serum flecainide concentration exceeded 1000 ng/mL *(128)*. Reference ranges and cots of monitoring of cardioactive drugs are summarized in Table 6.

### *7.3. Therapeutic Drug Monitoring of Antiasthmatic Drugs*

Theophylline is a bronchodilator and respiratory stimulant effective in the treatment of acute and chronic asthma. The drug is readily absorbed after oral absorption, but peak concentration may be observed much later with sustained release tablets. The bronchodilator effect of theophylline is proportional to the log of serum drug concentration over a range of 5–20 μg/mL *(129)*. Adverse reactions may be observed

**Table 6**
**Therapeutic Drug Monitoring of Cardioactive Drugs**

| *Drug* | *Specimen Requirement* | *Therapeutic Range*[b] *Trough* | *Cost*[a] |
|---|---|---|---|
| Amiodarone | Serum or plasma | 1.0–2.5 μg/mL | $$$ |
| Digoxin | Serum or plasma | 0.8–2.0 ng/mL | $ |
| Disopyramide | Serum or plasma | 1.5–5.0 μg/mL | $$$ |
| Flecainide | Serum or plasma | 0.2–1.0 μg/mL | $$ |
| Lidocaine | Serum or plasma | 1.5–5.0 μg/mL | $$ |
| Mexiletine | Serum or plasma | 0.5–2.0 μg/mL | $$$ |
| Propanolol | Plasma | 50–100 ng/mL | $$ |
| Procainamide | Serum or plasma | 4–10 μg/mL | $$ (both tests) |
| and NAPA | | 4–8 μg/mL | |
| Quinidine | Serum or plasma | 2–5 μg/mL | $ |
| Tocainide | Serum or plasma | 5–12 μg/mL | $$$ |
| Verapamil | Serum or plasma | 50–200 ng/mL | $$$ |

$, < $75; $$, < $100; $$$, > $100; $$$$, > $150.

[a] The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges based on ranges used in our hospital laboratory as well as published ranges in textbooks and test catalogues of reference laboratories. Reference ranges may vary significantly depending on patient population, disease states, and others.

at concentrations exceeding 20 μg/mL. However, serum therapeutic concentrations between 10 and 20 μg/mL have also been reported *(130)*. Theophylline is metabolized by hepatic cytochrome P450, and altered pharmacokinetics of theophylline in disease states have been reported. Clearance of theophylline is slow in neonates compared with that in adults, while theophylline metabolism is also altered in hepatic disease. Acute viral illness associated with fever may prolong the half-life of theophylline *(131)*. Patients with pneumonia and episodes of severe airways obstruction also may metabolize theophylline slowly *(132)*. Altered pharmacokinetics of theophylline in pregnancy and among smokers have been discussed earlier in this chapter. Treating debilitated elderly patients with a nasogastric tube may significantly alter the pharmacokinetics of theophylline. Berkovitch et al. reported that when similar doses of theophylline were administered through nasogastric tubes and orally, patients receiving theophylline through nasogastric tubes demonstrated unexpectedly low serum theophylline concentrations. For example, mean trough theophylline concentration was 3.78 μg/mL in patients receiving theophylline through nasogastric tubes compared with a mean theophylline plasma concentration of 8.63 μg/mL when patients received theophylline orally. Peak plasma concentration of theophylline also differed significantly between these two groups *(133)*. Reference ranges and costs of monitoring of antiasthmatic drugs are given in Table 7.

### *7.4. Therapeutic Drug Monitoring of Antidepressants*

TCAs, including amitriptyline, doxepin, nortriptyline, imipramine, desipramine, protriptyline, trimipramine, and clomipramine were introduced in the 1950s and the 1960s. These drugs have a narrow therapeutic window, and therapeutic drug monitoring is essential for efficacy of these drugs as well as to avoid drug toxicity. Issues in therapeutic drug monitoring of these drugs are discussed in Chapter 8. The efficacy of lithium in acute mania and for prophylaxis against recurrent episode of mania has been well established. Blood concentrations of lithium have been shown to parallel with total body water and brain concentration of lithium. Therapeutic drug monitoring of lithium is essential for efficacy as well as to avoid lithium toxicity. A guiding principle in the use of lithium salts in the treatment of mental illness is to maintain a serum

**Table 7**
**Therapeutic Drug Monitoring of Antiasthmatic Drugs**

| *Drug* | *Specimen Requirement* | *Therapeutic Range[b] Trough(μg/mL)* | *Cost[a]* |
|---|---|---|---|
| Theophylline | Serum or plasma | 10–20 | $ |
| Caffeine | Serum or plasma | 5–15 | $$ |

$, < $75; $$, < $100; $$$, > $100; $$$$; > $150.

[a] The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges based on ranges used in our hospital laboratory as well as published ranges in textbooks and test catalogues of reference laboratories. Reference ranges may vary significantly depending on patient population, disease states, and others.

**Table 8**
**Therapeutic Drug Monitoring of Antidepressants**

| *Drug* | *Specimen Requirement* | *Therapeutic Range[b] Trough* | *Cost[a]* |
|---|---|---|---|
| Amitriptyline and nortriptyline | Serum or plasma | 120–250 ng/mL (amitriptyline + nortriptyline)<br>50–150 ng/mL (nortriptyline alone) | $$$ |
| Clomipramine | Serum | 150–450 ng/mL | $$$ |
| Doxepin and nordoxepin | Serum or plasma | 150–250 ng/mL (doxepin and nordoxepin) | $$$ |
| Imipramine and desipramine | Serum or plasma | 150–250 ng/mL (imipramine and desipramine) | $$$ |
| Fluoxetine and norfluoxetine | Serum | 300–1000 ng/mL (fluoxetine and norfluoxetine) | $$$ |
| Paroxetine | Serum | 20–200 ng/mL | $$$$ |
| Sertraline | Serum or plasma | 30–200 ng/mL | $$$ |
| Lithium | Serum | 0.8–1.2 mEq/L | $ |
| Haloperidol | Serum | 2–15 ng/mL | $$$$ |

$, < $75; $$, < $100; $$$, > $100; $$$$, > $150.

[a] The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges based on ranges used in our hospital laboratory as well as published ranges in textbooks and test catalogues of reference laboratories. Reference ranges may vary significantly depending on patient population, disease states, and others.

lithium concentration between 0.8 and 1.2 mmol/L *(134)*. Lithium therapy has various neurological, cardiovascular, and renal side effects. Serum lithium concentration of 3.5 mmol/L or higher is considered potentially lethal and hemodialysis therapy is recommended *(135)*. More recently introduced antidepressants are selective serotonin reuptake inhibitors (SSRIs), including citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline. This class of drugs has a flat dose–response curve, thus a wide therapeutic index. Currently, most investigators agree that therapeutic drug monitoring of these drugs in the majority of patients is not essential *(136)*. The reference ranges and costs of monitoring antidepressants are listed in table 8.

### *7.5. Therapeutic Drug Monitoring of Immunosuppressant Drugs*

Blood concentrations of cyclosporine, tacrolimus, sirolimus, everolimus, and mycophenolic acid are routinely monitored at transplant centers for several reasons including avoiding rejection because of subtherapeutic levels of these drugs as well as to avoid drug toxicity. Although whole blood concentration of cyclosporine, tacrolimus, sirolimus, and everolimus is usually determined in clinical laboratories for therapeutic drug monitoring, usually serum concentration of mycophenolic acid is measured. Therapeutic drug monitoring of immunosuppressant drugs is discussed in detail in Chapter 9. Therapeutic ranges and costs of monitoring immunosuppressants are given in Table 9.

**Table 9**
**Therapeutic Drug Monitoring of Immunosuppressants**

| *Drug* | *Specimen Requirement* | *Therapeutic Range[b] Trough* | *Cost[a]* |
|---|---|---|---|
| Cyclosporine | Whole blood (EDTA) | 100–400 ng/mL | $$ |
| Tacrolimus | Whole blood (EDTA) | 5–15 ng/mL | $$$ |
| Mycophenolic acid | Serum or plasma | 1–3.5 μg/mL | $$$$ |
| Everolimus | Whole blood (EDTA) | 5–15 ng/mL | $$$$ |
| Sirolimus | Whole blood (EDTA) | 4–20 ng/mL | $$$ |

EDTA, ethylenediaminetetraacetic acid.

[a] $, < $75; $$, < $100; $$$, > $100; $$$$, > $150; The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges only and may alter significantly with transplant type and with combination of other immunosuppressants.

### *7.6. Therapeutic Drug Monitoring of Antibiotics*

The aminoglycoside antibiotics consist of two or more aminosugars joined by a glycosidic linkage to a hexose or an aminocyclitol. Streptomycin was the first aminoglycoside discovered in 1914. These drugs are used in the treatment of serious and often life-threatening systemic infections. However, aminoglycoside can produce serious nephrotoxicity and ototoxicity. Peak serum concentrations for amikacin and kanamycin above 32–34 μg/mL are associated with a higher risk of nephrotoxicity and ototoxicity *(137)*. Sustained peak concentrations above 12–15 μg/mL are associated with an increased risk of developing nephrotoxicity and ototoxicity for gentamicin, tobramycin, and sisomicin. For netilmicin, the toxicity is encountered at a peak concentration above 16 μg/mL. Peak concentration of streptomycin should not exceed 30 μg/mL *(138)*.

Aminoglycosides are poorly absorbed from the gastrointestinal track, and these drugs are administered intravenously or intramuscularly. The major route of elimination is through the kidney where 85–95% of the drugs are recovered unchanged. Patients with impaired renal function have lower aminoglycoside elimination rates and longer half-lives compared with patients with normal renal function. Moreover, elimination of aminoglycosides is slower in elderly patients, and many patients require prolonged dosing interval. Children have a higher clearance of aminoglycosides. Siber et al. reported that after 1 mg/kg dose of gentamicin, the mean peak plasma concentration was 1.58 μg/mL in children with age between 6 months and 5 years, 2.03 μg/mL in children between 5 and 10 years, and 2.81 μg/mL in children older than 10 years. Patients with fever showed shorter half-life and lower plasma concentrations of gentamicin *(139)*.

Patients with cystic fibrosis usually exhibit an altered pharmacokinetics of the antibiotics. After a conventional dose of an aminoglycoside, a patient with cystic fibrosis shows a lower serum concentration compared with a patient not suffering from cystic fibrosis. The lower serum concentrations of aminoglycoside in patients with cystic fibrosis may be due to increased total body clearance of these drugs combined with a larger $V_d$ *(140)*. Bosso et al. reported that mean clearance of netilmicin was higher in patients with cystic fibrosis compared with that in patients with no cystic fibrosis. Therefore, patients with cystic fibrosis required larger than normal dosages of netilmicin on a weight basis. The study also showed that the serum concentrations of netilmicin

should be monitored carefully to individualize dosage in these patients *(141)*. Another study indicated that the major route of elimination of gentamicin in patients with mild cystic fibrosis is through renal excretion, but aminoglycoside pharmacokinetics were changed with progression of disease *(142)*. Mann et al. *(143)* reported increased dosage requirement for tobramycin and gentamicin for treating *Pseudomonas pneumonia* in patients with cystic fibrosis. Dupuis et al. observed significant differences in pharmacokinetics of tobramycin in patients with cystic fibrosis before and after lung transplantation in a group of 29 patients who received at least one dosage of tobramycin before and after lung transplant. The clearance of tobramycin was decreased by 40% and the half-life was increased by 141% after transplant compared with pre-transplant values *(144)*. Patients with cystic fibrosis are also susceptible to renal impairment from repeated intravenous use of aminoglycosides, and these drugs should be cautiously used in these patients with regular monitoring of renal function *(145)*.

Renal clearance of penicillin is enhanced in cystic fibrosis because of the greater affinity of the renal secretory system for these drugs *(146)*. Another study involving 11 patients with cystic fibrosis and 11 controls demonstrated that mean elimination half-life of ticarcillin in serum was 70.8 min in control subjects and 53.1 min in subjects with cystic fibrosis. The non-renal clearance of ticarcillin was also higher in patients with cystic fibrosis compared with that in controls. The authors concluded that the shorter elimination half-life and higher total body clearance of ticarcillin in patients with cystic fibrosis are because of an increase in both renal and non-renal elimination *(147)*.

Therapeutic drug monitoring is also frequently employed during vancomycin therapy. The drug is excreted in the urine with no metabolism, and there is no known pharmacogenetic problem. Vancomycin has a low therapeutic index with both nephrotoxicity and ototoxicity complicating the therapy *(148)*. It is necessary to monitor both peak and trough concentration of vancomycin. Ranges for peak concentrations of 20–40 μg/mL have been widely quoted *(149)*. The given trough range of 5–10 μg/mL has reasonable literature support. Trough concentration above 10 μg/mL has been associated with an increased risk of nephrotoxicity *(150,151)*. For infants, Tan et al. recommended a conservative therapeutic range of 5–10 μg/mL for the trough and 20–40 μg/mL for the peak concentration. A less conservative range is 5–12 μg/mL for trough and 15–60 μg/mL for peak *(152)*. However, de Hoog et al. *(153)* recommended a trough concentration between 5 and 15 μg/mL and a peak concentration below 40 μg/mL in neonates. Zimmermann et al. *(154)* reported that patients were more likely to become afebrile within 72 h if the peak and trough vancomycin concentrations were greater than 20 and 10 μg/mL, respectively. Although the dispositions of many antibiotics are altered in patients with cystic fibrosis, patients with cystic fibrosis exhibit a disposition of vancomycin similar to that exhibited by healthy adults, and thus, cystic fibrosis does not alter pharmacokinetic parameters of vancomycin *(155)*. Reference ranges and costs for monitoring antibiotics are summarized in Table 10.

### *7.7. Therapeutic Drug Monitoring of Antiretroviral Drugs*

HIV is the virus that causes acquired immunodeficiency syndrome (AIDS). Four classes of drugs are used today to treat people with AIDS including nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine, non-NRTIs (NNRTIs), which include nevirapine, delavirdine, and efavirenz; and protease inhibitors (PIs), such as

**Table 10**
**Therapeutic Drug Monitoring of Antibiotics**

| *Drug* | *Specimen Requirement* | *Therapeutic Range*[b] | *Cost*[a] |
|---|---|---|---|
| Amikacin | Serum or plasma | <5 μg/mL (trough)<br>15–25 μg/mL (peak)[c] | $$ |
| Gentamicin | Serum or plasma | 1–2 μg/mL (trough)<br>4–8 μg/mL (peak)[c] | $$ |
| Tobramycin | Serum or plasma | 1–2 μg/mL (trough)<br>4–8 μg/mL (peak)[c] | $$ |
| Vancomycin | Serum or plasma | 5–15 μg/mL (trough)<br>30–40 μg (peak) | $$ |
| Cefazolin | Serum or plasma | 60–120 μg (peak) | $$$ |
| Ciprofloxacin | Serum or plasma | 3–5 μg/mL (peak)<br>0.5–2 μg/mL (trough) | $$$ |

[a] $, < $75; $$, < $100; $$$, > $100; $$$$, > $150. The costs are based on published charge for these tests in our hospital laboratory and reference laboratories.

[b] Therapeutic ranges are suggested ranges based on ranges used in our hospital laboratory as well as published ranges in textbooks and test catalogues of reference laboratories. Reference ranges may vary significantly depending on patient population, disease states, and others.

[c] Based on traditional dosing of aminoglycosides. Extended intravenous dosing may produce higher peak values.

saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, and atazanavir. Therapeutic drug monitoring of these drugs is discussed in Chapter 10. Suggested reference ranges and costs of monitoring antiretroviral drugs are given in Table 11.

### *7.8. Therapeutic Drug Monitoring of Antineoplastic Drugs*

Methotrexate is a competitive inhibitor of dihydrofolate reductase, a key enzyme for biosynthesis of nucleic acid. The cytotoxic activity of this drug was discovered in 1955. The use of leucovorin to rescue normal host cells has permitted the higher doses of methotrexate therapy in clinical practice. Methotrexate is used in the treatment of acute lymphoblastic leukemia (ALL), osteogenic sarcoma, brain tumors, and carcinomas of the lung. Most of the toxicities of this drug are related to serum concentrations and pharmacokinetic parameters. Methotrexate is also approved for the treatment of refractory rheumatoid arthritis. Usually, low doses of methotrexate are used for treating rheumatoid arthritis (5–25 mg once weekly). One study found that splitting a weekly dose of 25–35 mg of methotrexate into spilt doses separated by 8 h improved the bioavailability of the drug *(156)*. Although toxicity from low-dose treatment is rare, toxic manifestation with low-dose methotrexate has been reported. Izzedine et al. commented that permanent discontinuation of methotrexate therapy in 1 of 10 patients occurs because of toxicity. Moreover, nephrotoxicity, which is common with high doses of methotrexate, may also occur with low doses of therapy in patients receiving methotrexate *(157)*. A frequent adverse reaction seen is myelosuppression, which manifests as leucopenia and thrombocytopenia. Therapeutic drug monitoring is strongly recommended during high-dose treatment of methotrexate.

**Table 11**
**Therapeutic Drug Monitoring of Antiretrovirals**

| *Drug* | *Specimen Requirement* | *Therapeutic Range*[b] *Trough (ng/mL)* | *Cost*[a] |
|---|---|---|---|
| Amprenavir | Serum | 150–400 | |
| Atazanavir | Serum | 100 | |
| Indinavir | Serum | 80–120 | $$$$ |
| Lopinavir | Serum | 700 | |
| Nelfinavir | Serum | 700–1000 | $$$$ |
| Saquinavir | Serum | 100–250 | $$$$ |
| Nevirapine | Serum | 150–400 | $$$$ |
| Efavirenz | Serum | 100 | |

[a] $$$$; >$150. The costs are based on published charge for these tests according to the 2006 catalog price of Mayo Medical Laboratories, Rochester, MN, which offers therapeutic drug monitoring of these antiretroviral drugs.

[b] Therapeutic ranges are courtesy of Jennifer King, Pharm.D., assistant professor at the division of Clinical Pharmacology, University of Alabama at Birmingham. Reference ranges are recommended ranges only and may vary between institutions. The Department of Laboratory Medicine of Children's Hospital National Medical Center, Washington, DC, performs monitoring of antiretroviral drugs. Jewish Medical Center at Denver also offers therapeutic drug monitoring of antiretroviral drugs. This service may be available in other medical centers and reference laboratories in the USA, which the author may not be aware of.

The elimination half-life of methotrexate is 7–11 h, and on administration, less than 10% is oxidized to 7-hydroxymethotrexate irrespective of the route of administration. The protein-binding ranges vary from 30–70%, and albumin is the major binding protein in the serum *(158)*. Peak serum concentration of methotrexate correlates with the outcome in the treatment of osteosarcoma. Modification of the dosage to achieve a peak serum concentration between 700 and 1000 μmol/L has been recommended *(159)*. Omeprazole may delay elimination of methotrexate, and therefore, when prescribing methotrexate to a patient, an alternative to omeprazole should be used *(160)*. One case study reported that amoxicillin decreased the renal clearance of methotrexate probably by competition at common tubular secretion system and by secondary methotrexate-induced renal impairment *(161)*.

The platinum derivative cisplatin is used in the treatment of testicular cancer. In most studies determining pharmacokinetic parameters of cisplatin, free fractions were measured in plasma or tumor. There is a high variability between individual patients, and the therapeutic window is narrow. Dosage is often based on body surface area. Recently, Salas et al. *(162)* described therapeutic drug monitoring of cisplatin using total platinum measurement in plasma. Gietema et al. *(163)* reported that platinum was detectable in plasma in patients 20 years after being cured from metastatic testicular cancer following cisplatin therapy. Impaired bioavailability of phenytoin in a 24-year-old woman treated with cisplatin, vinblastine, and bleomycin has been reported. Data revealed mean phenytoin absorption of 32% (normal greater than 80%) establishing malabsorption of phenytoin because of cancer chemotherapy *(164)*. Since the 1960s, 5-fluorouracil has been used either alone or as part of a combination therapy with other drugs to treat various solid tumors and is also a standard therapy for colorectal cancer. Pharmacoki-

netic studies showed that clinical response as well as toxicity of 5-fluorouracil are related to AUC. Individual dosage adjustments based on pharmacokinetic monitoring lead to higher response rate of this drug as well as survival rates associated with tolerability. A limited sampling strategy using just two plasma concentrations can be used to predict AUC of 5-fluorouracil *(165)*. Pharmacokinetic monitoring of other anticancer drugs, such as doxorubicin, etoposide, mitoxantrone, mensa and dimensa, taxol, aminoglutethimide, tamoxifen and acrolein, and cyclophosphamide, may also be beneficial *(166)*.

There is a narrow therapeutic window between suboptimal therapy and toxicity in the treatment with antineoplastic drugs. Genetic polymorphism in phase I and phase II enzymes is present in the population and may explain in part the variations in the pharmacokinetic parameters of a particular drug between individual patients. The potential for applying pharmacogenetic screening before cancer chemotherapy may have applications with several cytochrome P450 enzymes, in particular with CYP2B6 (cyclophosphamide treatment), CYP2C8 (paclitaxel therapy), and CYP3A5 *(167)*.

## 8. CONCLUSIONS

There are many effective drugs in the clinical practice today, which are also highly toxic because of a narrow therapeutic window. Successful therapy with such drugs require individualization of dosages based on serum drug concentrations, and therapeutic monitoring of such drugs are routinely offered in most hospital-based clinical laboratories. Moreover, reference laboratories, as well as academic-based hospital laboratories, usually offer a wider menu of drugs that can be monitored compared with community hospital-based laboratories. Therapeutic drug monitoring is also useful to identify non-compliant patients. Moreover, therapeutic drug monitoring is cost effective in health care *(168)*. However, despite cost effectiveness and demonstrated clinical utility, therapeutic drug-monitoring service is underutilized in patient care. A recent report by Raebel et al. based on 17,748 ambulatory patients at 10 health maintenance organizations indicated that 50 percent or more patients receiving digoxin, theophylline, procainamide, quinidine, or primidone were not monitored and 25–50% of patients receiving divalproex, carbamazepine, phenobarbital, phenytoin, or tacrolimus were not monitored. The authors concluded that a substantial proportion of ambulatory patients receiving drugs with narrow therapeutic ranges did not have serum drug concentrations monitored during 1 year of use *(169)*. Therefore, more patient education is needed to utilize therapeutic drug monitoring in patient management for maximum therapeutic benefit of a drug with a narrow therapeutic window.

## REFERENCES

1. Peterson GM, Khoo BH, von Witt RJ. Clinical response in epilepsy in relation to total and free serum levels of phenytoin. *Ther Drug Monit* 1991; 13: 415–419.
2. Rajendran SD, Rao YM, Thanikachalam S, Muralidharan TR, et al. Comparison of target concentration intervention strategy with conventional dosing of digoxin. *Indian Heart J* 2005; 57: 265–267.
3. Watson I, Potter J, Yatscoff R, Fraser A, et al. Therapeutic drug monitoring. *Ther Drug Monit [Editorial]* 1997; 19: 125.
4. No author listed. Italian collaborative study on the utilization of therapeutic drug monitoring in hospital departments: Italian collaborative group on the therapeutic drug monitoring. *Ther Drug Monit* 1988; 10: 275–279.

5. Ried LD, Horn JR, McKenna DA. Therapeutic drug monitoring reduces toxic drug reactions; a meta-analysis. *Ther Drug Monit* 1990; 12: 72–78.
6. Levine B, Cohen SS, Birmingham PH. Effect of pharmacist intervention on the use of serum drug assays. *Am J Hosp Pharm* 1981; 38: 845–851.
7. Crist KD, Nahata MC, Ety J. Positive impact of a therapeutic drug monitoring program on total aminoglycoside dose and cost of hospitalization. *Ther Drug Monit* 1987; 9: 306–310.
8. Florence AT, Jani PU. Novel oral drug formulations: their potential in modulating adverse effects. *Drug Saf* 1994; 10: 233–266.
9. McCormick CG. Regulatory challenges for new formulation of controlled substances in today's environment. *Drug Alcohol Depend* 2006; 83: S63–S67.
10. Qiu LY, Bae YH. Polymer architecture and drug delivery. *Pharm Res* 2006; 23: 1–30.
11. Dandagi PM, Mastiholimath VS, Patil MB, Gupta MK. Biodegradable microparticulate system of captopril. *Int J Pharm* 2006; 307: 83–88.
12. Fedorak RN, Bistritz L. Targeted delivery, safety, and efficacy of oral enteric-coated formulations of budesonide. *Adv Drug Deliv Rev* 2005; 57: 303–316.
13. Tedesco-Silva H, Bastien MC, Choi L, Felipe C, et al. Mycophenolic acid metabolite profile in renal transplant patients receiving enteric coated mycophenolate sodium or mycophenolate mofetil. *Transplant Proc* 2005; 37: 852–855.
14. El-Harati AA, Charcosset C, Fessi H. Influence of formulation for solid lipid nanoparticles prepared with a membrane contactor. *Pharm Dev Technol* 2006; 11: 153–157.
15. Lennartz P, Mieleck JB. Minitabletting: improving the compactability of paracetamol powder mixtures. *Int J Pharm* 1998; 173: 75–85.
16. Keith S. Advances in psychotropic formulations. *Prog Neuropsychopharmacol Biol Psychiatry* 2006; 30: 996–1008.
17. Silvestry FE, St John Sutton MG. Sustained release calcium channel antagonists in cardiovascular disease: pharmacology and current therapeutic use. *Eur Heart J* 1998; 19 (Suppl I): I8–I14.
18. Weisler EH. Carbamazepine extended release capsules: a new treatment option for bipolar I disorder. *Expert Rev Neurother* 2005; 5: 587–595.
19. Kernitsky L, O'Hara KA, Jiang P, Pellock JM. Extended release divalproex in child and adolescent outpatients with epilepsy. *Epilepsia* 2005; 46: 440–443.
20. Robinson JR, Gauger LJ. Formulation of controlled release products. *J Allergy Clin Immunol* 1986; 78: 676–681.
21. Kerin NZ, Meengs WL, Timmis HGC, Salerno D, et al. Activity of Procanbid, procainamide twice daily formulation to suppress ventricular premature depolarizations. The study group investigators. *Cardiovasc Drug Ther* 1997; 11: 169–175.
22. McCormack PL, Keating GM. Prolonged release nicotinic acid: a review of its use in the treatment of dyslipidaemia. *Drugs* 2005; 65: 2719–2740.
23. van Hoogdalem E, de Boer AG, Breimer DD. Pharmacokinetics of rectal drug administration, Part I: general considerations and clinical applications of centrally active drugs. *Clin Pharmacokinet* 1991; 21: 11–26.
24. Fischer JH, Patel TV, Fischer PA. Fosphenytoin: clinical pharmacokinetics and comparative advantages in the acute treatment of seizures. *Clin Pharmacokinet* 2003; 42: 33–58.
25. Benson HA. Transdermal drug delivery: penetration enhancement techniques. *Curr Drug Deliv* 2005; 2: 23–33.
26. Feldmann RJ, Maibach HI. Regional variation in precutaneous absorption of $^{14}C$ cortisol in man. *J Invest Derm* 1967; 48: 181–183.
27. Brown MB, Martin GP, Jones SA, Akomeah FK. Dermal and transdermal drug delivery systems: current and future prospects. *Drug Deliv* 2006; 13: 175–187.
28. Pajouhesh H, Lenz GR. Medicinal chemicals properties of successful central nervous system drugs. *NeuroRx* 2005; 2: 541–553.
29. Walton NY, Uthman BM, El Yafi K, Kim JM, Treiman DM. Phenytoin penetration into brain after administration of phenytoin or fosphenytoin. *Epilepsia* 1999; 40: 153–156.
30. Ningaraj NS. Drug delivery to brain tumors: challenges and progress. *Expert Opin Drug Deliv* 2006; 3: 499–509.

31. Morgan MM, Khan DA, Nathan RA. Treatment for allergic rhinitis and chronic idiopathic urticaria: focus on oral antihistamines. *Ann Pharmacother* 2005; 39: 2056–2064.
32. Hoffman RS, Henry GC, Howland MA, Weisman RS, et al. Association between life-threatening cocaine toxicity and plasma cholinesterase activity. *Ann Emerg Med* 1992; 21: 247–253.
33. Tam YK. Individual variation in first pass metabolism. *Clin Pharmacokinet* 1993; 25: 300–328.
34. Rollins DE, Klaassen CD. Biliary excretion of drugs in man. *Clin Pharmacokinet* 1979; 4: 368–379.
35. Angelin B, Arvidsson A, Dahlqvist R, Hedman A, et al. Quinidine reduces biliary clearance of digoxin in man. *Eur J Clin Invest* 1987; 17: 262–265.
36. Kurt H, Wolk M, Scherman R, McDowell F. Insufficient parahydroxylation as a cause of diphenylhydantoin toxicity. *Neurology* 1964; 14: 542–548.
37. Wilkinson GR. Cytochrome P450 (CYP3A4) metabolism: prediction of in vivo activity in humans. *J Pharmacokinetic Biopharm* 1996; 24: 475–490.
38. Vizirianakis IS. Challenges in current drug delivery from the potential application of pharmacogenomics and personalized medicine in clinical practice. *Curr Drug Deliv* 1004; 1: 73–80.
39. de Leon J. Amplichip CYP450 test: personalized medicine has arrived in psychiatry. *Expert Rev Mol Diagn* 2006; 6: 277–286.
40. Schwartz JB. The influence of sex on pharmacokinetics. *Clin Pharmacokinet* 2003; 42: 107–121.
41. Donovan MD. Sex and racial differences in pharmacological response: effect of route of administration and drug delivery system on pharmacokinetics. *J Women Health (Larchmt)* 2005; 14: 30–37.
42. Meibohm B, Beierle I, Derendorf H. How important are gender differences in pharmacokinetics? *Clin Pharmacokinetic* 2002; 41: 329–342.
43. Miller MA. Gender differences in the toxicity of pharmaceuticals-the Food and Drug Administration's perspective. *Int J Toxicol* 2001; 20: 149–152.
44. Clark RA, Squires KE. Gender specific considerations in the antiretroviral management of HIV-infected women. *Expert Rev Anti Infect Ther* 2005; 3: 213–227.
45. Fleisch J, Fleisch MC, Thurmann PA. Women in early phase clinical drug trials: have things changed in the past 20 years? *Clin Pharmacol Ther* 2005; 78: 445–452.
46. Nafziger AN, Bertino JS. Sex related differences in theophylline pharmacokinetics. *Eur J Clin Pharmacol* 1989; 37: 97–100.
47. Rugstad HE, Hundal O, Holme I, Herland OB, et al. Piroxicam and naproxen plasma concentrations in patients with osteoarthritis: relation to age, sex, efficacy and adverse events. *Clin Rheumatol* 1986; 5: 389–398.
48. Travers RD, Reynolds EH, Gallagher BB. Variation in response to anticonvulsants in a group of epileptic patients. *Arch Neurol* 1972; 27: 29–33.
49. Hooper WD, Qing MS. The influence of age and gender on the stereoselective metabolism and pharmacokinetics of mephobarbital in humans. *Clin Pharmacol Ther* 1990; 48: 633–640.
50. Gex-Fabry M, Balant-Georgia AE, Balant LP, Garrone G. Clomipramine metabolism: model based analysis of variability factors from drug monitoring data. *Clin Pharmacokinet* 1990; 19: 241–255.
51. Walle T, Walle UK, Cowart TD, Conradi EC. Pathway selective sex differences in the metabolic clearance of propranolol in human subjects. *Clin Pharmacol Ther* 1989; 46: 257–263.
52. Lew KH, Ludwig EA, Milad MA, Donovan K. Gender based effects on methylprednisolone pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther* 1993; 54: 402–414.
53. Wolbold R, Klein K, Burk O, Nussler AK, et al. Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* 2003; 38: 978–988.
54. Bebia Z, Buch SC, Wilson JW, Frye RF, et al. Bioequivalence revisited: influence of age and sex on CYP enzymes. *Clin Pharmacol Ther* 2004; 76: 618–627.
55. Miners JO, Attwood J, Birkett DJ. Influence of sex and oral contraceptives on paracetamol metabolism. *Br J Clin Pharmacol* 1983; 16: 503–509.
56. Koski A, Vuori E, Ojanpera I. Relation of postmortem blood alcohol and drug concentrations in fetal poisonings involving amitriptyline, propoxyphene and promazine. *Hum Exp Toxicol* 2005; 24: 389–396.
57. Song BJ. Ethanol inducible cytochrome P450 (CYP2E1): biochemistry, molecular biology and clinical relevance-an update. *Alcoholism Clin Exp Res* 1996; 20 (Suppl 8): 136A–146A.

58. Jimenez-Lopez JM, Cederbaum AI. CYP2E1 dependent oxidative stress and toxicity: role in ethanol induced liver injury. *Expert Opin Drug Metab Toxicol* 2005; 1: 671–685.
59. Weathermon R, Crabb DW. Alcohol and medication interactions. *Alcohol Res Health* 1999; 23: 40–54.
60. Caballeria J, Baraona E, Deulofeu R, Hernandez-Munoz R, et al. Effect of H-32 receptor agonists on gastric alcohol dehydrogenase activity. *Digest Dis Sci* 1991; 36: 1673–1679.
61. DiPadova C, Roine R, Frezza M, Gentry RT, et al. Effects of ranitidine on blood alcohol levels after ethanol ingestion: comparison with H-2 receptor antagonists. *JAMA* 1992; 267: 83–86.
62. Brown AS, James OF. Omeprazole, ranitidine and cimetidine have no effect on peak blood ethanol concentrations, first pass metabolism or area under the time-ethanol curve under real life drinking conditions. *Aliment Pharmacol Ther* 1998; 12: 141–145.
63. Battiston L, Tulissi P, Moretti M, Pozzato G. Lansoprazole and ethanol metabolism: comparison with omeprazole and cimetidine. *Pharmacol Toxicol* 1997; 81: 247–252.
64. Havrda DE, Mai T, Chonlahan J. Enhanced antithrombotic effect of warfarin associated with low dose alcohol consumption. *Pharmacotherapy* 2005; 25: 303–307.
65. Zevin S, Benowitz NL. Drug interactions with tobacco smoking: an update. *Clin Pharmacokinetic* 1999; 36: 425–438.
66. Kroon LA. Drug interactions and smoking: raising awareness for acute and critical care provider. *Crit Care Nurs Clin North Am* 2006; 18: 53–62.
67. Lee BL, Benowitz NL, Jacob P. Cigarette abstinence, nicotine gum and theophylline disposition. *Ann Intern Med* 1987; 1964(4): 553–555.
68. Colucci VJ, Knapp JE. Increase in international normalization ratio associated with smoking cessation. *Ann Pharmacother* 2001; 35: 385–386.
69. Evans M, Lewis GM. Increase in international normalization ratio after smoking cessation in a patient receiving warfarin. *Pharmacotherapy* 2005; 25: 1656–1659.
70. Benowitz NL, Gourlay SG. Cardiovascular toxicity of nicotine: implications of nicotine replacement therapy. *J Am Coll Cardiol* 1997; 29: 1422–1431.
71. Perkins KA, Lerman C, Keenan J, Fonte C, Coddington S. Rate of nicotine onset from nicotine replacement therapy and acute response in smokers. *Nicotine Tob Res* 2004; 6: 501–507.
72. Creekmore FM, Lugo RA, Weiland KJ. Postoperative opiate analgesic requirements for smokers and non smokers. *Ann Pharmacother* 2004; 38: 949–953.
73. Cheng PY, Morgan ET. Hepatic cytochrome P 450 regulation in disease states. *Curr Drug Metab* 2001; 2: 165–183.
74. Man XB, Tang L, Qui XH, Yang LQ, et al. Expression of cytochrome P4502E1 gene in hepatocellular carcinoma. *World J Gastroenterol* 2004; 10: 1565–1568.
75. Trotter JF, Osborne JC, Heller N, Christians U. Effect of hepatitis C infection on tacrolimus does and blood levels in liver transplant recipients. *Aliment Pharmacol Ther* 2005; 22: 37–44.
76. Zimmermann JJ, Lasseter KC, Lim HK, Harper D, et al. Pharmacokinetics of sirolimus (rapamycin) in subjects with mild to moderate hepatic impairment. *J Clin Pharmacol* 2005; 45: 1368–1372.
77. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; 16: 31–41.
78. Terrell KM, Heard K, Miller DK. Prescribing to older ED patients. *Ann Emerg Med* 2006; 24: 468–478.
79. Schuck O, Teplan V, Sibova J, Stollova M. Predicting the glomerular filtration rate from serum creatinine, serum cystatin C and the Cockcroft and Gault formula with regard to drug dosage adjustments. *Int J Clin Pharmacol Ther* 2004; 42: 93–97.
80. O'Riordan S, Ouldred E, Brice S, Jackson SH, Swift GC. Serum cystatin C is not a better marker of creatinine or digoxin clearance than serum creatinine. *Br J Clin Pharmacol* 2002; 53: 398–402.
81. Hoppe A, Seronie-Vivien AM, Thomas F, Delord JP, et al. Serum cystatin C is a better marker of topotecan clearance than serum creatinine. *Clin Cancer Res* 2005; 11: 3038–3044.
82. Croxson MS, Ibbertson HK. Serum digoxin in patients with thyroid disease. *Br Med J* 1975; 3(5983): 566–568.

83. Sarich TC, Wright JM. Hypothyroxinemia and phenytoin toxicity: a vicious circle. *Drug Metabol Drug Interact* 1996; 13: 155–160.
84. Horii K, Fujitake J, Tatsuoka Y, Ishikawa K, et al. A case of phenytoin intoxication induced by hypothyroidism. *Rinsho Shinkeigaku* 1991; 31: 528–533 [in Japanese].
85. Nishimura M, Yamada K, Matsushita K, Saisu T, et al. Changes in trough levels of whole blood cyclosporine and graft function of a kidney transplant recipient with onset of hypothyroidism after transplantation. *Transplantation* 1996; 62: 1509–1511.
86. Haas M, Kletzmayer J, Staudinger T, Bohmig G, et al. Hypothyroidism as a cause of tacrolimus intoxication and acute renal failure: a case report. *Wien Klin Wochenschr* 2000; 112: 939–941.
87. Kurnik D, Loebstein R, Farfel Z, Ezra D, et al. Complex drug-drug disease interactions between amiodarone, warfarin and the thyroid gland. *Medicine (Baltimore)* 2004; 83: 107–113.
88. Kellett HA, Sawers JS, Boulton FE, Cholerton S, et al. Problems of anticoagulation with warfarin in hyperthyroidism. *Q J Med* 1986; 58: 43–51.
89. Benowitz NL, Meister W. Pharmacokinetics in patients with cardiac failure. *Clin Pharmacokinet* 1976; 1: 389–405.
90. Shammas FV, Dickstein K. Clinical pharmacokinetics in heart failure: an updated review. *Clin Pharmacokinetic* 15: 94–113.
91. Kotake T, Takada M, Komamura K, Kamakura S, et al. Heart failure elevates serum levels of cibenzoline in arrhythmic patients. *circ J* 2006; 70: 588–592.
92. Boucher BA, Wood GC, Swanson JM. Pharmacokinetic changes in critical illness. *Crit Care Clin* 2006; 22: 255–271.
93. Loebstein R, Koren G. Clinical relevance of therapeutic drug monitoring during pregnancy. *Ther Drug Monit* 2002; 24: 15–22.
94. Rakhmanina N, van den Anker, Soldin SJ. Safety and pharmacokinetics of antiretroviral therapy during pregnancy. *Ther Drug Monit* 2004; 26: 110–115.
95. Tomson T. Gender aspects of pharmacokinetics of new and old AEDs: pregnancy and breast feeding. *Ther Drug Monit* 2005; 27: 718–721.
96. Pennell PB, Newport DJ, Stowe ZN, Helmers SL, et al. The impact of pregnancy and childbirth on the metabolism of lamotrigine. *Neurology* 2004; 27: 292–295.
97. Petrenaite V, Sabers A, Hansen-Schwartz J. Individual changes in lamotrigine plasma concentrations during pregnancy. *Epilepsy* 2005; 65: 185–188.
98. Soldin OP. Thyroid function testing in pregnancy and thyroid diseases: trimester-specific reference intervals. *Ther Drug Monit* 2006; 28: 8–11.
99. Koren G, Soldin O. Therapeutic drug monitoring of antithyroid drugs during pregnancy. *Ther Drug Monit* 2006; 28: 12–13.
100. Oesterheld JR. A review of developmental aspects of cytochrome P 450. *J Child Adolesc Psychopharmacol* 1998; 8: 161–174.
101. Perucca E. Pharmacokinetics variability of new antiepileptic drugs at different age. *Ther Drug Monit* 2005; 27: 714–717.
102. Paap CM, Nahata MC. Clinical pharmacokinetics of antibacterial drugs in neonates. *Clin Pharmacokinet* 1990; 19: 280–318.
103. Williams BS, Ransom JL, Gal P, Carlos RQ, et al. gentamicin pharmacokinetics in neonates with patent ductus arteriosus. *Crit Care Med* 1997; 25: 272–275.
104. Brazier JL, Salle B. Conversion of theophylline to caffeine by the human fetus. *Semin Perinatol* 1981; 5: 315–320.
105. Kraus DM, Fischer JH, Reitz SJ, Kecskes SA. Alterations in theophylline metabolism during the first year of life. *Clin Pharmacol Ther* 1993; 54: 351–359.
106. Jonkman JH, Berg WC, deVries K, de Zeeuw RW, et al. Disposition and clinical pharmacokinetics of theophylline after administration of a new sustained release tablet. *Eur J Clin Pharmacol* 1981; 21: 39–44.
107. French J. The long term therapeutic management of epilepsy. *Ann Intern Med* 1994; 120: 411–422.
108. Maheshwari M, Padmini R. Role of Carbamazepine in reducing polypharmacy in epilepsy. *Acta Neurol* 1981; 64: 22–28.

109. Szymura-Olenksiak J, Waska E, Wasieczko A. Pharmacokinetic interaction between Carbamazepine in patients with major depression. *Psychopharmacology* 2001; 154: 38–42.
110. Stein G. Drug treatment of personality disorder. *Br J Psychiatry* 1992; 161: 167–184.
111. Small JG. Anticonvulsants in affective disorders. *Psychopharmacol Bull* 1990; 26: 25–36.
112. Sindrup SH, Jensen TS. Pharmacological treatment of pain in polyneuropathy. *Neurology* 2000; 55: 915–920.
113. Potter JM, Donnelly A. Carbamazepine 10, 11-epoxide monitoring in therapeutic drug monitoring. *Ther Drug Monit* 1998; 20: 652–657.
114. Hermida J, Tutor JC. How suitable are currently used Carbamazepine immunoassays for quantifying Carbamazepine 10, 11-epoxide in serum samples? *Ther Drug Monit* 2003; 25: 384–388.
115. Parant F, Bossu H, Gagnieu MC, Lardet G, Moulsma M. Cross reactivity assessment of Carbamazepine 10, 11-epoxide, oxacarbazepine, and 10 hydroxy carbazepine in two automated Carbamazepine immunoassay: PENTINA and EMIT 2000. *Ther Drug Monit* 2003; 25: 41–45.
116. Spina E, Perugi G. Antiepileptic drugs: indications other than epilepsy. *Epileptic Disord* 2004; 6: 57–75.
117. Asnis GM, Kohn SR, Henderson M, Brown NL. SSRIs versus non-SSRIs in post-traumatic stress disorders: an update with recommendations. *Drugs* 2004; 64: 383–404.
118. Bialer M. The pharmacokinetics and interactions of new antiepileptic drugs. *Ther Drug Monit* 2005; 27: 722–726.
119. LaRoche S, Helmers S. The new antiepileptic drugs: scientific review. *JAMA* 2004; 291: 605–614.
120. Johannessen S, Battino D, Berry D, Bialer M, et al. Therapeutic drug monitoring of the newer antiepileptic drugs. *Ther Drug Monit* 2003; 25: 347–363.
121. Lima JJ, Wenzke SC, Boudoulas H, Schaal SF. Antiarrhythmic activity and unbound concentrations of disopyramide enantiomers in patients. *Ther Drug Monit* 1990; 12: 23–28.
122. Echizen H, Saima S, Ishizaki T. Disopyramide protein binding in plasma from patients with nephritic syndrome during the exacerbation and remission phases. *Br J Clin Pharmacol* 1987; 24: 199–206.
123. Campbell NPS, Pantridge JF, Adgey AAJ. Long term antiarrhythmic therapy with mexiletine. *Br Heart J* 1978; 40: 796–801.
124. Koch-Weser J. Serum procainamide level as therapeutic guide. *Clin Pharmacokinet* 1977; 2: 389–402.
125. Lima JJ, Goldfarb AL, Conti DR, Golden LH, et al. Safety and efficacy of procainamide infusion. *Am J Cardiol* 1979; 43: 98–105.
126. Kim SY, Benowitz NL. Poisoning due to class IA antiarrhythmic drugs: Quinidine, procainamide and disopyramide. *Drug Saf* 1990; 5: 393–420.
127. Doki K, Homma M, Kuga K, Kusano K, et al. Effect of CYP2D6 genotype on flecainide pharmacokinetics in Japanese patients with supraventricular tachyarrhythmia. *Eur J Clin Pharmacol* 2006; 62: 919–926.
128. Homma M, Kuga K, Doki K, Katori K, et al. Assessment of serum flecainide trough levels in patients with tachyarrhythmia. *J Pharm Pharmacol* 2005; 57: 47–51.
129. Mitenko P, Ogilvie R. Rational intravenous doses of theophylline. *N Engl J Med* 1973; 289: 600–603.
130. Zeildman A, Gardyn J, Frandin Z, Fink G, et al. Therapeutic and toxic theophylline levels in asthma attacks-is there a need for additional theophylline? *Harefuah* 1997; 133: 3–5 [in Hebrew].
131. Chang K, Bell TD, Lauer B, Chai H. Altered theophylline pharmacokinetics during acute respiratory viral illness. *Lancet* 1978; 1(8074): 1132–1133.
132. Vozeh S, Powell JR, Riegelman S, Costello JF, et al. Changes in theophylline clearance in acute illness. *JAMA* 1978; 240: 1882–1884.
133. Berkovitch M, Dafni O, Leiboviz A, Mayan H, et al. Therapeutic drug monitoring of theophylline in frail elderly patients: oral compared with nasogastric tube administration. *Ther Drug Monit* 2002; 24: 594–597.
134. Sashidharan SP. The relationship between serum lithium levels and clinical response. *Ther Drug Monit* 1982; 4: 249–264.
135. Gadallah MF, Feinstein EI, Massry SG. Lithium intoxication: clinical course and therapeutic considerations. *Miner Electrolyte Metab* 1988; 14: 146–149.

136. Rasmussen BB, Brosen K. Is therapeutic drug monitoring a case for optimizing clinical outcome and avoiding interactions of selective serotonin reuptake inhibitors? *Ther Drug Monit* 2000; 22: 143–154.
137. Black RE, Lau WK, Weinstein RJ, Young LS, Hewitt WL. Ototoxicity of amikacin. *Antimicrob Agents Chemother* 1976; 9: 956–961.
138. Erlason P, Lundgren A. Ototoxicity side effects following treatment with streptomycin, dihydrostreptomycin and kanamycin. *Acta Med Scand* 1964; 176: 147–163.
139. Siber GR, Echeverria P, Smith AL, Paisley JW, et al. Pharmacokinetics of gentamicin in children and in adults. *J Infect Dis* 1975; 132: 637–651.
140. Horrevorts AM, Driessen OM, Michel MF, Kerrebijin KF. Pharmacokinetics of antimicrobial drugs in cystic fibrosis. Aminoglycoside antibiotics. *Chest* 1988; 94: 120S–125S.
141. Bosso J, Townsend PL, Herbst JJ, Masten JM. Pharmacokinetics and dosage requirements of netilmicin in cystic fibrosis patients. *Antimicrob Agents Chemother* 1985; 28: 829–831.
142. McDonald NE, Anas NG, Peterson RG, Schwartz RH, et al. Renal clearance of gentamicin in cystic fibrosis. *J Pediatr* 1983; 103: 985–990.
143. Mann HJ, Canafax DM, Cipolle RJ, Daniels CE, et al. Increased dosage requirement of tobramycin and gentamicin for treating Pseudomonas pneumonia in patients with cystic fibrosis. *Pediatr Pulmonol* 1985; 1: 238–243.
144. Dupuis RE, Sredzienski ES. Tobramycin pharmacokinetics in patients with cystic fibrosis preceding and following lung transplantation. *Ther Drug Monit* 1999; 21: 161–165.
145. Al-Aloul M, Miller H, Alapati S, Stockton PA, et al. Renal impairment in cystic fibrosis patients due to repeated intravenous aminoglycoside use. *Pediatr Pulmonol* 2005; 39: 15–20.
146. Wang JP, Unadkat JD, al-Habet SM, O'Sullivan TA, et al. Disposition of drugs in cystic fibrosis IV: mechanisms for enhanced renal clearance of ticarcillin. *Clin Pharmacol Ther* 1993; 54: 293–302.
147. de Groot R, Hack BD, Weber A, Chaffin D, et al. Pharmacokinetics of ticarcillin in patients with cystic fibrosis: a controlled prospective study. *Clin Pharmacol Ther* 1990; 47: 73–78.
148. Duffull SB, Begg EJ. Vancomycin toxicity: What is the evidence for dose dependence? *Adverse Drug React Toxicol Rev* 1994; 13: 103–114.
149. Begg EJ, Barclay ML, Kirkpatrick C. The therapeutic monitoring of antimicrobial agents. *Br J Clin Pharmacol* 1999; 47: 23–30 [Review].
150. Ryback MJ, Albrecht LM, Boike SC, Chandrasekar PH. Nephrotoxicity of vancomycin: alone or with an aminoglycoside. *J Antimicrob Chemother* 1990; 25: 679–687.
151. Cimino MA, Rotstein C, Slaughter RL, Emrich LJ. Relationship of serum antibiotic concentrations to nephrotoxicity in cancer patients receiving concurrent aminoglycoside and vancomycin therapy. *Am J Med* 1987; 83: 1091–1096.
152. Tan WH, Brown N, Kelsaff AW, McClure RJ, et al. Dose regimen for vancomycin not needing serum peak levels? *Arch Dis Child Fetal Neonatal Ed* 2002; 87: F214–216.
153. de Hoog M, Schoemaker RC, Mooton JW, van der Anker JN. Vancomycin population pharmacokinetics in neonates. *Clin Pharmacol Ther* 2000; 67: 360–367.
154. Zimmermann AE, Katona BG, Plaisance KI. Association of vancomycin serum concentrations with outcomes in patients with gram positive bacteremia. *Pharmacotherapy* 1995; 15: 85–91.
155. Pleasant RA, Michalets EL, Williams DM, Samuelson WM, et al. Pharmacokinetics of vancomycin in adult cystic fibrosis patients. *Antimicrob Agents Chemother* 1996; 40: 186–190.
156. Hoekstra M, Haagsma C, Neef C, Proost J, et al. Splitting high dose oral methotrexate improves bioavailability: a pharmacokinetic study with rheumatoid arthritis. *J Rheumatol* 2006; 33: 481–485.
157. Izzedine H, Launay-Vacher V, Karie S, Caramella C, et al. Is low dose methotrexate nephrotoxic? Case report and review of literature. *Clin Nephrol* 2005; 64: 315–319.
158. Endo L, Bressolle F, Gomeni R, Bologna C, et al. Total and free methotrexate pharmacokinetics in rheumatoid arthritis patients. *Ther Drug Monit* 1996; 18: 128–134.
159. Zelcer S, Kellick M, Wexler LH, Shi W, et al. Methotrexate levels and outcome in osteosarcoma. *Pediatr Blood Cancer* 2005; 44: 638–642.
160. Beorlegui B, Aldaz A, Ortega A, Aquerreta I, et al. Potential interaction between methotrexate and omeprazole. *Ann Pharmacother* 2002; 34: 1024–1027.

161. Ronchera CL, Hernandez T, Peris JE, Torres F, et al. Pharmacokinetic interaction between high dose methotrexate and amoxicillin. *Ther Drug Monit* 1993; 15: 375–379.
162. Salas S, Mercier C, Cicccolini J, Pourroy B, et al. Therapeutic drug monitoring for dose individualization of cisplatin in testicular cancer patients based upon total platinum measurement. *Ther Drug Monit* 2006; 28: 532–539.
163. Gietema JA, Meinardi MT, Messerschmidt J, Gelevert T, et al. Circulating plasma platinum more than 10 years after cisplatin treatment for testicular cancer. *Lancet* 2000; 355(9209): 1075–1076.
164. Sylvester RK, Lewis FB, Caldwell KC, Lobell M, et al. Impaired bioavailability secondary to cisplatinum, vinblastine, and bleomycin. *Ther Drug Monit* 1984; 6: 302–305.
165. Gusella M, Ferrazzi E, Ferrari M, Padrini R. New limited sampling strategy for determination of 5-fluorouracil area under the concentration curve-time curve after rapid intravenous bolus. *Ther Drug Monit* 2002; 425–431.
166. el-Yazigi A, Ezzat A. Pharmacokinetic monitoring of anticancer drugs at King Faisal Specialist Hospital, Riyadh, Saudi Arabia. *Ther Drug Monit* 1997; 19: 390–393.
167. van Schaik RH. Implications of cytochrome P 450 genetic polymorphisms on the toxicity of antitumor agents. *Ther Drug Monit* 2004; 26: 236–240.
168. Touw DJ, Neef C, Thomson AH, Vinks AA, et al. Cost effectiveness of therapeutic drug monitoring: a systematic review. *Ther Drug Monit* 2005; 27: 10–17.
169. Raebel MA, Carroll NM, Andrade SE, Chester EA, et al. Monitoring of drugs with a narrow therapeutic range in ambulatory care. *Am J Manag care* 2006; 12: 268–274.

# 2 Monitoring Free Drug Concentration

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***Contents***



**Summary**

Measurement of serum drug concentration may be misleading for a strongly protein-bound drug because a drug bound to protein is inactive and only unbound or free drug is pharmacologically active. Although free drug concentration can be estimated from total concentration in most cases, under certain pathophysiological conditions such as uremia, liver disease, and hypoalbuminemia free drug concentration may be significantly elevated even if the concentration of the total drug is within therapeutic range. Drug–drug interactions may also lead to a disproportionate increase in free drug concentrations. Elderly patients usually show increased free drug concentrations because of hypoalbuminemia. Elevated free phenytoin concentrations have also been reported in patients with AIDS and pregnancy. Currently, free drug concentrations of anticonvulsants such as phenytoin, carbamazepine, and valproic acid are widely measured in clinical laboratories. Newer drugs such as mycophenolic acid mofetil and certain protease inhibitors are also considered as candidates for monitoring free drug concentration.

**Key Words:** Free drugs; anticonvulsants; immunosuppressant; protein binding; clinical utility.

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## 1. INTRODUCTION

Therapeutic drug monitoring is defined as the management of a patient's drug regime based on serum, plasma, or whole blood concentration of a drug. Therapeutic drug monitoring is valuable when the drug in question has a narrow therapeutic index and toxicity may be encountered at a concentration slightly above the upper end of the therapeutic range. The protein binding of a drug can be low, moderate, or high (> 80%). Some drugs such as ethosuximide and lithium are not even bound to serum proteins (0% binding). Albumin, $\alpha_1$-acid glycoprotein and lipoproteins are major drug-binding proteins in serum. Drugs exist in peripheral circulation as free (unbound) and bound to protein forms following the principle of reversible equilibrium and law of mass action. Only free drug can bind with the receptor for pharmacological action, and concentrations of active drug molecule at the receptor site is generally considered as related to unbound (free) drug concentration in plasma *(1)*.

In general, there is equilibrium between free drug and protein-bound drug.

$$[D] + [P] = [DP]$$

$$K = [DP]/[D][P]$$

[D] is unbound drug concentration, [P] is binding protein concentration, [DP] represents drug/protein complex, and $K$ is the association constant (liters/mole). The greater the affinity of the protein for the drug, the higher is the $K$ value. The free fraction of a drug represents the relationship between bound and free drug concentration and is often referred as "$F_u$".

$$F_u = \frac{\text{Free drug concentration}}{\text{Total drug concentration (bound + free)}}$$

Free fraction ($F_u$) does not vary with total drug concentration because protein-binding sites usually exceed the number of drug molecules present. Therefore, unbound concentration of a drug can be easily calculated by multiplying total drug concentration with $F_u$, and there maybe no need to measure free drug directly.

$$\text{Free drug concentration} = F_u \times \text{Total drug concentration}$$

For example, phenytoin is 10% free ($F_u = 0.1$). Therefore, if total phenytoin concentration is 10 μg/mL, the free concentration should be 1 μg/mL. However, for certain drugs, the number of protein-binding sites may approach or be less than the number of drug molecules. Valproic acid exhibits saturable protein binding at the upper end of the therapeutic range and as a result the $F_u$ of valproic acid is subject to more variation than other highly protein-bound antiepileptic drugs *(2,3)*. For example, albumin concentration of 4.0 gm/dL is equivalent to an albumin concentration of 597 μmol/L because the molecular weight of albumin is 67,000 D. The therapeutic range of valproic acid is 50–100 μg/mL or 347–693 μmol/L. Therefore, the upper end of therapeutic molar concentration of valproic acid exceeds molar concentration of albumin and not enough binding sites are available to bind valproic acid. Other factors also may influence the $F_u$ such as displacement of a strongly protein-bound drug by another strongly protein-bound drug or endogenous factors.

## 2. DRUGS WHICH ARE CANDIDATE FOR FREE DRUG MONITORING

If the protein binding of a drug is less than 80%, it is not considered a candidate for free drug monitoring because variation in protein binding may not have clinically significant effect in altered free drug concentrations. For example, if a drug is 90% protein bound and the protein binding is decreased by 10%, the $F_u$ is increased by 90%. If the drug is 80% protein bound, reduction of protein to 72% will result in an $F_u$ increase from 2.0 μg/mL (assuming again total drug concentration is 10 μg/mL) to 2.8 μg/mL, a 40% increase. If the drug is only 50% bound to serum protein, a 10% reduction in protein binding will only alter free drug concentration by 10% (Fig. 1). An exception is free digoxin monitoring (digoxin is only 25% protein bound), which is very useful in patients overdosed with digoxin and being treated with digibind, the Fab fragment of antidigoxin antibody. Protein binding of some commonly monitored therapeutic drugs is given in Table 1. In today's practice, free drug monitoring is most common with anticonvulsants.

The first comprehensive report demonstrating the clinical utility of free drug monitoring dated back to 1973 *(4)*. In a population of 30 epileptic patients, the authors found a better correlation with toxicity (in coordination, ataxia and nystagmus) and free drug concentrations. Blum et al. described a uremic patient who was well controlled on a total phenytoin concentration of 3 μg/mL, which was far below the recommended

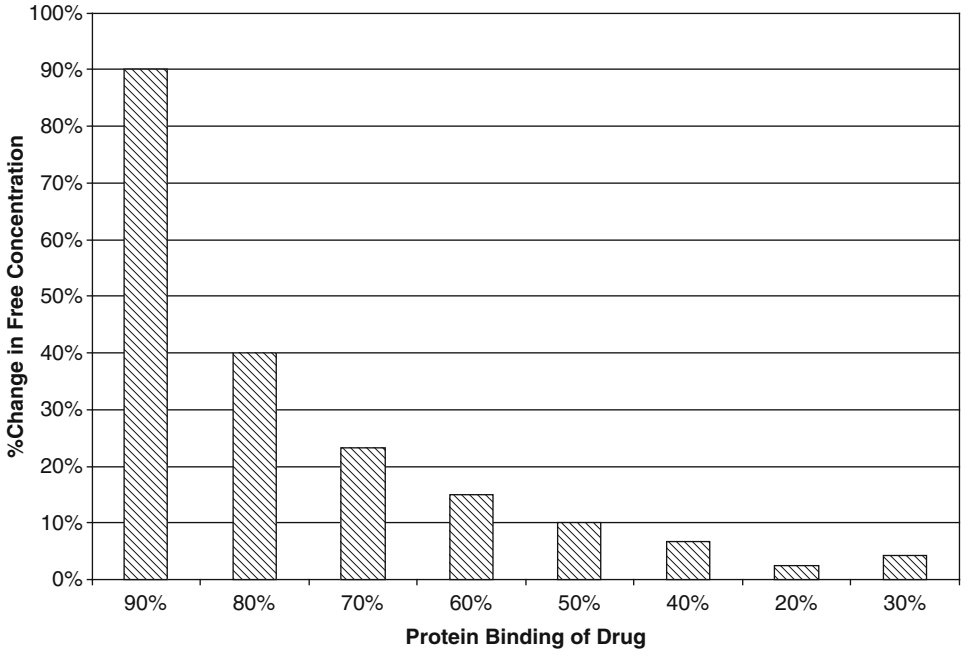


**Fig. 1.** Percentage change in free drug concentration because of a 10% reduction in protein binding of a drug. Calculations are based on a total drug concentration of 10 μg/mL. Range of protein binding of drug is from 20 to 90%.

**Table 1**
**Protein Binding of Commonly Monitored Therapeutic Drugs**

| *Drug* | *Protein Binding (%)* | *Protein Type* | *Free Drug Monitoring* |
|---|---|---|---|
| Amikacin | <5 | No | |
| Kanamycin | <5 | No | |
| Ethosuximide | 0 | No | |
| Procainamide | 10–15 | Albumin | No |
| Theophylline | 40 | Albumin | No |
| Phenobarb | 40 | Albumin | No |
| Phenytoin | 90 | Albumin | Yes |
| Carbamazepine | 80 | Albumin | Yes |
| Valproic acid | 90–95 | Albumin | Yes |
| Primidone | 15 | Albumin | No |
| Digoxin | 25 | Albumin | Yes |
| Quinidine | 80 | α-1-acid glycoprotein | Yes |
| Lidocaine | 60–80 | α-1-acid glycoprotein | Yes |
| Cyclosporine | 98 | Lipoproteins | Yes |
| Tacrolimus | 97 | Lipoprotein | Yes |
| Mycophenolic acid | 92 | Albumin | Yes |

therapeutic range of 10–20 μg/mL *(5)*. The most likely cause of this observation was significantly elevated $F_u$ of phenytoin because of uremia.

Albumin and $\alpha_1$-acid glycoprotein are the major drug-binding proteins in serum. At least two drug-binding sites with different domains have been identified in albumin, and a drug may bind to one or both sites. Basic drugs usually bind to either a single protein such as albumin or an $\alpha_1$-acid glycoprotein. Alternatively, basic drugs may also bind to several proteins including lipoproteins. The concentration of $\alpha_1$-acid glycoprotein may increase in several disease states thus may cause reduced $F_u$ of a free basic drugs. Such conditions are given in Table 2. Acidic drugs predominately bind to albumin, although interactions with $\alpha_1$-acid glycoprotein have also been reported. The concentration of album decreases significantly under several pathophysiological conditions leading to an increase in $F_u$ of acidic drugs. Such conditions are summarized in Table 3.

Drug protein binding may also change with temperature and pH. For basic drugs, the percentage of unbound drug decreases with increasing pH, but for acidic drugs, the unbound fraction may increase, decrease, or remain independent of pH change *(6)*.

### *2.1. Free Drug Monitoring of Lidocaine, Quinidine, and Other Drugs Bound to $\alpha_1$-Acid Glycoprotein*

Routledge et al. *(7)* reported in 1980 inter-individual variation in free lidocaine concentration. The percentage of unbound lidocaine was decreased in patients with uremia compared with that in controls (20.8% in uremic patients vs. 30.8% in control)

**Table 2**
**Pathophysiological Conditions that Alter α-1-acid Glycoprotein Concentration**

| *Elevated α-1-Acid Glycoprotein* | *Reduced α-1-Acid Glycoprotein* |
|---|---|
| Acute myocardial infarction | Liver cirrhosis |
| Renal failure | |
| Stroke | |
| Burn patient | |
| Inflammation | |
| Infection | |
| Pulmonary edema | |
| Rheumatoid arthritis | |
| Malignancy | |
| Advanced phase of chronic myelogenous leukemia | |
| Crohn's disease | |
| Pulmonary edema | |
| Trauma | |
| Intensive care unit patients | |
| Acute pancreatitis | |
| Hypertensive patients | |
| Smokers | |

as well as in renal transplant recipients. The cause of increased protein binding of lidocaine in these patients was significant increases in concentration of $\alpha_1$-acid glycoprotein (134.9 mg/dL in patients vs. 66.3 mg/dL in controls), the binding protein of lidocaine in human serum. In contrast, unbound concentrations of diazepam increased

**Table 3**
**Pathophysiological Conditions that Reduce Albumin Concentration Leading to an Increase in Free Fraction of Acidic Drugs**

| *Reduced Albumin Concentrations* |
|---|
| Uremia |
| Pregnancy |
| Intensive care unit patients |
| Trauma patients |
| Liver disease |
| Hyperthyroidism |
| Burn patient |
| Elderly (> 75years) |
| Cirrhosis |
| Hepatic disease |
| Malnutrition |
| AIDS patients |

significantly in patients with uremia. Authors concluded that binding of basic drugs in uremic patients may increase or decrease depending on the binding protein *(8)*. Shand commented that in situations where $\alpha_1$-acid glycoprotein concentration is altered (particularly myocardial infarction), the usual therapeutic range of total lidocaine may not apply and monitoring free concentration is more appropriate *(9)*. Routledge et al. *(10)* devised a free lidocaine index based on $\alpha_1$-acid glycoprotein concentration and concluded that free drug index is useful in rapidly assessing unbound lidocaine concentrations in plasma. Displacement of lidocaine from protein binding by disopyramide may result in elevated free lidocaine concentration because disopyramide has a stronger binding affinity for $\alpha_1$-acid glycoprotein *(11)*.

Although the frequency of common alleles of $\alpha_1$-acid glycoprotein is the same among Caucasians and African-Americans, the concentration of $\alpha_1$-acid glycoprotein is significantly lower in the Caucasian population. However, there was no significant relationship between $\alpha_1$-acid glycoprotein phenotype and the protein binding of lidocaine. In contrast, quinidine-unbound fraction was related to $\alpha_1$-acid glycoprotein phenotype *(12)*. Edwards et al. reported that $F_u$ of quinidine ($0.129 \pm 0.019$) was constant throughout the therapeutic range in healthy volunteers. Moreover, patients with hyperlipidemia had $F_u$ of quinidine similar to healthy volunteers, but patient suffering from traumatic head injury had a significant increase in $\alpha_1$-acid glycoprotein concentration and a decreased quinidine $F_u$ ($0.075 \pm 0.019$) *(13)*.

The protein binding of the short-acting narcotic analgesic alfentanil is affected by disease. This drug is mainly bound to $\alpha_1$-acid glycoprotein, the concentration of which can be significantly increased in patients with renal failure, myocardial infarction, and rheumatoid arthritis and also in intensive care unit patients. Interestingly, protein binding of alfentanil was only increased in patients with myocardial infarction. In patients with liver cirrhosis, concentrations of both albumin and $\alpha_1$-acid glycoprotein were reduced resulting in decreased protein binding of alfentanil. Dispyramide was able to displace alfentanil from protein binding leading to an increased $F_u$ whereas other strongly $\alpha_1$-acid glycoprotein-bound drugs quinidine, lidocaine, and bupivacaine had no effect *(14)*.

### *2.2. Analytical Considerations*

Equilibrium dialysis technique was used by several investigators to estimate free lidocaine concentration. Routledge et al. subjected two 1 ml aliquots of plasma to equilibrium dialysis using a Teflon equilibrium dialysis cell. The dialysis was performed against Sorenson's phosphate buffer (containing 0.5% w/v sodium chloride), and the pH was adjusted to 7.4 to which lidocaine hydrochloride was added (3 μg/mL buffer). This concentration was achieved by adding unlabeled lidocaine hydrochloride (2.8 μg/mL) to radioactive $^{14}$C lidocaine (200 ng/mL). The buffer and plasma compartment were separated by a Spectrapor dialysis membrane with a molecular weight cut-off range of 12,000–14,000, and the cells were rotated in a water bath for 3 h at 37°C. The authors demonstrated that equilibrium was achieved in 3 h and the binding was similar in heparinized plasma, citrated plasma, and serum. After dialysis, 300 μl of aliquots were withdrawn from each side of the cells, scintillation fluid was added, and the radioactivity was measured. Quench correction was made

**Table 4**
**Technical Aspects of Monitoring Free Drug Concentration**

| *Free Drug* | *Separation from Bound* | *Analytical Technique* |
|---|---|---|
| Phenytoin | Ultrafiltration | Immunoassay |
| Carbamazepine | Ultrafiltration | Immunoassay |
| Valproic Acid | Ultrafiltration | Immunoassay |
| Digoxin | Ultrafiltration | Immunoassay[a] |
| Lidocaine | Ultrafiltration[b] | Immunoassay |
| Quinidine | Ultrafiltration[b] | Immunoassay |
| Cyclosporine | Equilibrium dialysis | HPLC/MS |
| Tacrolimus | Equilibrium dialysis | HPLC/MS |
| Mycophenolic Acid | Ultrafiltration | Immunoassay/ HPLC |
| Indinavir | Ultrafiltration | LC/MS |
| Amprenavir | Ultrafiltration/ equilibrium dialysis | HPLC/UV |

HPLC, high-performance liquid chromatography; UV, ultraviolet.

[a] Because free digoxin is almost 75% of total digoxin concentration, commercially available immunoassays usually have enough sensitivity for accurate determination of free digoxin.

[b] Methods may also include equilibrium dialysis.

by the external standard ratio method, and the percentage of unbound drug in plasma was calculated as the ratio of the absolute disintegration rates in buffer and plasma multiplied by 100 *(7)*. Other studies published later also used equilibrium dialysis for estimating unbound lidocaine concentration *(8,10)*.

Edwards et al. studied protein binding of quinidine in human plasma. The quinidine $F_u$ was two- to threefold higher when blood was collected in evacuated blood collection glass syringes. Other factors that affect protein binding of quinidine include addition of heparin in vitro, condition of equilibrium dialysis, and the presence of dihydroquinidine, which may be a common impurity in quinidine preparation. The authors subjected 400 µl of phosphate buffer solution (pH 7.4, 0.134 M) to equilibrium dialysis against an equal volume of serum in plexiglass cells for 5 h at 37°C. Postdialysis, quinidine concentrations on each side of the dialysis membrane were determined by using liquid scintillation counting *(13)*.

McCollam et al. used ultrafiltration technique and fluorescence polarization immunoassay (FPIA) for determination of unbound concentration of lidocaine and quinidine. For this purpose, serum pH was adjusted to 7.4 ± 0.5 by bubbling carbon dioxide through 1 ml of the specimen. Then, ultrafiltration was performed to separate bound fraction from $F_u$ using a Centricon-10-ultrafiltration device (Amicon, Baverly, MA) at 2500 × *g* for 45 min. This ultrafiltration device has a molecular weight cut-off of 10,000, which prevents $\alpha_1$-acid glycoprotein to pass through the column. The FPIA assays were performed by using a TDx analyzer (Abbott Laboratories, Abbot Park, IL) *(12)*. Analytical conditions for monitoring free drug concentrations are summarized in Table 4.

### 2.3. Monitoring Free Concentrations of Immunosuppressant Drugs

Immunosuppressants such as cyclosporine, tacrolimus, and mycophenolic acid mofetil are strongly bound to serum proteins. Cyclosporine is strongly bound in plasma mainly to lipoproteins, and the unbound fraction is usually 2%. Some evidence also indicates that the unbound concentration of cyclosporine has a closer association with both kidney and heart allograft rejection than total concentrations. However, measuring free cyclosporine concentration is inherently complex and not usually performed in clinical laboratories *(15)*. Mendonza et al. described a liquid chromatography—tandem mass spectrometric technique for determination of cyclosporine concentrations in saliva. For a highly protein-bound drug such as cyclosporine, saliva offered a simple way to determine free cyclosporine concentration *(16)*.

Another widely used immunosuppressant drug, tacrolimus, is also strongly bound to erythrocytes and serum proteins. Warty et al. *(17)* studied the distribution of tacrolimus in 13 transplant recipients and reported that in plasma 64 ± 8% of tacrolimus was associated with lipoprotein-deficient plasma 21.0 ± 8% associated with high-density lipoprotein, 3.0 ± 3% associated with low-density lipoprotein, and 11.0 ± 3% associated with very low-density lipoprotein. Piekoszewski et al. *(18)* reported that the plasma protein binding of tacrolimus was on average 72%. Zahir et al. reported that unbound concentrations of tacrolimus were significantly lower during episodes of rejection, and in patients experiencing tacrolimus-related side effects, only the unbound concentrations of tacrolimus were found to be significantly higher. Blood distribution and protein binding of tacrolimus also change significantly in the posttransplant period, leading to changes in unbound tacrolimus concentrations *(19)*. Another study also confirmed that unbound concentrations of tacrolimus were lower in liver transplant recipients experiencing rejection *(20)*.

Mycophenolate mofetil is a prodrug which is converted into mycophenolic acid. The plasma protein binding increases with time after liver transplant from 92 to 98% causing intraindividual variation in liver transplant recipients *(21)*. Atcheson et al. reported that mycophenolic acid $F_u$ varied 11-fold from 1.6 to 18.3% whereas the metabolite glucuronide fraction varied threefold from 17.4 to 54.1%. There were positive correlations between urea and creatinine concentrations and free mycophenolic acid concentrations, whereas there was a negative correlation between albumin concentration and free mycophenolic acid concentration. The authors further reported that on average free mycophenolic acid $F_u$ was 70% higher in patients with albumin concentrations below 3.1 gm/dL than in patients with normal albumin concentrations. Patients with marked renal impairment showed higher free concentrations of mycophenolic acid. The exposure to unbound mycophenolic acid was significantly related to infections and hematological toxicity but neither free nor total mycophenolic acid concentration was related to rejection episodes *(22)*. High-unbound mycophenolic acid concentration was also encountered in a hematopoietic cell transplant patient with sepsis, renal, and hepatic dysfunction *(23)*. Concentrations of free mycophenolic acid can be measured in protein-free ultrafiltrate after solid phase extraction using SPE C-18 cartridges and liquid chromatography combined with tandem mass spectrometry *(24)*.

### *2.4. Analytical Consideration*

Although research indicates that there are advantages in monitoring free concentration of immunosuppressant drugs, technically it is very difficult and not offered as a routine test in clinical laboratories. First of all, unlike free anticonvulsants where commercially available kits can be used easily combined with ultrafiltration technique for monitoring, free phenytoin, free carbamazepine, and free valproic acid concentration, there is no commercially available kit for monitoring free concentrations of immunosuppressant drugs. Therefore, to achieve analytical reproducibility to detect such low concentrations of drug, high-performance liquid chromatography combined with tandem mass spectrometry (HPLC/MS/MS) is the preferred technique although there are few reports in the literature of use of HPLC combined with ultraviolet (UV) detection to achieve monitoring of free immunosuppressant drug concentrations.

Akhlaghi used equilibrium dialysis technique to determine the $F_u$ of cyclosporine. The authors used radioactive [$^3$H] cyclosporine purified by HPLC for this purpose. Equilibrium dialysis experiments were performed using a Spectrum equilibrium dialysis apparatus and cellulose dialysis membrane (SpectraPor 2, Spectrum Medical Instrument, Los Angeles, CA) with a molecular weight cut-off of 12,000–14,000. To avoid non-specific binding of cyclosporine to surfaces of the dialysis cell, original cells were replaced with cells constructed from medical grade stainless steel and having volume of 1.36 ml per half-cell. For the equilibrium dialysis experiment, 1 ml of plasma was supplemented with [$^3$H] cyclosporine and then dialyzed against isotonic phosphate buffer at 37°C for 18 h. After dialysis, aliquots of plasma and buffer were removed simultaneously using two glass syringes and radioactivity of specimens was measured using liquid scintillation counter. The volume shift was determined from total plasma protein before and after dialysis using a biuret method. Then the $F_u$ was calculated after correction of volume shift between cells *(25)*. The $F_u$ values reported by these authors matched well with the values reported by Henricsson *(26)* who also used equilibrium dialysis in stainless steel cells but in general lower than those observed by Legg et al. who used ultracentrifugation method and plasma from patients who had renal transplantation *(27)*.

Legg et al. used ultracentrifugation technique for determination of unbound cyclosporine concentrations. The authors added radiolabeled cyclosporine (approximately 60 ng/mL) to plasma, and after performing the ultracentrifugation, individual fractions were frozen and then sliced to measure high-density lipoprotein (HDL, measured in the bottom section), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL, top section), and chylomicrons. The unbound fraction of lipoprotein was measured in the middle protein-free section *(27)*. The authors also proposed a mathematical model to calculate for $F_u$ based on cholesterol and triglyceride concentration.

$$F_u = \frac{1}{1.346\ \text{triglyceride (mM/L)} + 2.815\ \text{cholesterol (mM/L)} + 1}$$

However, the authors also commented that direct measurement of unbound cyclosporine is more accurate than the calculated value *(27)*. Akhlaghi et al. *(25)* also proposed a model for calculating $F_u$ based on lipid parameters, but this model also had some inherent problem.

Several authors also used the equilibrium dialysis technique for determination of the unbound fraction of tacrolimus. Zahir et al. used the equilibrium dialysis technique to study the protein binding of tacrolimus using siliconized stainless steel dialysis cells to avoid non-specific binding of tacrolimus. The authors used [$^3$H] dihydro tacrolimus as a tracer compound and commented that although ultrafiltration is a simple technique to separate bound from the free, it may produce erroneous results *(19)*. Iwasaki et al. *(28)* also used the equilibrium dialysis technique to study protein binding of tacrolimus and reported that the binding was 99%. Piekoszewski et al. *(18)* used ultracentrifugation technique to determine the unbound concentration of tacrolimus.

Ultrafiltration is a suitable technique for separating the bound from the unbound fraction of mycophenolic acid. Ensom et al. used ultrafiltration technique and HPLC for measuring free mycophenolic acid concentration. Total mycophenolic acid concentration was also measured using HPLC coupled with UV detection *(29)*. Akhlaghi et al. *(30)* also used ultrafiltration technique to separate free mycophenolic acid from the bound form and then measured free mycophenolic acid in the protein-free ultrafiltrate using liquid chromatography and tandem mass spectrometry. Atcheson et al. placed 500 µl of plasma specimen in a sealed ultrafiltration tube and centrifuged at $3000 \times g$ for 20 min at 20°C, and free mycophenolic acid concentration was determined in the protein-free ultrafiltrate using liquid chromatography combined with tandem mass spectrometry. The unbound concentration of mycophenolic acid glucuronide was measured using liquid chromatography combined with UV detection. Authors used the Centrifree Micropartition system consisting of a 1-ml reservoir and a membrane with a 30,000 D molecular weight cut-off (Amicon, Danvers, MA) *(31)*.

### *2.5. Monitoring Free Concentrations of Protease Inhibitors*

Antiretroviral drugs used in treating patients with AIDS demonstrate wide variations in serum protein bindings. Protease inhibitors with the exception of indinavir are strongly protein bound (>90%) mainly to $\alpha_1$ acid glycoprotein. Efavirenz, a non-nucleoside reverse transcriptase inhibitor, is more than 99% bound to serum protein (mainly albumin). The pharmacological effect of antiretroviral drugs is dependent upon the unbound concentration of drugs capable of entering cells that harbor human immunodeficiency virus (HIV) *(32)*. Protein binding of indinavir varied between 54 and 70% in eight men with a mean protein binding of 61%. However, the variability of protein binding was concentration-dependent *(33)*. Although determination of indinavir concentrations in saliva using HPLC is useful in determining compliance of patients with indinavir therapy, salivary concentrations of indinavir do not correlate with unbound concentration of indinavir in plasma if saliva collection was stimulated, but correlate well with unbound concentration in plasma if saliva collection was not stimulated *(34)*.

The mean free plasma-unbound amprenavir concentration was 8.6% (range 4.4–20%) in one study. Moreover, lopinavir was able to displace amprenavir from protein binding in vitro, but another strongly protein-bound protease inhibitor ritonavir had no effect *(35)*. Boffito et al. studied lopinavir protein binding in vivo through a 12-h dosing interval and measured free lopinavir concentrations using HPLC-MS/MS. The mean unbound lopinavir concentration was 0.92% when measured using ultrafiltration but 1.32% using equilibrium dialysis. The unbound percentage of lopinavir was also

found to be higher after 2 h than at baseline *(36)*. However, therapeutic drug monitoring of free concentrations of antiretroviral drugs are at this point in the preliminary stage, and more studies are needed with a larger patient base as well as clinical correlations to establish guidelines for monitoring the free drug concentration of antiretroviral drugs.

### *2.6. Monitoring Free Digoxin Concentration*

Digoxin is a cardioactive drug which is only 25% bound to serum proteins (mainly albumin). Monitoring free digoxin concentration can be useful only under special circumstances: (a) in patients overdosed with digoxin and being treated with Fab fragment of antidigoxin antibody (digibind) and (b) to eliminate interference of endogenous digoxin-like immunoreactive factors on serum digoxin measurement. Certain Chinese medicines such as Chan Su, Dan Shen, and Ginsengs may interfere with digoxin immunoassays. Moreover, therapy with spironolactone or potassium canrenoate can cause significant interference in serum digoxin measurement using immunoassays. These issues are discussed in detail in Chapter 6.

## 3. MONITORING CONCENTRATIONS OF FREE ANTICONVULSANTS

Anticonvulsants such as phenytoin, carbamazepine, and valproic acid are strongly protein bound, mainly to albumin. Clinical utility of monitoring free phenytoin, free carbamazepine, and free valproic acid has been well documented in the literature, and many clinical laboratories offer free phenytoin, carbamazepine, and valproic acid determinations in their test menu. Moreover, the College of American Pathologists also has free anticonvulsant levels in their external survey specimens, and assay kits are commercially available for monitoring free levels of phenytoin, carbamazepine, and valproic acid.

### *3.1. Free Valproic Acid Concentrations and Clinical Outcome*

Significant inter-individual variations can be observed in the $F_u$ of phenytoin, carbamazepine, and valproic acid, especially in the presence of uremia and liver disease. Drug–drug interactions can also lead to elevated free drug concentration. When binding is changed, the total concentration no longer reflects the pharmacologically active free drug in the plasma. Measuring free drug concentrations for antiepileptic drugs eliminates a potential source of interpretative errors in therapeutic drug monitoring using traditional total drug concentrations *(37)*. Usually, total drug concentrations (free + protein bound) are measured in the laboratory because it is technically easier than free drug monitoring, but changes in the extent of protein binding for highly protein-bound drugs are clinically significant *(38)*. For example, if the binding of a drug changes from 98 to 96%, the total drug concentration is unaltered, but the concentration of $F_u$ is doubled *(39)*.

Valproic acid (therapeutic range of 50–100 μg/mL) is extensively bound to serum proteins, mainly albumin *(40)*. Fluctuations in protein binding occur within the therapeutic range because of the saturable binding phenomenon leading to variations of the $F_u$ from 10 to 50% *(41)*. Moreover, unbound valproic acid concentration may also vary during one dosing interval in patients already stabilized on valproic acid *(42)*,

and several studies have reported problems associated with predicting a therapeutic response of valproic acid from total serum concentrations *(43,44)*.

Gidal et al. reported a case where markedly elevated plasma-free valproic acid in a hypoalbuminemic patient contributed to neurotoxicity. The total valproic acid concentration was 103 μg/mL, but the free valproic acid concentration was 26.8 μg/mL. This unexpected elevation was due to a low albumin level (3.3 gm/dL) of the patient *(45)*. Haroldson et al. reported a case demonstrating the importance of monitoring free valproic acid in a heart transplant recipient with hypoalbuminemia. When the valproic acid dose was adjusted based on the free valproic acid concentration rather than total valproic acid concentration, the patient improved and was eventually discharged from the hospital *(46)*. Lenn and Robertson demonstrated that the concentration of free valproic acid has clinical significance in management of seizure as well as avoiding undesirable side effects. The authors recommended using free valproic acid concentration for routine patient management *(47)*. Diurnal fluctuations in free and total plasma concentrations of valproic acid at steady state have been reported *(48,49)*. Ahmad et al. reported that total valproic acid concentrations show higher interindividual variation and tend to underestimate the effect of poor compliance, but the use of free valproic acid concentration offers an advantage in therapeutic drug monitoring *(50)*. Although unbound valproic acid concentration mirrors CSF valproic acid concentration, Rapeport et al. *(51)* reported a lack of dc correlation between free levels and pharmacological effects. Valproic acid is strongly protein bound and is considered not to be removable by extracorporeal means. In the case of severe overdose with valproic acid where free concentration is high because of disproportionate protein binding, extracorporeal means such as hemodialysis and hemoperfusion can be used *(52)*.

### *3.2. Free Phenytoin Concentrations and Clinical Outcome*

Soldin *(39)* reported that in his personal experience, free phenytoin is the most requested free drug level by clinicians. Phenytoin is 90% bound to serum proteins, mainly albumin. Phenytoin does not show any concentration-dependent binding within the therapeutic range. Kilpatrick et al. *(53)* reported that unbound phenytoin concentration (1.2–2.5 μg/mL) reflected the clinical status of a patient equally or better than the total phenytoin concentration. Booker *(4)* earlier reported that free phenytoin concentration correlated better with toxicity, and the authors observed no toxicity at free phenytoin concentration of 1.5 μg/mL or less. In patients with greatly decreased albumin levels, free phenytoin is the better indicator of effective plasma concentrations (therapeutic arrange: 0.8–2.1 μg/mL) *(54)*. Dutkiewicz et al. showed that in hypercholesterolemia and in mixed hyperlipidemia, the blood level of free phenytoin was elevated. The effect was probably related to displacement of phenytoin by free fatty acids *(55,56)*. In eclampsia, free phenytoin levels are usually abnormally high although total phenytoin levels are within therapeutic range. Unfortunately, neither total nor free phenytoin levels are good predictors of seizure control *(57)*. The binding of phenytoin to serum albumin can be altered significantly in uremia. The lower protein-binding capacity of phenytoin in uremia can be related to hypoalbuminemia, structural modification of albumin, and accumulation of uremic compound in blood that displaces phenytoin from protein-binding sites *(58–60)*.

Monitoring free phenytoin concentration is very important in patients with hypoalbuminemia to avoid toxicity. Lindow et al. *(61)* described severe phenytoin toxicity associated with hypoalbuminemia in critically ill patients that was confirmed by direct measurement of free phenytoin. Zielmann et al. reported that in 76% of 38 trauma patients, the free phenytoin fraction was increased to as high as 24% compared with 10% $F_u$ in otherwise healthy subjects. The major causes of elevated free phenytoin were hypoalbuminemia, uremia, and hepatic disease *(62)*. The authors recommended monitoring of free phenytoin in such patients. Thakral et al. reported a case where a 19-year-old man who developed blurred vision and xanthopsia after administration of phenytoin for status epilepticus. The free phenytoin level was found to be toxic. Phenytoin was withheld, and the patient experienced partial recovery. The authors concluded that phenytoin toxicity as revealed by an elevated free phenytoin concentration contributed to this acute visual dysfunction *(63)*. Burt et al. studied total and free phenytoin levels in 139 patients. Free phenytoin concentrations were 6.8–35.3% of total phenytoin concentrations (expected range 8–12%). Clinical indications responsible for variations were hypoalbuminemia, drug interactions, uremia, pregnancy, and age. The authors concluded that monitoring total phenytoin is not as reliable as free phenytoin as a clinical indicator for therapeutic concentrations and recommended that therapeutic monitoring of phenytoin should be only the free concentration *(64)*. Recently, Iwamoto et al. *(65)* also reiterated the need of free phenytoin monitoring in patients receiving phenytoin monotherapy because free phenytoin fraction was significantly influenced by aging, mean creatinine clearance, and serum albumin levels in the patient population they studied. Deleu *(66)* recommended a dose of 6.1 mg/kg to achieve a free phenytoin concentration of 1.5 μg/mL.

### *3.3. Free Carbamazepine Concentrations and Clinical Outcome*

Carbamazepine is effective in the treatment of primary or secondary generalized tonic-clonic epilepsy, all variety of partial seizure, and myoclonic epilepsy. The plasma protein binding of carbamazepine is 70–80%. The primary and active metabolite 10,11-epoxide is only 50% bound to serum proteins. There seems to be less variability in the protein binding of carbamazepine compared with that of phenytoin and valproic acid *(67)*. Froscher et al. *(68)* showed that in patients with carbamazepine monotherapy, there was no closer relationship between free concentration and pharmacological effects compared with total concentration and pharmacological effects. Lesser et al. *(69)* found a broad overlapping of unbound carbamazepine causing toxicity and no toxicity. Because 10,11-epoxide has a greater percentage of $F_u$ and is almost equipotent to carbamazepine, epoxide probably contributes significantly to the pharmacological effects of carbamazepine. Therapeutic monitoring of epoxide along with carbamazepine may be useful especially in patients taking valproic acid or lamotrigine *(70,71)*.

## 4. WHEN SHOULD FREE ANTICONVULSANT BE MONITORED?

For strongly protein-bound anticonvulsants, such as phenytoin, valproic acid, and carbamazepine, free drug monitoring is strongly recommended in the following cases:

1. Uremic patients
2. Patients with chronic liver disease

3. Patients with hypoalbuminemia (burn patients, elderly, pregnancy, AIDS, etc.)
4. Suspected drug–drug interactions where one strongly protein-bound drug can displace another strongly protein-bound anticonvulsant.

## 5. ELEVATED FREE ANTICONVULSANT CONCENTRATIONS IN UREMIA

Unexpected elevated concentrations of free valproic acid, free phenytoin, and free carbamazepine are encountered in uremia. In uremia, the $F_u$ of valproic acid can be as high as 20–30% compared with 8.45% as observed in healthy volunteers. In uremia, $F_u$ of phenytoin can be as high as 30% whereas in normal volunteers the $F_u$ is usually 10%. Uremia also modifies the disposition of a highly metabolized drug by changes in plasma protein binding or hepatic metabolism *(72)*.

High free drug concentrations in uremia are related to hypoalbuminemia, as well as to the presence of endogenous uremic compounds that can displace strongly protein-bound drugs from protein-binding sites. Monaghan et al. studied in detail the relationship between serum creatinine, blood urea nitrogen, albumin, and the unbound fraction of phenytoin in patients who have undergone renal transplant. The authors concluded that the estimation of $F_u$ of phenytoin in patients with a history of uremia and hypoalbuminemia should not be based on measurement of serum creatinine and albumin *(73)*.

Hippuric acid and indoxyl sulfate, the two other compounds that are present in elevated concentrations in uremia, can cause displacement of strongly protein-bound drugs *(74)*. Takamura identified 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF) as the major uremic toxin that causes impaired protein binding of furosemide. Oleate also plays a role *(75)*. Other uremic compounds such as guanidine, methyl guanidine, and guanidinosuccinic acid do not cause any displacement of drug from protein binding. Another study indicates that several endogenous compounds with small molecular weights (<500) play significant roles in displacement of strongly protein-bound drugs, but mid-molecular uremic toxins do not displace drugs *(76)*. Otagiri *(77)* in a recent review on drug protein binding commented that reduced protein binding of drugs in uremia can be explained by a mechanism that involves a combination of direct displacement by free fatty acids as well as a cascade of effects from free fatty acids and unbound uremic toxins.

### *5.1. Elevated Free Anticonvulsant Levels in Hepatic Disease*

Patients with hepatic disease usually have hypoalbuminemia. Because albumin is the major binding protein for phenytoin, valproic acid, and carbamazepine, elevated free anticonvulsant concentration is expected in patients with liver disease. Elevated free phenytoin concentration occurs in patients with hepatic disease because of hypoalbuminemia *(78)*. In hepatic failure, the hepatic clearance of unbound phenytoin may also be reduced because of hepatic tissue destruction and a reduction in hepatic enzyme activities responsible for metabolism of phenytoin. When this occurs, a reduction of phenytoin dose is necessary to maintain unbound phenytoin concentration below toxic level. Prabhakar and Bhatia *(79)* reported that free phenytoin levels are elevated in patients with hepatic encephalopathy. Fosphenytoin is a phosphate ester prodrug of phenytoin developed as an alternative to phenytoin for acute treatment

of seizures. Fosphenytoin is rapidly converted to phenytoin in vivo with a half-life of 7–15 min. The unbound plasma phenytoin concentrations achieved with standard fosphenytoin intravenous loading doses are similar to that achieved by equivalent phenytoin administration. However, earlier and higher unbound phenytoin concentrations and thus an increase in systemic adverse effect may occur following intravenous fosphenytoin-loading doses in patients with impairment in fosphenytoin (93–98% bound to protein) and phenytoin protein binding (hepatic or renal disease, hypoalbuminemia, and elderly) *(80)*.

Hepatic disease can alter pharmacokinetic parameters of valproic acid. Klotz et al. reported that alcoholic cirrhosis and viral hepatitis decreased valproic acid protein binding from 88.7 to 70.3 and 78.1%, respectively, with a significant increase in volume of distribution. Elimination half-life was also prolonged *(81)*. An increase in unbound concentration of carbamazepine has been reported in patients with hepatic disease *(82)*.

### *5.2. Free Anticonvulsant Concentrations in Patients with AIDS*

Seizures are a common manifestation of central nervous system disease in patients with HIV infection. The incidence is approximately 10% in a population of hospitalized patients with an advanced stage of disease *(83)*. Phenytoin is widely prescribed in the treatment of tonic-clonic seizures and other forms of epilepsy. Burger et al. investigated serum concentrations of phenytoin in 21 patients with AIDS. The total phenytoin concentrations were significantly lower in patients with AIDS than in the control population, although phenytoin doses were significantly higher in patients with AIDS. Calculation of Michaelis–Menten parameters demonstrated that $V_{max}$ values were similar in patients with AIDS and the control group, but a non-significant trend of lower $K_m$ values was observed in patients with HIV. The authors demonstrated that unbound phenytoin concentrations were significantly higher in patients with HIV and concluded that the lower protein binding of phenytoin in patients with AIDS could be related to hypoalbuminemia. Because unbound phenytoin is the pharmacologically active fraction, authors recommended monitoring unbound phenytoin concentrations for patients with HIV infection receiving phenytoin *(84)*. In vitro experiments also confirmed the findings of Burger et al. Concentrations of free phenytoin and free valproic acid were significantly elevated in serum pools prepared from patients with AIDS and supplemented with phenytoin or valproic acid compared with serum pools prepared from normal subjects and also supplemented with the same amount of phenytoin or valproic acid. Hypoalbuminemia alone did not explain the elevation of free phenytoin or free valproic acid. Drug–drug interactions probably play a major role because an average patient with AIDS is receiving more than 10 medications per day *(85)*. Toler et al. *(86)* also described severe phenytoin toxicity because of decreased protein binding of phenytoin in a patient with AIDS leading to an elevated free phenytoin concentration of 4.9 μg/mL.

### *5.3. Free Anticonvulsants in Pregnancy*

The pharmacokinetics of many anticonvulsants undergo important changes in pregnancy because of modification in body weight, altered plasma composition,

hemodynamic alteration, hormonal influence, and contribution of the fetoplacental unit to drug distribution and disposition. Pregnancy thus affects absorption of drugs, binding to plasma protein, distribution, metabolism, and elimination *(87)*. At constant dosages, plasma levels of anticonvulsants such as phenytoin, valproic acid, carbamazepine, phenobarbital, and primidone tend to decrease during pregnancy and then return to normal within the first or second month after delivery. Marked decrease in total phenytoin concentrations (about 40% of pre-pregnancy level) have been reported while free phenytoin level decreased to a much lesser extent *(88)*. Reports on the decline in total and free carbamazepine during pregnancy are conflicting. One study reports a 42% decline in total carbamazepine concentration and 22% decrease in free carbamazepine concentration from pregnancy to delivery in 22 patients *(89)*, whereas another study reported only a slight decrease in total carbamazepine concentration and an insignificant decrease in free carbamazepine concentration from pregnancy to delivery. For valproic acid, no significant change in free concentration was observed despite reduction in total valproic acid concentration. Tomson *(90)* commented that for highly protein-bound drugs such as phenytoin and valproic acid, total plasma concentrations may be misleading during pregnancy, underestimating the pharmacological effects of the drug.

## 6. DRUG–DRUG INTERACTIONS AND ELEVATED FREE ANTICONVULSANT CONCENTRATIONS

Sandyk *(91)* reported a case where phenytoin toxicity was induced by ibuprofen. This was due to displacement of phenytoin from protein binding by a strongly protein-bound drug ibuprofen. Tsanaclis et al. studied plasma protein binding of phenytoin in nine epileptic patients before and during addition of sodium valproate to the drug therapy. The mean $F_u$ of phenytoin increased from a mean value of 13.5–18.2%. The total phenytoin concentrations were reduced. The authors concluded that valproic acid displaces phenytoin from plasma protein-binding sites but does not inhibit its metabolism *(92)*. Pospisil and Perlik *(93)* demonstrated in vivo significant decreases in phenytoin protein binding because of the presence of valproic acid or primidone. Penicillins including oxacillin and dicloxacillin were effective in displacing phenytoin from its binding sites. In vivo, the total phenytoin concentration in serum decreased during penicillin administration while the free phenytoin concentrations increased *(94)*. However, phenytoin–oxacillin interaction is not significant at a lower dose of oxacillin usually prescribed in oral therapy. However, the interaction is significant at higher oxacillin doses especially in patients with hypoalbuminemia *(95)*. In vitro and in vivo displacement of phenytoin by antibiotics ceftriaxone, nafcillin, and sulfamethoxazole also have been reported *(96)*. Strongly protein-bound drugs that may displace phenytoin from protein-binding sites are summarized in Table 5.

Several non-steroidal anti-inflammatory drugs such as salicylate, ibuprofen, tolmetin, naproxen, mefenamic acid, and fenoprofen can displace phenytoin, valproic acid, and carbamazepine from protein-binding sites *(97)*. Blum et al. reported that tenidap sodium (an anti-inflammatory drug) 120 mg/day at steady state increased the percentage of protein binding of phenytoin in plasma by 25%. The authors concluded that because tenidap increases the percentage of unbound phenytoin in plasma, when monitoring plasma phenytoin concentration, free phenytoin concentrations also should

**Table 5**
**Common Strongly Protein-Bound Drugs that Displace Phenytoin from Protein-Binding Sites and May Lead to a Higher Free Fraction**

| *Drugs that May Displace Phenytoin from Protein Binding* |
|---|
| Valproic acid |
| Tolmetin |
| Fenoprofen |
| Oxacillin |
| Nafcillin |
| Salicylate |
| Naproxen |
| Tenidap |
| Dicloxacillin |
| Sulfamethoxazole |
| Ibuprofen |
| Mefenamic acid |
| Penicillin |
| Ceftriaxone |

be considered *(98)*. Reduced interaction between phenytoin and valproic acid with non-steroidal anti-inflammatory drugs in uremia has been described. The reduced interactions may be due to the presence of inhibitors *(97)*. Unexpected suppression of free phenytoin concentration by salicylate in uremic sera because of the presence of inhibitor had also been reported *(99)*. Carbamazepine-salicylate and digitoxin–valproic acid interactions are also reduced in uremic sera because of the presence of inhibitors *(100,101)*.

## 7. SALIVA AND TEARS: ALTERNATIVE TO SERUM FOR THERAPEUTIC DRUG MONITORING

Drugs that are not ionizable or are un-ionized within the salivary pH range (phenytoin, carbamazepine, and theophylline) are candidates for salivary therapeutic drug monitoring *(102)*. Salivary flow rates vary significantly between individuals and under different conditions. The use of stimulated saliva has advantage over resting saliva. The salivary flow rate, pH and sampling condition, and other pathophysiological factors may influence the concentration of a particular drug in saliva. However, under well-controlled and standardized conditions, saliva can be used as an alternative matrix for monitoring of carbamazepine, phenytoin, primidone, and ethosuximide. One report also concluded that monitoring of salivary phenytoin and carbamazepine proved to be a realistic alternative to plasma-free level monitoring because excellent correlations were found between salivary levels and serum-unbound levels of both phenytoin and carbamazepine *(103)*. However, a poor correlation was observed between serum and salivary methadone concentration *(104)*. Controversy also exists for valproic acid and phenobarbital *(105)*. Nakajima et al. *(106)* compared tear valproic acid concentrations

with total and free valproic acid concentrations in serum and concluded that the tear valproic acid concentrations correlated well with free valproic acid concentrations in serum.

Berkovitch et al. reported that there was no correlation between total or free digoxin plasma digoxin levels and salivary digoxin concentrations in children. The authors also observed no correlation between plasma gentamicin concentration and salivary levels when gentamicin was administered three times a day. In contrast, when gentamicin was administered once a day, a good correlation was found between plasma and salivary gentamicin concentrations *(107)*. Madsen et al. *(108)* reported that tobramycin could not be detected in saliva within the first 6 h of therapy in patients with cystic fibrosis. On the contrary, the average indinavir concentration in plasma is 70% of the serum concentration, and saliva can be used as an alternative matrix for the therapeutic drug monitoring of indinavir *(109)*. Ryan et al. *(110)* reported that despite wide inter-patient variability in saliva and serum ratio of lamotrigine, there is usually a good relation between serum and salivary concentrations of lamotrigine.

### *7.1. Analytical Considerations*

Many investigators used immunoassays available for monitoring serum concentrations of various drugs to determine salivary concentrations of drugs. al Za'abi et al. used the respective FPIA and TDx analyzer (Abbott Laboratories) for the determination of free carbamazepine, valproic acid, and phenytoin concentrations in serum as well as for measuring salivary concentrations of the respective drugs *(103)*. Madsen et al. used the Beckman Synchron CX system and Beckman immunoassay for determination of plasma as well as salivary concentration of tobramycin *(108)*. However, for drugs where immunoassays are not available, HPLC can be used for determination of both serum and salivary concentration of a drug. Ryan et al. *(110)* used HPLC for determination of both serum and salivary concentrations of lamotrigine.

## 8. ASSAY TECHNIQUES FOR FREE ANTICONVULSANTS

Ultrafiltration using Centrifree Micropartition System is the most common technique for monitoring free drug concentrations in clinical laboratories. Usually, 0.8–1.0 ml of serum is centrifuged for 15–20 min to prepare the ultrafiltrates. Then, free drug concentrations are measured in the protein-free ultrafiltrates. The time of centrifuging to prepare ultrafiltrates is crucial for measuring free drug concentrations. Liu et al. demonstrated that there is a significant difference between measured free valproic acid concentration in ultrafiltrates prepared by centrifuging specimens for 5 versus 10 or 20 min. The measured free concentrations were low if the specimen was centrifuged for 5 min. Therefore, authors recommended centrifugation of specimens for at least 15 min *(111)*. McMillin et al. recently reported that ultrafiltrate volumes were directly proportional to the centrifugation time (15–30 min) and were inversely proportional to albumin concentrations of serum. Although ultrafiltrate volume was significantly increased with increasing centrifugation time, free phenytoin values did not change significantly indicating that equilibrium was maintained between the ultrafiltrate and serum retained in the ultrafiltration device *(112)*. Another issue is the add-on request for free drug on specimens analyzed previously for total drug concentrations. Usually,

specimens after analysis are stored for 3–7 days at 4˚C. In our experience, if the specimen is removed from a refrigerator and the ultrafiltration is started immediately, the concentrations of free drugs are slightly lower. However, this problem can be easily circumvented if specimens are allowed to equilibrate at the room temperature for at least 15 min before the preparation of ultrafiltrates for free drug measurement *(113)*.

Although immunoassays are commercially available for determination of free phenytoin, valproic acid, and carbamazepine concentrations in protein-free ultrafiltrates, liquid chromatography combined with tandem mass spectrometry can also be applied for determination of free phenytoin concentration in protein-free ultrafiltrate *(114)*.

### *8.1. Pitfalls of Using Equations for Predicting Free Phenytoin Concentrations*

Beck et al. compared free phenytoin concentrations predicted by three different equations with measured values. The authors used the Gugler method, Sheiner–Tozer equation, and the Sheiner–Tozer nomogram for predicting free phenytoin concentrations from measured total phenytoin concentration and albumin concentration. Authors concluded that all three methods for predicting free phenytoin concentrations suffered from bias and should not be used for predicting free phenytoin concentrations. The authors recommended direct measurement of free phenytoin concentration *(115)*. Dager et al. *(116)* concluded that although the Sheiner–Tozer equation underestimates the measured free phenytoin concentration by an average of 12.4%, the equation still has some reliability in normalizing total phenytoin reliably in patients with hypoalbuminemia. Tadon et al. also reported that in patients with serum albumin level in the hyper and hypoalbuminemic range, corrected phenytoin levels for albumin using Sheiner–Tozer equation were better indicator for clinical outcome than simply total phenytoin level. The authors further commented that in developing countries such as India where direct measurement of free drug level is expensive such indirect measurement may have clinical value *(117)*. However, in our experience, direct measure of free level of phenytoin is always clinically more valuable than such indirect measures to correct phenytoin levels.

May et al. reported that although free phenytoin measured by HPLC correlated well with free phenytoin measured by FPIA using the TDx analyzer, the free phenytoin concentrations obtained by using FPIA showed positive bias compared with HPLC values. Moreover, free phenytoin determined by HPLC fits better with calculated phenytoin value in patients also taking valproic acid *(118)*. However, again our recommendation is to measure free phenytoin concentration directly instead of using any equation.

## 9. CONCLUSIONS

Therapeutic drug monitoring of strongly protein-bound antiepileptic drugs such as phenytoin, valproic acid, and carbamazepine is useful for patients with uremia, liver disease, and hypoalbuminemia. Drug–drug interactions may also increase $F_u$ of antiepileptic drugs without significantly altering total drug concentrations. Monitoring

free concentration of immunosuppressant drugs such as cyclosporine, tacrolimus, and mycophenolic acid may have clinical value but are technically more difficult to perform. Monitoring free protease inhibitors may be useful but need further studies for establishing guidelines. Monitoring free lidocaine and free quinidine concentrations may be beneficial for certain patient populations.

## REFERENCES


1. Chan S, Gerson B. Free drug monitoring. *Clin Lab Med* 1987; 7; 279–287.
2. Klotz U, Antonin KH. Pharmacokinetics and bio-availability of sodium valproate. *Clin Pharmacol Ther* 1977; 21: 736–743.
3. Meinardi H, Vander Kleijn E, Meijer JWA. Absorption and distribution of anti-epileptic drugs. *Epilepsia* 1982; 23: 23–26.
4. Booker HE, Darcey B. Serum concentrations of free diphenylhydratoin and their relationship to clinical intoxication. *Epilepsia* 1973; 2: 177–184.
5. Blum MR, Riegelman S, Becker CE. Altered protein binding of diphenylhydamtoin in uremic plasma. *N Engl J Med* 1972; 286: 109.
6. Hinderling PH, Hartmann D. The pH dependency of the binding of drugs to plasma proteins in man. *Ther Drug Monit* 2005; 27: 71–85.
7. Routledge PA, Barchowsky A, Bjornsson TD, Kitchell BB, Shand DG. Lidocaine plasma protein binding. *Clin Pharmacol Ther* 1980; 27: 347–351.
8. Grossman SH, Davis D, Kitchell BB, Shand DG, Routledge PA. Diazepam and lidocaine plasma protein binding in renal disease. *Clin Pharmacol Ther* 1982; 31: 350–357.
9. Shand DG. Alpha 1-acid glycoprotein and plasma lidocaine binding. *Clin Pharmacokinet* 1984; 9 (Suppl. 1): 27–31.
10. Routledge PA, Lazar JD, Barchowsky A, Stargel WW, Wagner GS, Shand DG. A free lignocaine index as a guide to unbound drug concentration. *Br J Clin Pharmacol* 1985; 20: 695–698.
11. Bonde J, Jenen NM, Burgaard P, et al. Displacement of lidocaine from human plasma proteins by disopyramide. *Pharmacol Toxicol* 1987; 60: 151–155.
12. McCollam PL, Crouch MA, Arnaud P. Caucasian versus African American differences in orosomucoid: potential implication for therapy. *Pharmacotherapy* 1998; 18: 620–626.
13. Edwards DJ, Axelson JF, Slaughter RL, Elvin AT, Lalka D. Factors affecting quinidine protein binding in humans. *J Pharm Sci* 1984; 73: 1264–1267.
14. Belpaire FM, Bogaert MG. Binding of alfentanil to human alpha-1 glycoprotein, albumin and serum. *Int J Clin Pharmacol* 1991; 29(3): 96–102.
15. Akhlaghi F, Trull AK. Distribution of cyclosporine in organ transplant recipients [Review]. *Clin Pharmacokinet* 2002; 41: 615–637.
16. Mendonza A, Gohh R, Akhlaghi F. Determination of cyclosporine in saliva using liquid chromatography-tandem mass spectrometry. *Ther Drug Monit* 2004; 26: 569–575.
17. Warty V, Venkataramanan R, Zendehrough P, et al. Distribution of FK 506 in plasma lipoproteins in transplant patients. *Transplant Proc* 1991; 23: 954–955.
18. Piekoszewski W, Jusko WJ. Plasma protein binding of tacrolimus in humans. *J Pharm Sci* 1993; 82: 340–341.
19. Zahir H, McCaughan G, Gleeson M, Nada RA, McLachlan AJ. Changes in tacrolimus distribution in blood and plasma protein binding following liver transplant. *Ther Drug Monit* 2004; 26: 506–515.
20. Zahir H, McCaughan G, Gleeson M, Nada RA, McLachlan AJ. Factors affecting variability in distribution of tacrolimus in liver transplant recipients. *Br J Clin Pharmacol* 2004; 57: 298–309.
21. Pisupati J, Jain A, Burckart G, et al. Intraindividual and interindividual variation in the pharmacokinetics of mycophenolic acid in liver transplant patients. *J Clin Pharmacol* 2005; 45: 34–41.
22. Atcheson BA, Taylor PJ, Mudge DW, et al. Mycophenolic acid pharmacokinetics and related outcomes early after renal transplant. *Br J Clin Pharmacol* 2005; 59: 271–280.

23. Jacobson P, Long J, Rogosheske J, Brunstein C, Eweisdorf D. High unbound mycophenolic acid concentrations in a hematopoietic cell transplantation patient with sepsis and renal and hepatic dysfunction [Letter]. *Biol Blood Marrow Transplant* 2005; 11: 977–978.
24. Patel CG, Mendonza AE, Akhlaghi F, et al. Determination of total mycophenolic acid and its glucuronide metabolite using liquid chromatography with ultraviolet detection and unbound mycophenolic acid using tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; 813(1–2): 287–294.
25. Akhlaghi F, Ashley JJ, Keogh AM, Brown KF. Indirect estimation of the unbound fraction of cyclosporine in plasma. *Ther Drug Monit* 1998; 20: 301–308.
26. Henricsson S. A new method for measuring the free fraction of cyclosporine in plasma by equilibrium dialysis. *J Pharm Pharmacol* 1987; 39: 384–385.
27. Legg B, Gupta SK, Rowaland M. A model to account for the variation in cyclosporine binding to plasma lipids in transplant patients. *Ther Drug Monit* 1988; 10: 20–27.
28. Iwasaki K, Miyazaki Y, Termura Y, Kawamura A, Tozuka Z, Hata T, Undre N. Binding of tacrolimus (FK 506) with human plasma proteins reevaluation and effect of mycophenolic acid. *Res Commun Mol Pathol Pharmacol* 1996; 94: 251–257.
29. Ensom M, Partovi N, Decarie D, Ignaszewski AP, Fradet GJ, Levy RD. Mycophenolate pharmacokinetics in early period following lung or heart transplant. *Ann Pharmacother* 2003; 37: 1761–1767.
30. Akhlaghi F, Patel CG, Zuniga XP, Halilovic J, Preis IS, Gohh RY. Pharmacokinetics of mycophenolic acid and metabolites in diabetic kidney transplant recipients. *Ther Drug Monit* 2006; 28: 95–101.
31. Atcheson B, Taylor PJ, Mudge DW, Johnson DW, Pillans PI, Tett SE. Quantification of free mycophenolic acid and its glucuronide metabolite in human plasma by liquid chromatography using mass spectrometric and ultraviolet absorbance detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; 799 (1): 157–163.
32. Boffito M, Black DJ, Blaschke TF, et al. Protein binding in antiretroviral therapies [Review]. *AIDS Res Hum Retroviruses* 2003; 19(9): 825–835.
33. Anderson PL, Brundage RC, Bushman L, Kakuda TN, Remmel RP, Flecher CV. Indinavir plasma protein binding in HIV-1 infected adults. *AIDS* 2000;14: 2293–2297.
34. Hugen PW, Burger DM, de Graaff M, et al. Saliva as a specimen for monitoring compliance but not for predicting plasma concentrations in patients with HIV treated with indinavir. *Ther Drug Monit* 2000; 22: 437–435.
35. Barrail A, Tiec CL, Paci-Bonaventure S, Furlan V, Vicent I, Taburet AM. Determination of amprenavir total and unbound concentrations in plasma by high performance liquid chromatography and ultrafiltration. *Ther Drug Monit* 2006; 28: 89–94.
36. Boffito M, Hoggard PG, Lindup WE, et al. Lopinavir protein binding in vivo through 12-hour dosing interval. *Ther Drug Monit* 2004; 26: 35–39.
37. Perucca E. Free level monitoring of antiepileptic drugs: Clinical usefulness and case studies. *Clin Pharmacokinet* 1984; 9(Suppl 1) 71–78.
38. Kwong TC. Free drug measurements: methodology and clinical significance. *Clin Chim Acta* 1985; 151: 193–216.
39. Soldin SJ. Free drug measurements; when and why? An overview. *Arch Pathol Lab Med* 1999; 123: 822–823.
40. Urien S, Albengres E, Tillement JP. Serum protein binding of valproic acid in healthy subjects and in patients with liver disease. *Int J Clin Pharmacol* 1981; 19: 319–325.
41. Bowdle TA, Patel IH, Levy RH, Wilensky AJ. Valproic acid dosage and plasma protein binding and clearance. *Clin Pharmacol Ther* 1980; 28: 486–492.
42. Marty JJ, Kilpatrick CJ, Moulds RFW. Intra-dose variation in plasma protein binding of sodium valproate in epileptic patients. *Br J Clin Pharmacol* 1982; 14: 399–404.
43. Gugler R, Von Unruh GE. Clinical pharmacokinetics of valproic acid. *Clin Pharmacokinet* 1980; 5:67–83.
44. Chadwick DW. Concentration-effect relationship of valproic acid. *Clin Pharmacokinet* 1985; 10: 155–163.

45. Gidal BE, Collins DM, Beinlich BR. Apparent valproic acid neurotoxicity in a hypoalbuminemic patient. *Ann Pharmacother* 1993; 27: 32–35.
46. Haroldson JA, Kramer LE, Wolff DL, Lake KD. Elevated free fractions of valproic acid in a heart transplant patient with hypoalbuminemia. *Ann Pharmacother* 2000; 34: 183–187.
47. Lenn NJ, Robertson M. Clinical utility of unbound antiepileptic drug blood levels in the management of epilepsy. *Neurology* 1992; 42: 988–990.
48. Bauer LA, Davis R, Wilensky A, Raisys VA, Levy RH. Diurnal variation in valproic acid clearance. *Clin Pharmacol Ther* 1984; 35: 505–509.
49. Bauer LA, Davis R, Wilensky A, Raisys VA, Levy RH. Valproic acid clearance: unbound fraction and diurnal variation in young and elderly patients. *Clin Pharmacol Ther* 1985; 37: 697–700.
50. Ahmad AM, Douglas Boudinot F, Barr WH, Reed RC, Garnett WR. The use of Monte Carlo stimulation to study the effect of poor compliance on the steady state concentrations of valproic acid following administration of enteric-coated and extended release divalprox sodium formulation. *Biopharm Drug Dispos* 2005; 26: 417–425.
51. Rapeport WG, Mendelow AD, French G, et al. Plasma protein binding and CSF concentration of valproic acid in man following acute oral dosing. *Br J Clin Pharmacol* 1983; 8: 362–371.
52. Al Aly Z, Yalamanchili P, Gonzalez E. Extracorporeal management of valproic acid toxicity: a case report and review of literature. *Semin Dial* 2005; 18: 62–66.
53. Kilpatrick CJ, Wanwimolruk S, Wing LMH. Plasma concentrations of unbound phenytoin in the management of epilepsy. *Br J Clin Pharmacol* 1984; 17: 539–546.
54. Fedler C, Stewart MJ. Plasma total phenytoin: a possible misleading test in developing countries. *Ther Drug Monit* 1999; 21: 155–160.
55. Dutkiewicz G, Wojcicki J, Garwronska-Szklarz B. The influence of hyperlipidemia on pharmacokinetics of free phenytoin. *Neurochir Pol* 1995; 29: 203–211.
56. Dasgupta A, Crossey MJ. Elevated free fatty acid concentrations in lipemic sera reduce protein binding of valproic acid significantly more than phenytoin. *Am J Med Sci* 1997; 313: 75–79.
57. Naidu S, Moodley J, Botha J, et al. The efficacy of phenytoin in relation to serum levels in severe pre-eclampsia and eclampsia. *Br J Obstet Gynaecol* 1992; 99: 881–886.
58. Sjoholm I, Kober A, Odar-Cederlof I, Borga O. Protein binding of drugs in uremia and normal serum: the role of endogenous binding inhibitors. *Biochem Pharmacol* 1976; 25: 1205–1213.
59. McNamara PI, Lalka D, Gibaldi M. Endogenous accumulation products and serum protein binding in uremia. *J Lab Clin Med* 1981; 98: 730–740.
60. Reidenberg MM, Drayer DE. Alteration of drug protein binding in renal disease. *Clin Pharmacokinet* 1984; 9 (Suppl. 1): 18–26.
61. Lindow J, Wijdicks EF. Phenytoin toxicity associated with hypoalbuminemia in critically ill patients. *Chest* 1994; 105: 602–604.
62. Zielmann S, Mielck F, Kahl R, et al. A rational basis for the measurement of free phenytoin concentrations in critically ill trauma patients. *Ther Drug Monit* 1994; 16: 139–144.
63. Thakral A, Shenoy R, Deleu D. Acute visual dysfunction following phenytoin-induced toxicity. *Acta Neurol Belg* 2003; 103: 218–220.
64. Burt M, Anderson D, Kloss J, Apple F. Evidence based implementation of free phenytoin therapeutic drug monitoring. *Clin Chem* 2000; 46: 1132–1135.
65. Iwamoto T, Kagawa Y, Natio Y, Kuzuhara S, Okuda M. Clinical evaluation of plasma free phenytoin measurement and factors influencing its protein binding. *Biopharm Drug Dispos* 2005; 27: 77–84.
66. Deleu D, Aarons L, Ahmed IA. Estimation of population pharmacokinetic parameter of free phenytoin in adult epileptic patients. *Arch Med Res* 2005; 36: 49–53.
67. Bertilsson L, Tomson T. Clinical pharmacokinetics and pharmacological effects of carbamazepine and carbamazepine 10,11-epoxide. *Clin Pharmacokinetic* 1986; 11: 177–198.
68. Froscher W, Burr W, Penin H, et al. Free level monitoring of carbamazepine and valproic acid: clinical significance. *Clin Neuropharmacol* 1985; 8: 362–371.
69. Lesser RP, Pippenger CE, Luders H, Dinners DS. High dose monotherapy in treatment of intractable seizure. *Neurology* 1984; 34: 707–711.

70. Al-Qudah AA, Hwang PA, Giesbrecht E, Soldin SJ. Contribution of 10,11-epoxide to neurotoxicity in epileptic children on polytherapy. *Jordan Med J* 1991; 25: 171–177.
71. Potter JM, Donnelly A. Carbamazepine 10,11-epoxide in therapeutic drug monitoring. *Ther Drug Monit* 1998; 20: 652–657.
72. Yuan R, Venitz J. Effect of chronic renal failure on the disposition of highly hepatically metabolized drugs. *Int J Clin Pharmacol* 2000; 38: 245–253.
73. Monaghan MS, Marx MA, Olsen KM, Turner PD, Bergman KL. Correlation and prediction of phenytoin using standard laboratory parameters in patients after renal transplantation. *Ther Drug Monit* 2001; 23: 263–267.
74. Gulyassy PF, Jarrard E, Stanfel L. Roles of hippurate and indoxyl sulfate in the impaired ligand binding by azotemic plasma. *Adv Exp Med Biol* 1987; 223: 55–58.
75. Takamura N, Maruyama T, Otagiri M. Effects of uremic toxins and fatty acids on serum protein binding of furosemide: possible mechanism of the binding defect in uremia. *Clin Chem* 1997; 43: 2274–2280.
76. Dasgupta A, Malik S. Fast atom bombardment mass spectrometric determination of the molecular weight range of uremic compounds that displace phenytoin from protein binding: absence of midmolecular uremic toxins. *Am J Nephrol* 1994; 14: 162–168.
77. Otagiri M. A molecular functional study on the interactions of drugs with plasma proteins [Review]. *Drug Metab Pharmacokinetic* 2005; 20: 309–323.
78. Reidenberg MM, Affirme M. Influence of disease on binding of drugs to plasma proteins. *Ann NY Acad Sci* 1973; 226: 115–126.
79. Prabhakar S, Bhatia R. Management of agitation and convulsions in hepatic encephalopathy. *Indian J Gastroenterol* 2003; 22 (Suppl. 2): S54–S58.
80. Fischer JH, Patel TV, Fischer PA. Fosphenytoin: clinical pharmacokinetics and comparative advantage in the acute treatment of seizure. *Clin Pharmacokinet* 2003; 42: 33–58.
81. Klotz U, Rapp T, Muller WA. Disposition of VPA in patients with liver disease. *Eur J Clin Pharmacol* 1978; 13: 55–60.
82. Hooper W, Dubetz D, Bochner F, et al. Plasma protein binding of carbamazepine. *Clin Pharmacol Ther* 1975; 17: 433–440.
83. Wong MC, Suite NDA, Labar DR. Seizures in human immunodeficiency virus infection. *Arch Neurol* 1990; 47: 640–642.
84. Burger D, Meenhorst PL, Mulder JW, et al. Therapeutic drug monitoring of phenytoin in patients with the acquired immunodeficiency syndrome. *Ther Drug Monit* 1994; 16: 616–20.
85. Dasgupta A, McLemore J. Elevated free phenytoin and free valproic acid concentrations in sera of patients infected with human immunodeficiency virus. *Ther Drug Monit* 1998; 20: 63–67.
86. Toler SM, Wilkerson MA, Porter WH, Smith AJ, Chandler MH. Severe phenytoin intoxication as a result of altered protein binding in AIDS. *DICP: Ann Pharmacother* 1990; 24: 698–700.
87. Pennell PB. Antiepileptic drug pharmacokinetics during pregnancy and lactation. *Neurology* 2003; 61 (Suppl. 2): S35–S42.
88. Tomson T, Lindbom U, Ekqvist B, et al. Epilepsy and pregnancy: a prospective study on seizure control in relation to free and total concentrations of carbamazepine and phenytoin. *Epilepsia* 1994; 35: 122–130.
89. Yerby MS, Friel PN, McCormick K. Antiepileptic drug disposition during pregnancy. *Neurology* 1992; 42 (Suppl. 5): 12–16.
90. Tomson T. Gender aspect of pharmacokinetics of new and old AEDs; pregnancy and breast feeding. *Ther Drug Monit* 2005; 27: 718–721.
91. Sandyk R. Phenytoin toxicity induced by interaction with ibuprofen. *S Afr Med J* 1982; 62: 592.
92. Tsanaclis LM, Allen J, Perucca E, Routledge PA, Richens A. Effect of valproate on free plasma phenytoin concentrations. *Br J Clin Pharmacol* 1984; 18: 17–20.
93. Pospisil J, Perlik F. Binding parameters of phenytoin during monotherapy and polytherapy. *Int J Clin Pharmacol Ther Toxicol* 1992; 30: 24–28.
94. Arimori K, Nanko M, Otagiri M, Uekama K. Effect of penicillins on binding of phenytoin to plasma proteins in vitro and in vivo. *Biochem Drug Dispos* 1984; 5: 219–227.

95. Dasgupta A, Sperelakis A, Mason A, Dean R. Phenytoin-oxacillin interactions in normal and uremic sera. *Pharmacotherapy* 1997; 17: 375–378.
96. Dasgupta A, Dennen DA, Dean R, McLawhon RW. Displacement of phenytoin from serum protein carriers by antibiotics: studies with ceftriaxone, nafcillin and sulfamethoxazole. *Clin Chem* 1991; 37: 98–100.
97. Dasgupta A, Emerson L. Interaction of valproic acid with nonsteroidal anti-inflammatory drugs mefenamic acid and fenoprofen in normal and uremic sera: lack of interaction in uremic sera due to the presence of endogenous factors. *Ther Drug Monit* 1996; 18: 654–659.
98. Blum RA, Schentag JJ, Gardner MJ, Wilner KD. The effect of tenidap sodium on the disposition and plasma protein binding of phenytoin in healthy male volunteers. *Br J Clin Pharmacol* 1995; 39 (Suppl. I): 35S–38S.
99. Biddle D, Wells A, Dasgupta A. Unexpected suppression of free phenytoin concentration by salicylate in uremic sera due to the presence of inhibitors: MALDI mass spectrometric determination of molecular weight range of inhibitors. *Life Sci* 2000; 66L: 143–151.
100. Dasgupta A, Thompson WC. Carbamazepine-salicylate interaction in normal and uremic sera: reduced interaction in uremic sera. *Ther Drug Monit* 1995; 17: 199–202.
101. Dasgupta A, Paul A, Wells A. Uremic sera contain inhibitors that block digitoxin-valproic acid interaction. *Am J Med Sci* 2001; 322: 204–208.
102. Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva: an update. *Clin Pharmacokinetic* 1992; 23: 365–379.
103. al Za'abi M, Deleu D, Batchelor C. Salivary free concentrations of anti-epileptic drugs: an evaluation in a routine clinical setting. *Acta Neurol Belg* 2003; 103: 19–23.
104. Shiran MR, Hassanzadeh-Khayyat M, Iqbal MZ, et al. Can saliva replace plasma for the monitoring of methadone? *Ther Drug Monit* 2005; 27: 580–586.
105. Liu H, Delgado MR. Therapeutic drug concentration monitoring using saliva samples: focus on anticonvulsants. *Clin Pharmacokinetic* 1999; 36: 453–460.
106. Nakajima M, Yamato S, Shimada K, et al. Assessment of drug concentrations in tears in therapeutic drug monitoring I: determination of valproic acid in tears by gas chromatography/mass spectrometry with EC/NCI mode. *Ther Drug Monit* 2000; 22: 716–722.
107. Berkovitch M, Bistritzer T, Aladjem M, Burtin P, Dagan T, Chen-Levi Z, Freedom R, Koren G. Clinical relevance of the therapeutic drug monitoring of digoxin and gentamicin in saliva of children. *Ther Drug Monit* 1998; 20: 253–256.
108. Madsen V, Lind A, Rasmussen M, Coulthard K. Determination of tobramycin in saliva is not suitable for therapeutic drug monitoring of patients with cystic fibrosis. *J Cyst Fibros* 2004; 3: 249–252.
109. Wintergerst U, Kurowski M, Rolinski B, Muller M, Wolf E, Jaeger H. Use of saliva specimens for monitoring indinavir therapy in human immunodeficiency virus infected patients. *Antimicrob Agents Chemother* 2000; 44: 2572–2574.
110. Ryan M, Grim SA, Miles MV, et al. Correlation of lamotrigine concentrations between serum and saliva. *Pharmacotherapy* 2003; 23: 1550–1557.
111. Liu H, Montoya JL, Forman LJ, et al. Determination of free valproic acid: evaluation of centrifree system and comparison between high performance liquid chromatography and enzyme immunoassay. *Ther Drug Monit* 1992; 14: 513–521.
112. McMillan GA, Juenke J, Dasgupta A. Effect of ultrafiltrate volume on the determination of free phenytoin concentration. *Ther Drug Monit* 2005; 27: 630–633.
113. Dasgupta A, Bard D, Pro S, Blackwell W. Effect of storing serum specimens at 4°C on add-on request for free drug concentration [Abstract]. *Clin Chem* 1995; 41 (Suppl.): S117.
114. Bardin S, Ottinger JC, Breau AP, O'Shea TJ. Determination of free levels of phenytoin in human plasma by liquid chromatography/tandem mass spectrometry. *J Pharm Biomed Anal* 2000; 23: 573–579.
115. Beck DE, Farringer JA, Ravis WR, Robinson CA. Accuracy of three methods for predicting concentrations of free phenytoin. *Clin Pharm* 1987; 6: 888–894.
116. Dager WE, Inciardi JF, Howe TL. Estimating phenytoin concentrations by the Sheiner-Tozer method in adults with pronounced hypoalbuminemia. *Ann Pharmacother* 1995; 29: 667–670.

117. Tandon M, Pandhi P, Garg SK, Prabhakar SK. Serum albumin-adjusted phenytoin levels: an approach for predicting drug efficacy in patients with epilepsy, suitable for developing countries. *Int J Clin Pharmacol Ther* 2004; 42: 5450–555.
118. May TW, Rambeck B, Jurges U, Blankenhorn V, Jurgens U. Comparison of total and free phenytoin serum concentrations measured by high performance liquid chromatography and standard TDx: implications for the prediction of free phenytoin serum concentrations. *Ther Drug Monit* 1998; 20: 619–623.

# 3 Analytical Techniques for Measuring Concentrations of Therapeutic Drugs in Biological Fluids

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***Contents***



**Summary**

Different types of assays are used in clinical laboratories for determination of concentrations of various drugs in biological fluids for therapeutic drug monitoring. Historically, concentrations of various anticonvulsants such as phenytoin, carbamazepine, phenobarbital, and primidone in serum or plasma were measured using gas chromatography (GC) or high-performance liquid chromatography. Later, these assays were replaced by immunoassays because of automation as well as need for faster turnaround time. Minimal or no specimen pretreatment is needed for analysis of various drugs in sera using immunoassays. However, immunoassays are not available for all drugs monitored in clinical laboratories, for example lamotrigine, protease inhibitors, and new generation of anticonvulsants. For analysis of these drugs, GC, HPLC, or HPLC combined with tandem mass spectrometric techniques are used.

**Key Words:** Therapeutic drug monitoring; immunoassay; gas chromatography; high-performance liquid chromatography; mass spectrometry.

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## 1. INTRODUCTION

Usually, serum or plasma is used for the measurement of concentration of a drug of interest for the purpose of therapeutic drug monitoring (TDM). However, monitoring whole blood concentrations of certain drugs such as various immunosuppressants is clinically more relevant. During the 1950s, TDM of antiepileptic drugs was performed using gas chromatography (GC). Several bioassays were available for monitoring certain antibiotics. In the 1960s, extensive research took place to develop various assays for therapeutic drugs using high-performance liquid chromatography (HPLC). In the 1970s, immunoassays were available for accurate determination of concentrations of various drugs in serum and plasma, thus revolutionizing the field of TDM. Immunoassays are widely used in clinical laboratories for routine drug monitoring because of ease of operation, simplicity, and speed. However, immunoassays are not commercially available for all drugs currently monitored in clinical practice, and various sophisticated techniques such as HPLC combined with tandem mass spectrometry or GC combined with mass spectrometry are used for determination of concentrations of these drugs in biological matrix. Usually, such specialized tests are offered in reference laboratories, academic based hospital laboratories, and clinical laboratories of larger hospitals.

## 2. IMMUNOASSAYS IN TDM

Most TDM and drugs of abuse (DAU) testing are now performed by immunoassay methods on automated systems. Currently, there are over 25 immunoassays commercially available and are routinely used in TDM/DAU laboratory analysis. Most immunoassay methods use specimens without any pretreatment and are run on fully automated, continuous, random access systems. The assays require very small amounts of sample (mostly $<100\,\mu L$), reagents are stored in the analyzer, and most analyzers have stored calibration curves on the system. In immunoassays, the analyte is detected by its complexation with a specific binding molecule, which in most cases is an analyte-specific antibody (or a pair of specific antibodies). This reaction is further utilized in various formats and labels, giving a whole series of immunoassay technologies, systems, and options (Table 1).

With respect to assay design, there are two formats of immunoassays: competition and immunometric (commonly referred as "sandwich"). Competition immunoassays work best for analytes with small molecular weight, requiring a single analyte-specific antibody. In contrast, sandwich immunoassays are mostly used for analytes with larger molecular weight, such as proteins or peptides, and use two different specific antibodies. Since most TDM immunoassays involve analytes of small molecular size, these assays employ the competition format. In this format, the analyte molecules in the specimen compete with analyte (or its analogues), labeled with a suitable tag provided in the reagent, for a limited number of binding sites provided by, for example, an analyte-specific antibody (also provided in the reagent). Thus, in these types of assays, the higher the analyte concentration in the sample, the less of label can bind to the antibody to form the conjugate. If the bound label provides the signal, which in turn is used to calculate the analyte concentration in the sample, the analyte concentration in the specimen is inversely proportional to the signal produced. If the free label provides the

**Table 1**
**Various Types of Commercial Immunoassay Kits**

| *Immunoassay types* | *Analyte* | *Assay format* | *Example* | *Assay Signal* |
|---|---|---|---|---|
| Competition | Small molecular weight (≤1000 D) | Homogeneous | FPIA (TDx® from Abbott, Abbott Park, IL, USA): TDM, DAU, other (e.g., T4, T3) | Fluorescence polarization |
| | | | EMIT® (Dade-Behring, Newark, DE, USA): TDM, DAU, other (e.g., T4, T3) | Colorimetry (enzyme modulation) |
| | | | CEDIA® (Microgenics, Fremont, CA, USA): TDM, DAU, other (e.g., T4, T3) | Colorimetry (enzyme modulation) |
| Competition and sandwich | Both small and large | Heterogeneous | ADVIA Chemistry: Siemens, Tarrytown, NY, USA TDM, DAU, other (HbA1c, plasma proteins, etc.) | Turbidimetry, latex micro-particle assisted |
| | | | Centaur® (Siemens): TDM, others (thyroid, steroid, etc.) | Chemiluminescence (acridinium ester label) |
| | | | ACCESS® (Beckman, Fullerton, CA, USA): TDM, others (thyroid, steroid, etc.) | Enzyme immunoassay (using chemiluminescent substrate) |
| | | | Elecsys® (Roche, Indianapolis, IN, USA): TDM, others (thyroid, steroid, etc.) | Electro-chemiluminescence |
| | | | AxSym® (Abbott): TDM, others (thyroid, steroid, etc.) | Enzyme immunoassay (using fluorescence substrate) |

signal, then signal produced is proportional to the analyte concentration. The signal is mostly optical—absorbance, fluorescence, or chemiluminescence.

There are several variations in this basic format. The assays can be homogeneous or heterogeneous. In the former, the bound label has different properties than the free label. For example, in fluorescent polarization immunoassay (FPIA), the free label has different Brownian motion than when the relatively small molecular weight (a few

hundreds to thousand Daltons) label is complexed with a large antibody (140,000 D). This results in difference in the fluorescence polarization properties of the label, which is utilized to quantify the bound label *(1)*. In another type of homogeneous immunoassay, an enzyme is used as the label, the activity of which is modulated differently in the free versus the complexed conditions with the antibody. This forms the basis of the enzyme-multiplied immunoassay technique (EMIT®) or cloned enzyme donor immunoassay (CEDIA®) technologies *(2,3)*. In the EMIT method, the label enzyme glucose 6-phosphate-dehydrogenase is active in the free form but inactive in the antigen–antibody complex. The active enzyme during reaction with the substrate also reduces the cofactor NAD to NADH, and the absorbance is monitored at 340 nm. Because antibody-bound enzyme is inactivated or reacts slowly with the substrate, the unbound or free portion is responsible for the signal. Therefore, at equilibrium, the amount of unbound enzyme-labeled analyte will be directly proportional to the drug concentration.

In the CEDIA method, two genetically engineered inactive fragments of the enzyme beta-galactosidase are coupled to the antigen and the antibody reagents. When they combine, the active enzyme is produced and the substrate, a chromogenic galactoside derivative, produces the assay signal. In a third commonly used format of homogeneous immunoassay (turbidimetric immunoassay or TIA), analytes (antigen) or its analogs are coupled to colloidal particles, for example, of latex *(4)*. As antibodies are bivalent, the latex particles agglutinate in presence of the antibody. However, in presence of free analytes in the specimen, there is less agglutination. In a spectrophotometer, the resulting turbidity can be monitored as end-point or as rate.

In heterogeneous immunoassays, on the contrary, the bound label is physically separated from the unbound labels, and its signal is measured. The separation is often done magnetically, where the reagent analyte (or its analog) is provided as coupled to paramagnetic particles (PMP), and the antibody is labeled. Conversely, the antibody may be also provided as conjugated to the PMP, and the reagent analyte may carry the label. After separation and wash, the bound label is reacted with other reagents to generate the signal. This is the mechanism in many chemiluminescent immunoassays (CLIA), where the label may be a small molecule which generates chemiluminescent signal *(5)*. The label also may be an enzyme [enzyme-linked immunosorbent assay (ELISA)], which generates chemiluminescent, fluorometric, or colorimetric signal. In older immunoassay formats, the labels used to be radioactive [radio-immunoassay (RIA)]. But because of safety and waste disposal issues, RIA is rarely used today. Another type of heterogeneous immunoassay uses polystyrene particles. If these particles are micro-sizes, that type of assay is called microparticle-enhanced immunoassay *(6)*. Microparticle enzyme immunoassay (MEIA) is also used for analysis of drugs in biological matrix.

The main reagent in the immunoassay is the binding molecule which is most commonly an analyte-specific antibody or its fragment. Several types of antibodies or their fragments are now used in immunoassays. There are polyclonal antibodies, which are raised in an animal when the analyte (as antigen) along with an adjuvant is injected into the animal. For small molecular weight analyte, it is most commonly injected as a conjugate of a large protein. Appearance of analyte-specific antibodies in the sera of animal is monitored, and when sufficient concentration of the antibody is reached, blood is collected from the animal. The serum can be used directly as the analyte-specific binder in an immunoassay

but common practice is to purify the antibody before use. As there are many clones of the antibodies specific for the analyte, these antibodies are called polyclonal. In newer technologies, however, a mast cell of the animal can be selected as producing the optimum antibody and then can be fused to an immortal cell. The resulting tumor cell grows uncontrollably producing only the single clone of the desired antibody. Such antibodies, termed as monoclonal antibody, may be grown in live animals or cell-culture. There are several benefits of the monoclonal antibodies over polyclonal ones: (a) the characteristics of polyclonal antibodies are dependent on the animal producing the antibodies, and when the source individual animal is changed, the resultant antibody may be quite different; (b) as polyclonal antibodies constitute many antibody clones, these antibodies are less specific compared with monoclonal antibodies for the analyte. Sometimes, instead of using the whole antibody, fragments of the antibody, generated by digestion of the antibody with peptidases, for example, Fab, Fab′ (or their dimeric complexes) are used as a reagent.

The other major component of the immunoassay reagents is the labeled antigen (or its analog). There are many different kinds of labels used in commercially available immunoassays, generating different kinds of signals. As described earlier, an enzyme may be also used as the label.

Even though the immunoassay methods are now widely used, there are few limitations of this technique. Antibody specificity is the major concern of an immunoassay. Many endogenous metabolites of the analyte (drug) may have very similar structural recognition motif as the analyte itself. There maybe other molecules unrelated to the analyte but producing comparable recognition motif as the analyte. These molecules are generally called cross-reactants. When present in the sample, these molecules may produce false results (both positive and negative interference) in the relevant immunoassay *(7–9)*. Other components in a specimen, such as bilirubin, hemoglobin, or lipid, may interfere in the immunoassay by interfering with the assay signal, thus producing incorrect results. These effects have been described in Chapter 5. A third type of immunoassay interference involves endogenous human antibodies in the specimen, which may interfere with components of the assay reagent such as the assay antibodies, or the antigen-labels. Such interference includes the interference from heterophilic antibodies or various human antianimal antibodies and is described in Chapter 12.

## 3. GAS CHROMATOGRAPHY

Gas–liquid chromatography also commonly referred to as GC is a separation technique first described in 1952 by James and Martin. In most GC column, the stationary phase is a liquid and the mobile phase is an inert gas, thus the name gas–liquid chromatography. Typically, the stationary phase has a low vapor pressure so that at column temperature it can be considered nonvolatile. Introduction of the capillary column dramatically improved resolution of peaks in GC analysis. Resolution equivalent to several hundred thousand theoretical plates can be achieved using a fused silica capillary column, which was originally derived from the fiber-optic technology. Depending on the stationary phase composition, a GC column may have low polarity, intermediate polarity, or high polarity. Research is ongoing to develop new polymers for use as a stationary phase in GC column. Mayer-Helm reported development of a novel dimethylsiloxane-based copolymer for use as stationary phase in GC column *(10)*. Microprocessor control of oven temperature and automatic sample injection techniques

also enhanced both performance and ease of automation of GC technique in clinical laboratories. The sensitivity and specificity of GC analysis depends on the choice of detector. Mass spectrometry can be used in combination with a gas chromatograph, and mass spectrometry is capable of producing a mass spectrum of any compound coming out of the column of gas chromatograph. Nitrogen phosphorus detector is specific for nitrogen- and phosphorus-containing compounds and is very sensitive. Electron capture detector can detect any halogen-containing compounds. Flame ionization and thermal conductivity detectors are also used in GC. The major limitation of GC is that this technique can only be applied to volatile substances with relatively low molecular weights. Polar compounds cannot be analyzed by this technique. However, a relatively polar compound can be chemically converted to a nonpolar compound (derivatization) for analysis by GC.

## 4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Application of GC as a separation technique is limited to volatile molecules. However, HPLC can be used for separation of both polar and nonpolar molecules. Usually, derivatization is not necessary for HPLC analysis. HPLC is based on the principle of liquid–liquid chromatography where both mobile phase and stationary phase are liquid. Liquid chromatography using column was first described in 1941. In normal liquid chromatography, the stationary phase is polar and the mobile phase (eluting solvent) is nonpolar. In reverse phase chromatography, the stationary phase is nonpolar and the mobile phase is polar. Several detectors can be used for monitoring elution of peaks from HPLC column including ultraviolet detector, fluorescence detector, conductivity detector, and refractive index detector. Ultraviolet detection is commonly used in clinical laboratories although other detection techniques such as fluorescence technique and electrochemical detection technique are also used.

## 5. MASS SPECTROMETRY

Mass spectrometer is capable of analyzing charged particle based on their mass. A typical mass spectrometer consists of an inlet system, which supplies the pure compound (separated from complex biological matrix by GC or HPLC) to the mass spectrometer, an ion source, a mass analyzer, and a detector. The ion source is responsible for fragmentation pattern of the compound of interest in characteristic pattern depending on the functional groups and other structural features of the molecule. The detector plots a chromatogram listing all ions generated and separated by their mass to charge ratios as well as abundance. Mass spectrum is usually considered as a molecular fingerprint of a compound, not only for relatively small molecules but also for macromolecules such as proteins and DNA *(11,12)*. Mass spectrometer is often used as a detector for compounds eluting from a gas chromatograph or column of an HPLC. Gas-chromatography–mass spectrometry (GC/MS) is widely used in clinical toxicology laboratories for detection and quantification of DAU in biological matrix such as urine because of its specificity, sensitivity, and the availability of larger number of mass spectra in standard drug libraries. Coupling of HPLC with mass spectrometry enables the analysis of thermally labile compounds, polar compounds, or compounds with high molecular weights that cannot be analyzed by GC or a combined GC/MS.

Electron ionization (EI) at 70 eV produces reproducible mass spectrum, which is a common ion source used in GC/MS analysis of therapeutic drugs especially DAU. As far as combining HPLC with a mass spectrometer, only moving belt and particle beam interfaces are compatible with EI *(13)*. The electrospray interface is very common in HPLC/MS analyzer used in clinical laboratories. The electrospray interface produces singly or multiple charged ions directly from a solvent system by creating a fine spray of highly charged droplets in the presence of a strong electric field with assistance from heat or from pneumatics. In this process, nonvolatile and polar compound can be ionized. The atmospheric pressure chemical ionization interface produces sample ions by charge transfer from reagent ions. The reagent ions are produced from solvent vapor of the mobile phase.

After producing charged particles from the analyte eluting from the column, a mass spectrum is produced by detecting these charged particles (positive or negative ion) in the detector of the mass spectrum. The major types of mass spectrometric analyzers are quadrupole analyzer, ion trap analyzers, and time-of-flight analyzer. Quadrupole detector can be used for quantification of drugs *(14)*. Mass spectrometer is usually operated in a selected ion-monitoring mode or single ion-monitoring mode.

## 6. APPLICATION OF GC, GC/MS, HPLC, AND HPLC/MS FOR DRUG ANALYSIS

Although immunoassays are widely used for routine TDM in clinical laboratories, other analytical techniques such as GC, HPLC, GC/MS, and HPLC/MS are also used for determination of concentrations of various drugs in clinical laboratories (Table 2). These alternative techniques, especially GC/MS and HPLC/MS, are very sensitive and specific for a chosen analyte whereas immunoassays suffer from multiple problems including interferences from compounds with similar structures, hook effect, and sensitivity. There is no commercially available immunoassay for analysis of antiretrovirals used in the treatment of patients with HIV infection. HPLC methods or HPLC combined with tandem mass spectrometry are the only available techniques for therapeutic monitoring of these drugs. GC, GC/MS, and HPLC are also preferred methods for TDM of tricyclic antidepressants because commercially available fluorescence polarization immunoassay (FPIA) cross-reacts with all tricyclic antidepressants and their metabolites. Although immunoassays are available for routine monitoring of certain immunosuppressant drugs, these assays suffer from many limitations and HPLC combined with tandem mass spectrometry can be used for therapeutic monitoring of these drugs *(15)*.

### *6.1. Analysis of Anticonvulsants*

Immunoassays are commercially available for TDM of antiepileptic drugs including phenytoin, carbamazepine, phenobarbital, ethosuximide, primidone, and valproic acid. Before development of immunoassay techniques, conventional antiepileptic drugs were analyzed by GC and HPLC in clinical laboratories. Kuperberg *(16)* described a GC method for quantitative analysis of phenytoin, phenobarbital, and primidone in plasma. Another GC method for simultaneous analysis of phenobarbital, primidone, and

**Table 2**
**Application of Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC) for Analysis of Drugs in Serum/Plasma Where There is No Commercially Available Immunoassay**

| *Drug* | *Class* | *Analysis* | *Reference* |
|---|---|---|---|
| Carbamazepine | Anticonvulsant | HPLC | *(20)* |
| and 10,11-epoxide | | GC/MS | *(21)* |
| Gabapentene | Anticonvulsant | HPLC | *(23)* |
| Lamotrigine, | Anticonvulsant | HPLC | *(24)* |
| Oxcarbazepine, Felbamate | | GC/MS | *(25)* |
| Lamotrigine | | | |
| Pregabalin | Anticonvulsant | HPLC | *(26)* |
| Mexiletine | Cardioactive | HPLC | *(33)* |
| | | GC/MS | *(34,35)* |
| Flecainide | Cardioactive | HPLC | *(37)* |
| | | GC/MS | *(38)* |
| Tocainide, Verapamil | Cardioactive | HPLC | *(30)* |
| Amiodarone and other drugs | Cardioactive | | |
| Encainide | | HPLC | *(31)* |
| Fluoxetine, Citalopram | Antidepressant | HPLC/MS | *(42)* |
| Paroxetine, Venlafaxine Paroxetine and 12 other | Antidepressant | HPLC and GC/MS | *(41)* |
| Sisomicin, Astromicin | Antibiotic | HPLC | *(47)* |
| Netilmicin, Micronomicin | Antibiotic | HPLC/MS | *(51)* |
| Metronidazole, Spiramycin | | | |
| Amphotericin B | Antibiotic | HPLC | *(52)* |
| 5-Fluorouracil | Antineoplastic | HPLC | *(56)* |
| Irinotecan | Antineoplastic | HPLC | *(57)* |
| Docetaxel, Paclitaxel | Antineoplastic | HPLC | *(58)* |
| Imatinib | Antineoplastic | HPLC | *(59)* |
| Doxorubicin and other | Antineoplastic | HPLC | *(60)* |
| Ifosfamide | Antineoplastic | GC/MS | *(62)* |

MS, mass spectrometry.

phenytoin in patient's sera utilized *N*, *N*-dimethyl derivatives of these drugs and "on-column" derivatization technique *(17)*. Atwell et al. developed an HPLC assay for determination of phenobarbital and phenytoin in plasma using a porous particle silicic acid column. The mobile phase was composed of chloroform dioxane–isopropanol–acetic acid (310:9:7:1.0:0.1 by volume). The elution of drugs was monitored at 254 nm *(18)*. Later, commercially available immunoassays took the place of these techniques for routine monitoring of anticonvulsants in clinical laboratories. Good correlations were observed between results obtained by immunoassays and GC analysis of phenytoin, phenobarbital, primidone, carbamazepine, and ethosuximide. Moreover, precision observed in these immunoassays for anticonvulsants was superior to GC analysis, and immunoassays were also relatively free from interfering substances *(19)*. However, immunoassays also have certain limitations. Carbamazepine is metabolized to an active metabolite carbamazepine 10, 11-epoxide. This active metabolite may accumulate in

patients with renal impairment and therapeutic monitoring of carbamazepine 10, 11-epoxide along with carbamazepine is recommended in certain patient populations. Currently, there is no commercially available immunoassay in the market to measure concentration of this metabolite. Moreover, cross-reactivity of this metabolite with carbamazepine immunoassays varied from very low (0–4%) to very high (94%) as discussed in Chapter 7. Berg and Buckley described an HPLC protocol for simultaneous determination of carbamazepine, carbamazepine 10, 11-epoxide, phenytoin, and phenobarbital in serum or plasma using a manual column-switching technique, an isocratic mobile phase, and UV detection. Diluted plasma or serum was injected directly to the system and reporting of results was achieved within 5 min *(20)*. A GC/MS method for simultaneous detection of carbamazepine and carbamazepine 10, 11-epoxide has also been reported. After microcolumn extraction of carbamazepine and its metabolite, the compounds were analyzed using a GC/MS. The capillary GC column used for the analysis was 25 m long with a 0.2-mm internal diameter and a film thickness of 0.33 μm (cross-linked with 5% phenyl-methylsilicone) *(21)*. A sensitive method for simultaneous determination of carbamazepine and its active metabolite carbamazepine 10, 11-epoxide has also been described using HPLC combined with tandem mass spectrometry. After liquid–liquid extraction, the specimen was analyzed using a Phenomenex Luna C18 column (150 × 2 mm, particle size 5 μm) using a mobile phase composition of acetonitrile, methanol, and 0.1 % formic acid (10:70:20 by volume). Detection was achieved by a Micromass Quattro Ultima mass spectrometer (LC-MS-MS) using electrospray ionization, monitoring the transition of protonated molecular ion for carbamazepine at m/z 237.05, and carbamazepine 10, 11-epoxide at m/z 253.09 to the predominant ions of m/z 194.09 and 180.04, respectively. Using only 0.5 mL plasma, authors achieved a detection limit of 0.722 ng/mL for carbamazepine and 5.15 ng/mL for carbamazepine 10, 11-epoxide *(22)*.

For newer antiepileptic drugs, HPLC or HPLC combined with mass spectrometry is used because of lack of commercially available immunoassays. Bahrami and Mohammadi *(23)* described an HPLC protocol for analysis of gabapentin in human serum after derivatization with 4-chloro-7-nitrobenzofuran, fluorescent-labeling agent. In this process, sensitivity was improved compared with *o*-phthalaldehyde derivatization. Contin et al. *(24)* described an HPLC method for simultaneous determination of lamotrigine, oxcarbazepine monohydroxy derivative, and felbamate in human plasma using only 0.5 mL specimen and a reverse phase HPLC column with UV detection. Although most reports for determination of lamotrigine concentration use HPLC, lamotrigine in serum can also be analyzed using GC/MS after extraction and conversion into *tert*-butyldimethylsilyl derivative *(25)*. Berry and Millington *(26)* reported an HPLC method for determination of a new antiepileptic drug pregabalin using a C8 column and derivatization of pregabalin with picryl sulfonic acid.

### *6.2. Analysis of Cardioactive Drugs*

Immunoassays are commercially available for many drugs including digoxin, procainamide, lidocaine, quinidine, and disopyramide. The FPIA for disopyramide was used for determination of both total and free disopyramide concentration in serum. Chen et al. *(27)* observed good correlation between total and free disopyramide concentrations determined by FPIA assay and HPLC protocol. The EMIT assay of disopyramide

can also be used for measuring both total and free concentrations of disopyramide *(28)*. Several immunoassays are commercially available from different diagnostic companies for determination of serum digoxin concentrations. However, these immunoassays are subjected interferences. This aspect is discussed in detail in Chapter 6. HPLC/MS method, which is very specific for digoxin measurement in biological matrix, is useful to investigate fatal poisoning of digoxin in medical legal situation *(29)*.

Verbesselt et al. described a rapid HPLC assay with solid phase extraction for analysis of 12 antiarrhythmic drugs in plasma: amiodarone, aprindine, disopyramide, flecainide, lidocaine, lorcainide, mexiletine, procainamide, propafenone, sotalol, tocainide, and verapamil. Because most of these drugs are basic compounds, alkalinization of column produced good absorption of these drugs in the extraction column. However, for amiodarone, an acidic pH (3.5) was maintained, and aprindine was eluted at neutral pH. After washing with water, the compounds were eluted with methanol except for amiodarone, which was eluted with acetonitrile and acetate buffer (8:5 by volume) at pH 5. Chromatographic separation was achieved by using a Spherisorb hexyl column ($150 \times 4.6$ mm ID with particle size of 5 μm) and mobile phase was composed of a mixture of acetonitrile or methanol with phosphate or acetate buffer at a different pH. Detection of peaks was achieved by either a UV detector or a fluorescence detector *(30)*.

Concentrations of encainide and its metabolites can be determined in human plasma by using HPLC *(31)*. A liquid chromatographic analysis of mexiletine in human serum with alternate application to determine concentrations of procainamide and its active metabolite *N*-acetyl procainamide has also been reported. The authors used *N*-propionyl procainamide as the internal standard *(32)*. McErlance described stereoselective analysis of mexiletine enantiomers using HPLC. Resolution of mexiletine enantiomers as their 2-napthol derivatives was achieved by using a Prikle type 1A chiral phase column and fluorescence detection *(33)*. However, concentration of mexiletine in serum can also be measured by GC couple with mass spectrometry with selected ion monitoring after derivatization of mexiletine and the internal standard. Minnigh et al. used *p*-chlorophenylalanine as the internal standard. The drug and the internal standard were extracted from plasma by a combination of ethyl acetate, hexane, and methanol (60:40:1 by volume) followed by evaporation of organic phase and derivatization to pentafluoropropyl derivatives. The mass spectrometer was operated in a selected ion-monitoring mode *(34)*. Other derivatization techniques for determination of mexiletine concentration in serum have been described. Mexiletine can be extracted from alkaline serum with dichloromethane followed by derivatization with 2,2,2-trichloroethyl chloroformate. The reaction was completed in 30 min at 70°C. *N*-propylamphetamine was used as the internal standard. The derivatized internal standard separated well from derivatized mexiletine. A representative total ion chromatogram showing analysis of mexiletine in serum is given in Fig. 1. The mass spectrometer was operated in selected ion-monitoring mode (m/z at 58, 102, 122, 232, 234 monitored for derivatized mexiletine and mz/z at 56, 91, 131, 260, and 262 for derivatized internal standard). The total ion chromatogram (EI) of mexiletine is given in Fig. 2. The assay was linear for serum mexiletine concentrations between 0.2 to 2.5 mg/L *(35)*. GC/MS analysis of mexiletine in human serum after extraction and derivatization with perfluorooctanoyl chloride has also been reported *(36)*. Although an FPIA assay for

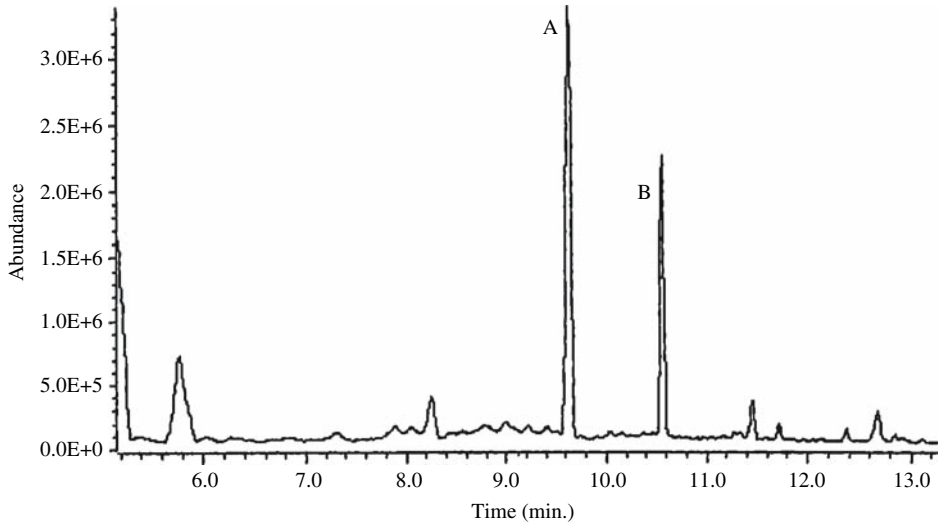


**Fig. 1.** Total ion chromatogram analysis of a patient's sample containing 0.6 mg/L of mexiletine. Peak A was derivatized internal standard (*N*-propylamphetamine) whereas Peak B was derivatized mexiletine (2,2,2,-trichloroethyl carbamate).

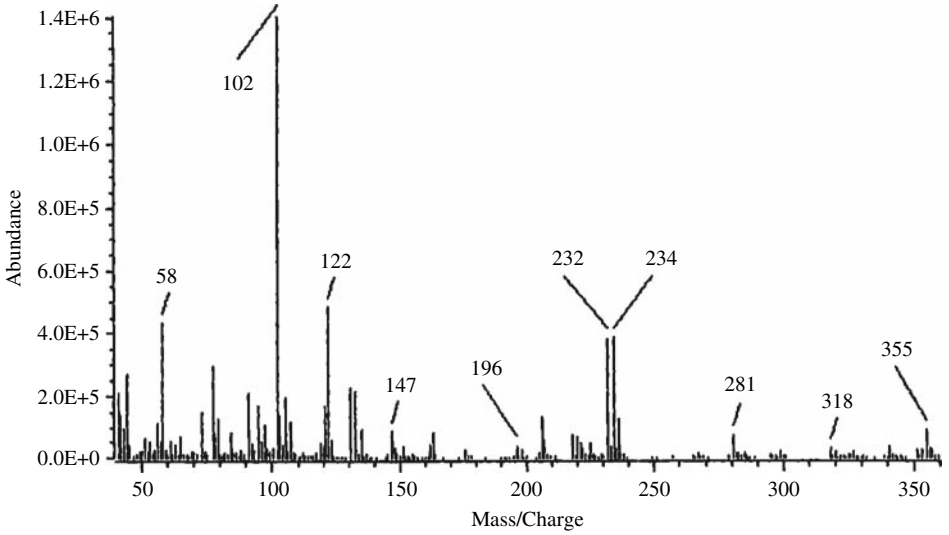


**Fig. 2.** Electron ionization full scan mass spectrum of 2,2,2-trichloroethyl carbamate derivative of mexiletine.

flecainide was commercially available from the Abbott Laboratories, Abbott Park, IL in the past, this assay is no longer available and HPLC is the preferred method of determination of flecainide concentration in serum. Determination of serum flecainide concentration along with its metabolites using HPLC can be achieved by using octadecylsilyl silica (ODS) column and fluorescence detection. Flecainide and its metabolites

were extracted from serum with ethyl acetate *(37)*. Another report described GC/MS validation of HPLC analysis of flecainide enantiomers in serum *(38)*.

### *6.3. Analysis of Antiasthmatic, Antidepressants, Immunosuppressants, and Antiretroviral Drugs*

Immunoassays are commercially available for TDM of antiasthmatic drugs theophylline and caffeine. The manufacturer of the FPIA assay for determination of total tricyclic antidepressants (TCA) in serum only recommends this assay for screening of TCA in serum in a patient with suspected overdose and cautions against using this assay for routine TDM of TCA. A patient overdosed with carbamazepine may show a falsely elevated TCA level if the FPIA assay is used because of cross-reactivity of carbamazepine with the antibody used in this immunoassay *(39,40)*. Both HPLC and GC or GC combined with mass spectrometry can be used for routine TDM of various TCA. Methods for TDM of TCA are discussed in detail in Chapter 8. Recently, Wille et al. *(41)* reported HPLC and GC/MS analysis of 13 new-generation antidepressants (venlafaxine, fluoxetine, viloxazine, fluvoxamine, mianserin, mirtazapine, melitracen, reboxetine, citalopram, maprotiline, sertraline, paroxetine, and trazodone) together with eight of their metabolites. Juan et al. reported HPLC combined with electrospray ionization mass spectrometric determination of fluoxetine, citalopram, paroxetine, and venlafaxine in plasma. These drugs were extracted by solid phase extraction from alkalinized plasma, and HPLC separation was achieved by a reverse phase C18 column *(42)*.

Although immunoassays are available for certain immunosuppressant drugs, HPLC and HPLC/MS are widely used in clinical laboratories for routine TDM of various immunosuppressants because of the superior sensitivity and specificity of these technique compared with various commercially avail immunoassays. This is discussed in depth in Chapter 9. Currently, there is no commercially available immunoassay for TDM of antiretroviral drugs, and HPLC or HPLC/MS are the only available techniques. This topic is discussed in Chapter 10.

### *6.4. Analysis of Antibiotics*

Immunoassays are commercially available for frequently monitored antibiotics including amikacin, gentamicin, tobramycin, and vancomycin. However, GC and in most cases HPLC assays are also available for determination of concentrations of antibiotics in biological matrix. Owing to lack of volatility, chromophore, and hydrophilicity of aminoglycosides, most methods require derivatization of these drugs before analysis. Packed column separation was generally used for GC methods. However, for HPLC methods, reverse phase, ion pair, ion exchange, and normal phase separation protocols have been reported *(43)*. Lai and Sheehan *(44)* described a reversed-phase HPLC method for determination of tobramycin using pre-column derivatization with *o*-phthalaldehyde followed by fluorescence detection. Another HPLC method for determination of amikacin used pre-column derivatization of amikacin with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. The derivatization reaction can be conducted in aqueous medium at room temperature with borate buffer at pH 8.0. The formation of the derivative is instantaneous, and the derivative is

stable for more than 36 h. Detection was performed by using UV absorption instead of fluorescence *(45)*. Nicoli and Santi used 1-fluoro-2,4-dinitrobenzene for derivatizing amikacin for analysis by HPLC using a C18 column and mobile phase composition of acetonitrile–water–acetic acid (47:53:0.1 by volume). The column temperature was maintained at 45°C *(46)*. Kawamoto et al. described an HPLC protocol for determination of serum concentrations of sisomicin, netilmicin, astromicin, and micronomicin using an amino acid analyzer system. The aminoglycosides were separated by reverse phase ion-pair chromatography on Zorbax C8 and ODS columns and detected by post column derivatization technique *(47)*. Soltes *(48)* reviewed HPLC techniques for determination of aminoglycoside concentrations in biological specimens developed in last two decades.

Aminoglycosides can also be determined by HPLC technique without derivatization using evaporative light scattering detection technique *(49)*. Some investigations also used liquid chromatography combined with mass spectrometry for analysis of aminoglycosides. Kim et al. analyzed kanamycin, neomycin, and gentamicin after derivatization with phenylisocyanate. The authors used a liquid chromatography combined with electrospray ionization mass spectroscopy for separation and final analysis of these aminoglycosides *(50)*. An analytical method using liquid chromatography combined with mass spectrometry has also been reported for simultaneous determination of metronidazole and spiramycin I in human plasma, saliva, and gingival crevicular fluid. Ornidazole was used as the internal standard. After liquid–liquid extraction, drugs were analyzed using a 5-μm Kromasil C18 column (150 × 4.6 mm ID, particle size 5 μm) and a mobile phase composed of acetonitrile, water, and formic acid with a solvent gradient *(51)*. Amphotericin B is used for the treatment of invasive and disseminated fungal infections. Lee et al. described a sensitive liquid chromatography tandem mass spectrometric method for quantification of total and free amphotericin B in biological matrices. The authors used a C18 column for HPLC analysis *(52)*. Azithromycin in serum can be analyzed by HPLC using electrochemical detection. Fouda and Schneider described an HPLC combined with atmospheric pressure chemical ionization mass spectrometric method for quantitative determination of azithromycin in human serum; deuterium-labeled azithromycin was used as the internal standard *(53)*.

### *6.5. Analysis of Antineoplastic Drugs*

The most widely monitored neoplastic drug is methotrexate, and commercially available immunoassays are usually used for routine TDM of methotrexate in clinical laboratories. However, trace level of this drug cannot be detected using immunoassays because of sensitivity issues. Truci et al. described an HPLC-tandem mass spectrometric technique for determination of trace amount of methotrexate in human urine of hospital personnel exposed to this neoplastic agent. After solid phase extraction, HPLC analysis was carried out using an octadecyl silica SPE column *(54)*. Concentrations of methotrexate along with its metabolite 7-hydroxy methotrexate can be determined using HPLC. HPLC methods are also available for TDM of other antineoplastic drugs including doxorubicin, etoposide, 5-fluorouracil, mitoxantrone, mensa and demensa, taxol, aminoglutethimide, tamoxifen, and acrolein. GC with a capillary column can be used for analysis of cyclophosphamide, lomustine, and carmustine *(55)*.

Using chlorouracil as the internal standard, Maring et al. *(56)* developed an HPLC method for the determination of concentration of 5-fluorouracil as well as the concentration of its metabolite 5,6-dihydrofluorouracil in plasma using a reversed-phase C18 column and UV diode array detector. Schoemaker et al. developed an HPLC protocol for simultaneous measurement of anticancer drug irinotecan (CPT-11) and its active metabolite SN-38 in human plasma after converting both CPT-11 and SN-38 to their carboxylate form by using 0.01 mol/L of sodium tetraborate. HPLC separation was achieved using a Zorbax SB-C18 column and detector used was a fluorescence detector. The detection limit was 5.0 ng/mL for CPT-11 and 0.5 ng/mL for SN-38 *(57)*. Determination of docetaxel and paclitaxel concentrations using HPLC with UV detection has also been reported after liquid–liquid extraction of these antineoplastic agents from human plasma *(58)*. Quantification of imatinib, a selective tyrosine kinase inhibitor, in human plasma using HPLC combined with tandem mass spectrometry has been reported. The authors used a reversed-phase C18 column with a gradient of acetonitrile-ammonium formate buffer (4 mmol/L at pH 4.0) for HPLC analysis. Imatinib was detected by electrospray ionization mass spectrometry with multiple-reaction monitoring mode *(59)*. Fogil et al. described a method for determination of daunorubicin, idarubicin, doxorubicin, epirubicin, and their 13-dihydro metabolites in human plasma using HPLC and spectrofluorometric detection of all these anthracyclines using excitation and emission wavelength of 480 and 560 nm, respectively. After extraction of these drugs from human plasma, HPLC analysis was carried out using a Supelcosil LC-CN (25 cm × 4.6 mm ID, particle size 5 μm, Supelco Bellefonte, PA) column *(60)*. Concentrations of these anthracyclines in human serum can also be determined using liquid chromatography combined with electrospray mass spectrometry. Lachatre et al. determined concentrations of epirubicin, doxorubicin, daunorubicin, idarubicin, and active metabolites (doxorubicinol, daunorubicinol, and idarubicinol) in human plasma using aclarubicin as the internal standard. After solid phase extractions, these drugs were analyzed using a reversed-phase C18 column *(61)*.

GC combined with nitrogen phosphorus detector or mass spectrometry can also be used for determination of specific neoplastic drugs. Kerbusch et al. determined concentration of ifosfamide, 2- and 3-dechloroethylifosfamide in human plasma by using gas chromatography coupled with nitrogen phosphorus detector or mass spectrometry without any derivatization. Sample preparation involved liquid–liquid extraction with ethyl acetate after adding trofosfamide as the internal standard and making the serum alkaline. The authors concluded that gas chromatography with nitrogen phosphorus detector was more sensitive for analysis of these compounds compared with gas chromatography combined with positive ion electron-impact ion trap mass spectrometry *(62)*.

## 7. OTHER ANALYTICAL TECHNIQUES

Lithium has been used in the treatment of manic-depressive disorder since 1970, and the narrow therapeutic index of lithium with the nonspecific nature of lithium toxicity prompted routine therapeutic monitoring for these patients. Methodologies available for routine determination of lithium in human serum or plasma include flame atomic emission spectroscopy, flame atomic absorption spectroscopy, potentiometry

with lithium ion-selective electrodes, and colorimetric methods with spectrophotometric determination of lithium concentration and reflectometry methods. Sampson et al. evaluated determination of lithium by five ion-selective electrodes and one colorimetric method by comparing the results obtained by flame atomic absorption and flame atomic emission spectroscopy. The colorimetric method was based on the shifting of absorbance of a crown ether chromophore when it binds lithium (Vitros; formerly Ektachem slide) *(63)*. For colorimetric determination of lithium, aromatic organic reagents such as crown ethers and amide ionophores are used. Crown ether and cryptands provide best selectivity for lithium. The cryptand phenol exhibits greater than 4000:1 selectivity for lithium compared with sodium for binding because of rigid configuration of a binding site for lithium to form the complex. This complex is also water-soluble. Crown ether with bulky groups inhibits formation of 2:1; crown : sodium complex while allowing 1:1; crown : lithium complex *(64)*.

The frequent use of platinum complexes in cancer therapy may require therapeutic monitoring of platinum in serum. Using 400 μL specimen, Kloft et al. described a rapid flameless atomic absorption spectrometric assay of platinum in serum or in the protein free ultrafiltrate (free platinum). The limit was detection for platinum was 40 ng/mL in serum and 20 ng/mL in ultrafiltrate *(65)*.

Supercritical fluid and microbore liquid chromatography may have potential application for drug analysis. In supercritical fluid chromatography (SFC), the mobile phase is a gas (for example, carbon dioxide) maintained at its supercritical state. This mobile phase has low viscosity of a gas and high diffusivity between that of a gas and liquid. SFC analysis can be performed with a GC or LC detector and mass spectrometry can certainly be used as a detector for SFC. Potential applications include analysis of a small amount of specimen (5–200 μL) for pediatric and neonatal TDM as well as drug confirmation for toxicology *(66)*. Graves et al. *(67)* used SFC for characterization of a labile digitalis-like factor.

## 8. APPLICATION OF CAPILLARY ELECTROPHORESIS FOR DRUG ANALYSIS

Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) have application in determining concentration of drugs and metabolites in body fluid. In CZE, nanoliter amount of specimen is applied at one end of a fused silica capillary column (usually 15–100 cm long with an internal diameter between 25 and 75 μm) filled with buffer. When a high-voltage DC field is applied, charged solutes migrated through the capillary column by the combined action of electrophoresis and electro-osmotic bulk flow and are separated and detected at the other end of the column. Common detection techniques applied are on-column direct and indirect absorbance, direct and indirect fluorescence detection, occasionally radiometry or amperometry, as well as off-column detection, such as mass spectrometry. Only charged particles can be separated by CZE and neutral compounds cannot be analyzed by this method. In MECC, the buffer contains charged micelles (such as dodecyl sulfate), and both charged and uncharged solutes are separated based on differential partitioning between the micelles and the surrounding buffer. The separation is of chromatographic nature and elution order is based on partitioning. For charged solutes, separation is based on both chromatography and charged effect

including electrophoresis. This technique can separate acidic, neutral, and basic drugs in human serum. Several drug levels determined by this technique correlated well with values obtained using immunoassay and HPLC *(68)*.

Teshima et al. used CZE for simultaneous determination of sulfamethoxazole and trimethoprim in human plasma. The authors used ethyl acetate to extract these drugs from human plasma and used UV detection at 220 nm. The analysis was performed at 20 kV and 25°C using 15 mM phosphate buffer (pH 6.2) *(69)*. Another report described CZE determination of methotrexate, leucovorin, and folic acid in human urine using a capillary column (60 cm × 75 μm) and 15 mM phosphate buffer solution. The analysis was carried it using 25 kV at 20°C *(70)*. A method of coupling CZE with electrospray ionization mass spectrometry has also been reported for TDM of lamotrigine in human plasma. Tyramine was used as the internal standard. The linearity of the assay was from 0.1 to 5.0 μg/mL of serum lamotrigine concentration, and the limit of detection of was 0.05 μg/mL. The run time was less than 6 min *(71)*. A solid phase extraction followed by CZE analysis of tobramycin in serum has also been reported *(72)*. Determination of several analgesic and anti-inflammatory drugs (acetaminophen, ibuprofen, indomethacin, and salicylic acid) using CZE and micellar electrokinetic chromatography has also been reported *(73)*. Huang et al. described a micellar electrokinetic chromatography with direct UV detection for determination of concentration of cisplatin in human serum. The main hydrolytic metabolite was determined using a CZE method *(74)*.

## 9. CONCLUSION

Although immunoassays are widely used for determination of therapeutic drug concentrations in clinical laboratories, other analytical techniques such as GC, HPLC, GC/MS, and HPLC combined with tandem mass spectrometry also have applications for TDM. Immunoassays can provide a result rapidly because of ease of operation, but these assays also suffer from specificity because of cross-reactants. Moreover, immunoassays are not available for all drugs monitored in clinical laboratories. Therefore, HPLC, GC/MS, and especially HPLC combined with tandem mass spectrometry are essential for TDM especially in reference laboratories and hospital laboratories of academic medical center and referral hospitals.

## REFERENCES

1. Jolley ME, Stroupe SD, Schwenzer KS, Wang CJ, et al. Fluorescence Polarization immunoassay III. An automated system for therapeutic drug determination. *Clin Chem* 1981; 27: 1575–1579.
2. Hawks RL, Chian CN, eds. *Urine Testing for Drugs of Abuse*. Rockville, MD: National Institute of Drug Abuse (NIDA), Department of Health and Human Services; 1986. NIDA research monograph 73.
3. Jeon SI, Yang X, Andrade JD. Modeling of homogeneous cloned enzyme donor immunoassay. *Anal Biochem* 2004; 333: 136–147.
4. Datta P, Dasgupta A. A new turbidimetric digoxin immunoassay on the ADVIA 1650 analyzer is free from interference by spironolactone, potassium canrenoate, and their common metabolite canrenone. *Ther Drug Monit* 2003; 25: 478–482.
5. Dai JL, Sokoll LJ, Chan DW. Automated chemiluminescent immunoassay analyzers. *J Clin Ligand Assay* 1998; 21: 377–385.

6. MEIA Montagne P, Varcin P, Cuilliere ML, Duheille J. Microparticle-enhanced nephelometric immunoassay with microsphere-antigen conjugate. *Bioconjugate Chem* 1992; 3: 187–193.
7. Datta P, Larsen F. Specificity of digoxin immunoassays toward digoxin metabolites. *Clin Chem* 1994; 40: 1348–1349.
8. Datta P. Oxaprozin and 5-(p-hydroxyphenyl)-5-phenylhydantoin interference in phenytoin immunoassays. *Clin Chem* 1997; 43: 1468–1469.
9. Datta P, Dasgupta A. Bidirectional (positive/negative) interference in a digoxin immunoassay: importance of antibody specificity. *Ther Drug Monit* 1998; 20: 352–357.
10. Mayer-Helm BX, Kahlig H, Rauter W. Tetramethyl-p,p'-sildiphenylene ether-dimethyl, diphenylsiloxane copolymer as stationary phase in gas chromatography. *J Chromatogr A* 2004; 1042: 147–154.
11. Gentil E, Banoub J. Characterization and differentiation of isomeric self-complementary DNA oligomers by electrospray tandem mass spectrometry. *J Mass Spectrom* 1996; 31: 83–94.
12. Zancani M, Peresson C, Biroccio A, Federici G, et al. Evidence for the presence of ferritin in plant mitochondria. *Eur J Biochem* 2004; 271: 3657–3664.
13. Marquet P, Lachatre G. Liquid chromatography-mass spectrometry in forensic and clinical toxicology. *J Chromatogr B* 1999; 7333: 93–118.
14. Taylor P. Therapeutic drug monitoring of immunosuppressant drugs by high performance liquid chromatography-mass spectrometry. *Ther Drug Monit* 2004; 26: 215–219.
15. Napoli KL. Is microparticle enzyme-linked immunoassay (MEIA) reliable for use of tacrolimus TDM? Comparison of MEIA to liquid chromatography with mass spectrometric detection using longitudinal trough samples from transplant recipients. *Ther Drug Monit* 2006; 28: 491–504.
16. Kuperberg HJ. Quantitative estimation of diphenylhydantoin, primidone and phenobarbital in plasma by gas liquid chromatography. *Clin Chim Acta* 1970; 29: 282–288.
17. Davis HL, Falk KJ, Bailey DG. Improved method for the simultaneous determinations of Phenobarbital, primidone and diphenylhydantoin in patients' serum by gas liquid chromatography. *J Chromatogr* 1975; 9: 61–66.
18. Attwell SH, Green VA, Haney WG. Development and evaluation of method for simultaneous determination of phenobarbital and diphenylhydantoin in plasma by high pressure liquid chromatography. *J Pharm Sci* 1975; 64: 806–809.
19. Dietzler DN, Hoelting CR, Leckie MP, Smith CH, et al. Emit assays for five major anticonvulsant drugs: an evaluation of adaptations to two discrete kinetic analyzers. *Am J Clin Pathol* 1980: 74: 41–50.
20. Berg JD, Buckley BM. Rapid measurement of anticonvulsant drug concentrations in the out-patient clinic using HPLC with direct injection of plasma. *Ann Clin Biochem* 1987; 24: 488–493.
21. Minkova G, Getova D. Determination of carbamazepine and its metabolite carbamazepine 10, 11-epoxide in serum with gas chromatography mass spectrometry. *Methods Find Exp Clin Pharmacol* 2001; 23: 481–485.
22. Van Rooyen GF, Badenhorst D, Swart KJ, Hundt HK, et al. Determination of carbamazepine in human plasma by tandem liquid chromatography-mass spectrometry with electron spray ionization. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 769: 1–7.
23. Bahrami G, Mohammadi B. Sensitive microanalysis of gabapentin by high performance liquid chromatography in human serum using pre column derivatization with 4-chloro-7-nitrobenzofuran: application to a bioequivalence study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; 837: 24–28.
24. Contin M, Balboni M, Callegati E, Candela C, et al. Simultaneous liquid chromatographic determination of lamotrigine, oxcarbazepine monohydroxy derivative and felbamate in plasma of patients with epilepsy. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; 828: 113–117.
25. Dasgupta A, Hart AP. Lamotrigine analysis in plasma by gas chromatography-mass spectrometry after conversion into a tert-butyldimethylsilyl derivative. *J Chromatogr B Biomed Sci Appl* 1997; 693: 101–107.
26. Berry D, Millington C. Analysis of pregabalin at therapeutic concentrations in human plasma/serum by reverse phase HPLC. *Ther Drug Monit* 2005; 27: 451–456.

27. Chen BH, Taylor EH, Pappas AA. Total and free disopyramide by fluorescence polarization immunoassay and relationship between free fraction and alpha-1-acid glycoprotein. *Clin Chim Acta* 1987; 163: 75–90.
28. Shaw LM, Doherty JU, Waxman HL, Josephson ME. The pharmacokinetic and pharmacodynamic effects of carrying the free fraction of disopyramide. *Angiology* 1987; 38: 192–197.
29. Scislowski M, Rojek S, Klys M, Wozniak K, et al. Application of HPLC/MS for evaluation of fatal poisoning with digoxin in the aspect of medico-legal evidence. *Arch Med Sadowej Kryminol* 2002; 53: 19–31 [in Polish].
30. Verbesselt R, Tjandramaga TB, de Schepper PJ. High performance liquid chromatographic determination of 12 antiarrhythmic drugs in plasma using solid phase extraction. *Ther Drug Monit* 1991; 13: 157–165.
31. Dasgupta A, Rosenzweig IB, Turgeon J, Raisys VA. Encainide and metabolites analysis in serum or plasma using a reversed-phase high performance liquid chromatographic technique. *J Chromatogr* 1990; 526: 260–265.
32. vasBinder E, Annesley T. Liquid chromatographic analysis of mexiletine in serum with alternate application to tocainide, procainamide and N-acetylprocainamide. *Biomed Chromatogr* 1991; 5: 19–22.
33. McErlance KM, Igwemezie L, Kerr CR. Stereoselective analysis of the enantiomers of mexiletine by high-performance liquid chromatography using fluorescence detection and study of stereoselective disposition in man. *J Chromatogr* 1987; 415: 335–346.
34. Minnigh MB, Alvin JD, Zemaitis MA. Determination of plasma mexiletine levels with gas chromatography-mass spectrometry and selected ion monitoring. *J Chromatogr B Biomed Appl* 1994; 662: 118–122.
35. Dasgupta A, Appenzeller P, Moore J. Gas chromatography-electron ionization mass spectrometric analysis of serum mexiletine concentration after derivatization with 2,2,2-trochloroethyl chloroformate: a novel derivative. *Ther Drug Monit* 1998; 20: 313–318.
36. Dasgupta A, Yousef O. Gas chromatographic-mass spectrometric determination of serum mexiletine concentration after derivatization with perfluorooctanoyl chloride, a new derivative. *J Chromatogr B Biomed Sci Appl* 1998; 705: 282–288.
37. Doki K, Homma M, Kuga K, Watanabe S, et al. Simultaneous determination of serum flecainide and its metabolites by using high performance liquid chromatography. *J Pharm Biomed Anal* 2004; 35: 1307–1312.
38. Fischer C, Buhl K. Gas chromatography/mass spectrometry validation of high performance liquid chromatography analysis of flecainide enantiomers in serum. *Ther Drug Monit* 1992; 14: 433–435.
39. Dasgupta A, McNeese C, Wells A. Interference of carbamazepine and carbamazepine 10, 11-epoxide in the fluorescence polarization immunoassays for tricyclic antidepressants: estimation of true tricyclic antidepressant concentrations in the presence of carbamazepine using a mathematical model. *Am J Clin Pathol* 2004; 121: 418–423.
40. Chattergoon DS, Verjee Z, Anderson M, Johnson D, et al. Carbamazepine interference with an immune assay for tricyclic antidepressants in plasma. *J Clin Toxicol* 1998; 36: 109–113.
41. Wille SM, Maudens KE, Van Peteghem CH, Lambert WE. Development of a solid phase extraction for 13 new generation antidepressants and their active metabolites for gas chromatographic-mass spectrometry analysis. *J Chromatogr A* 2005; 1098: 19–29.
42. Juan H, Zhiling Z, Huande L. Simultaneous determination of fluoxetine, citalopram, paroxetine, venlafaxine in plasma by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-MS/ESI). *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; 820: 33–39.
43. Isoherranen N, Soback S. Chromatographic methods for analysis of aminoglycoside antibiotics. *J AOAC Int* 1999; 82: 1017–1045.
44. Lai F, Sheehan T. Enhanced of detection sensitivity and cleanup selectivity for tobramycin through pre-column derivatization. *J Chromatogr* 1992; 609: 173–179.
45. Ovalles JF, Brunetto Mdel R, Gallignani M. A new method for the analysis of amikacin using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization and high performance liquid chromatography with UV detection. *J Pharm Biomed Anal* 2005; 39: 294–298.
46. Nicoli S, Santi P. Assay of amikacin in the skin by high performance liquid chromatography. *J Pharm Biomed Anal* 2006; 41: 994–997.

47. Kawamoto T, Mashimoto I, Yamauchi S, Watanabe M. Determination of sisomicin, netilmicin, astromicin and micronomicin in serum by high-performance liquid chromatography. *J Chromatogr* 1984; 305: 373–379.
48. Soltes L. Aminoglycoside antibiotics-two decades of their HPLC bioanalysis. *Biomed Chromatogr* 1999; 13: 3–10.
49. Galanakis EG, Megoulas NC, Solich P, Koupparis MA. Development and validation of a novel LC-non derivatization method for the determination of amikacin in pharmaceuticals based on evaporative light scattering detection. *J Pharm Biomed Appl* 2006; 40: 1114–1120.
50. Kim BH, Lee SC, Lee HJ, Ok JH. Reversed-phase liquid chromatographic method for the analysis of aminoglycoside antibiotics using pre-column derivatization with phenylisocyanate. *Biomed Chromatogr* 2003; 17: 396–403.
51. Sagan C, Salvador A, Dubreuil D, Poulet PP, et al. Simultaneous determination of metronidazole and spiramycin in human plasma, saliva and gingival crevicular fluid by LC-MS/MS. *J Pharm Biomed Anal* 2005; 38:298–306.
52. Lee JW, Peterson ME, Lin P, Dressler D, et al. Quantitation of free and total amphotericin B in human biologic matrices by a liquid chromatography tandem mass spectrometric method. *Ther Drug Monit* 2001; 23: 268–276.
53. Fouda HG, Schneider RP. Quantitative determination of the antibiotic azithromycin in human serum by high performance liquid chromatography (HPLC)-atmospheric pressure chemical ionization mass spectrometry: correlation with a standard HPLC electrochemical method. *Ther Drug Monit* 1995; 17: 179–183.
54. Truci R, Fiorentino ML, Sottani C, Minoia C. Determination of methotrexate in human urine trace levels by solid phase extraction and high performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2000; 14: 173–179.
55. el-Yazigi A, Ezzat A. Pharmacokinetic monitoring of anticancer drugs at King Faisal Specialist Hospital, Riyadh, Saudi Arabia. *Ther Drug Monit* 1997; 19: 390–393.
56. Maring JG, Schouten L, Greijdanus B, de Vries EG, et al. A simple and sensitive fully validated HPLC-UV method for the determination of 5-fluorouracil and its metabolite 5, 6-dihydrofluorouracil in plasma. *Ther Drug Monit* 2005; 27: 25–30.
57. Schoemaker NE, Rosing H, Jansen S, Schellens JH, et al. High performance liquid chromatographic analysis of the anticancer drug irinotecan (CPT-11) and its active metabolite SN-38 in human plasma. *Ther Drug Monit* 2003; 25: 120–124.
58. Zufia Lopez L, Aldaz Pastor A, Armendia Beitia JM, Arrobas Velilla J, et al. Determination of docetaxel and paclitaxel in human plasma by high performance liquid chromatography: validation and application to clinical pharmacokinetic studies. *Ther Drug Monit* 2006; 28: 199–205.
59. Titier K, Picard S, Ducint D, Teihet E, et al. Quantification of imatinib in human plasma by high performance liquid chromatography-tandem mass spectrometry. *Ther Drug Monit* 2005; 27: 634–640.
60. Fogil S, Danesi R, Innocenti F, Di Paolo A, et al. An improved HPLC method for therapeutic drug monitoring of daunorubicin, idarubicin, doxorubicin, epirubicin and their 13-dihydro metabolites in human plasma. *Ther Drug Monit* 1999; 21: 367–375.
61. Lachatre F, Marquet P, Ragot S, Gaulier JM, et al. Simultaneous determination of four anthracyclines and three active metabolites in human serum by liquid chromatography-electrospray mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2000; 738: 281–291.
62. Kerbusch T, Jeuken MJ, Derraz J, van Putten JW, et al. Determination of ifosfamide, 2 and 3-dechloroethylifosfamide using gas chromatography with nitrogen-phosphorus or mass spectrometry detection. *Ther Drug Monit* 2000; 22: 613–620.
63. Sampson M, Ruddel M, Elin RJ. Lithium determination evaluated in eight analyzers. *Clin Chem* 1994; 40: 869–872.
64. Christian GD. Analytical strategies for the measurement of lithium in biological samples. *J Pharm Biomed Anal* 1996; 14: 899–908.
65. Kloft C, Appelius H, Siegert W, Schunack W, et al. Determination of platinum complexes in clinical samples by a rapid flameless absorption spectrometry assay. *Ther Drug Monit* 1999; 21: 631–637.
66. Wong SH. Supercritical fluid chromatography and microbore liquid chromatography for drug analysis. *Clin Chem* 1989: 35: 1293–1298.

67. Graves SW, Markides KE, Hollenberg NK. Application of supercritical fluid chromatography to characterize a labile digitalis-like factor. *Hypertension* 2000; 36: 1059–1064.
68. Thormann W, Zhang CX, Schmutz A. Capillary electrophoresis for drug analysis in body fluid. *Ther Drug Monit* 1996; 18: 506–520.
69. Teshima D, Otsubo K, Makino K, Itoh Y, et al. Simultaneous determination of sulfamethoxazole and trimethoprim in human plasma by capillary zone electrophoresis. *Biomed Chromatogr* 2004; 18: 51–54.
70. Rodriguez Flores J, Penalvo GC, Mansilla AE, Gomez MJ. Capillary zone electrophoresis determination of methotrexate, leucovorin and folic acid in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; 819: 141–147.
71. Zheng J, Jann MW, Hon YY, Shamsi SA. Development of capillary zone electrophoresis-electrospray ionization mass spectrometry for the determination of lamotrigine in human plasma. *Electrophoresis* 2004; 25: 2033–2043.
72. Fonge H, Kaale E, Govaerts C, Desmet K, et al. Bioanalysis of tobramycin for therapeutic drug monitoring by solid phase extraction and capillary zone electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; 810: 313–318.
73. Makino K, Itoh Y, Teshima D, Oishi R. Determination of nonsteroidal anti-inflammatory drugs in human specimens by capillary zone electrophoresis and micellar electrokinetic chromatography. *Electrophoresis* 2004; 25: 1488–1495.
74. Huang Z, Timerbaev AR, Keppler BK, Hirokawa T. Determination of cisplatin and its hydrolytic metabolite in human serum by capillary electrophoresis technique. *J Chromatogr A* 2006; 1106: 75–79.

# 4 The Pre-Analytical Phase of Drug Testing

*From Specimen Collection to Analysis*

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***Contents***



**Summary**

The pre-analytical phase for samples collected for the analysis of therapeutic drugs and toxins is as critical as the analytical phase. One of the most important aspects to this phase is the knowledge of the time at which the sample was collected and its relationship to the time of dosing or ingestion. These pieces of information are absolutely necessary for interpretation of the results. In addition, samples must be collected using the proper devices and processed or stored under conditions that minimize alterations to the drug concentration. In this chapter, I will review the elements that have repeatedly led to investigations and issues within the laboratory.

**Key Words:** Pre-analytical; blood collection tube; anticoagulants; storage.

## 1. INTRODUCTION

Much focus is placed on the analytical portion of drug testing. Specifically, the instruments we use for these activities often attract more attention than any other aspect of the total process. Unfortunately, this means that the pre-analytical phase of the process is often downplayed or even forgotten—that is, until one spends hours troubleshooting what seems to be an analytical problem only to trace the issue back to

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an earlier step in the process. These steps are just as critical as those during the analysis to delivering valid results for therapeutic drug monitoring (TDM) and toxicology. Without attention to and knowledge of the conditions under which a given sample was obtained and prepared for testing, the data generated from its subsequent analysis may lead to erroneous conclusions and inappropriate, even dangerous, treatment. Thus, it is crucial that we pay close attention to the steps involved during method development or evaluation and that we implement processes to detect problems once a method is put into use. In this chapter, I will cover aspects of the pre-analytical phase key to TDM and toxicology testing and will give examples of problems that have been reported. These include issues associated with the timing of sample collection, sample containers, processing, and storage.

## 2. IT IS A MATTER OF TIME

Time is a critical factor in both TDM and toxicology, and its importance and roles repeat throughout discussions of these services. Results, including both serum drug concentrations and urine toxicology screens, are best interpreted with respect to the time of dosing or exposure and the time at which the samples are acquired. In TDM, sample collection is not a random process, but one that must be carefully controlled with collection taking place within specific windows of time in conjunction with the dosing regimen. Unfortunately, the steps involved in sample collection are often out of the hands of the analyst, a situation that represents a continuous source of frustration. This can somewhat be minimized by maintaining open lines of communication with, and providing detailed guidelines and resources to, the staff involved in sample collection.

Most commonly, the best window of collection takes place during the 30–60 min preceding a dose when circulating concentrations necessary for the drug to be effective are lowest. Samples collected at this time are referred to as trough samples. When a drug is most effective over a very narrow concentration range and toxicity is a concern, monitoring may be most effective using peak concentrations when maximal concentrations are achieved. This type of collection is difficult to control as the time at which peak concentrations are achieved vary with the individual for each drug collected in this manner and may even vary with the formula, dosing route, and coingestion of food.

Samples are also collected at other points in the dosing cycle, for example, as with the once-daily dosing protocols used for aminoglycoside or vancomycin administration. In these protocols, samples are collected at defined points within the dosing cycle. Some laboratories call these “random” samples because they are neither peak nor trough and the time of collection may vary, but this is an inaccurate use of the term because for the result to be useful, the collection time must be controlled and known—not random. The once-daily protocols for aminoglycosides differ from other protocols in another manner. Usually, peak samples are used to assess toxicity, but in these protocols, trough concentrations are used because at this time the patient should have completely cleared the drug. The finding that the aminoglycoside remains in the circulation at this time indicates reduced clearance and hence the likelihood of toxicity.

Over the years, numerous studies and surveys have shown that samples for digoxin monitoring are among the most problematic to collect at the optimal time. Because digoxin exhibits a long distribution phase, there is a significant lag in the time between

dosing and when the maximum pharmacological response is observed. As a result, samples for digoxin measurement should be collected no earlier than 6–8 h after dosing and preferably after 12 h. Samples collected before distribution is complete are often elevated. Although these elevated concentrations are not associated with toxicity, they are nevertheless confusing and lead to misinterpretation of the results. The problem of incorrect timing of sample collection is not new and has been described since digoxin testing began in earnest in the 1970s! In 1991 and in 2004, the College of American Pathologists conducted Q-probe surveys on the problem *(1,2)*. The recent study focused on outpatients, included results from 59 institutions, and correlated the collection time with the concentration measured for 1571 outpatients. It showed that for every five specimens for which toxic concentrations were obtained, three were inappropriately collected with respect to the dosing interval *(2)*. Unfortunately, this study shows no improvement in processes from the previous survey conducted in the early 1990s *(1)*. Given the interest in patient safety and utilization of health care resources, this is one area that cries out for attention. One institution looked at the problem closely and implemented a nighttime dosing regimen for both their inpatients and outpatients. This has reportedly worked well for this facility and could serve as an effective solution for others *(3)*.

In an effort to improve efficiency, prepare patients for discharge earlier in the day, and minimize the use of blood, many hospitals have consolidated phlebotomy collections and begin routine collection rounds often as early as 0400 h. This has led to some TDM samples being collected as part of these very early routine chemistry and hematology collections. Unfortunately, this means that the sample is often being collected prematurely if the drug is scheduled to be given mid-morning or later. Such samples do not represent true troughs, and although the results may fall within the therapeutic range, they pose the risk of toxicity if used for calculating subsequent doses.

An example of this is seen with a recent inquiry from one of our transplant coordinators regarding a patient who was receiving tacrolimus. Following admission, the patient was found to have an elevated concentration and the team contacted the laboratory to question whether the result could be falsely elevated as the patient's dose had not been altered. Although the team was certain that the sample had been appropriately collected as a trough, we were able to document that the sample had been collected 7.5 h early. With no change in the patient's dose, subsequent samples collected at the appropriate time were within the targeted range.

In contrast, samples for toxicology testing are typically random. For serum-based monitoring, i.e., in situations involving salicylate or acetaminophen ingestions, the patient may not be willing or able to recall and give an accurate time of ingestion *(4)*. For this reason, it may be useful to obtain more than one sample. Repeat measurements can be used to assess whether absorption is continuing as might happen with extended release preparations or when bezoars form and to calculate the half-life of the compound. Urine drug-screening results pose interesting dilemmas in that some health care providers often wish to use the results to estimate when the ingestion may have taken place. As a random urine collection represents a snapshot of the individual's metabolic and excretion pattern, this is simply not possible. At best, one can apply

average windows of detection reported for drugs with the caution that variations occur related to differences in pharmacokinetics and pharmacogenetics.

## 3. CONTAINERS: DEALING WITH GLASS, PLASTICS, GELS, AND ADDITIVES

Continuing in the pre-analytical phases of TDM and toxicology, it is important to think about the sample type needed and collection container. For most chemistry analyses, serum or heparinized plasma is the preferred specimen. To facilitate later steps in sample handling and testing, laboratories prefer to collect blood for these in separator or gel-barrier tubes, as the barrier formed between the serum or the plasma and the erythrocytes during centrifugation protects and stabilizes some of the measured chemistry constituents. Additionally, most laboratories have also transitioned from glass tubes to plastic for safety reasons. The previous generations of gels and other materials used in these tubes were found to adsorb many commonly monitored drugs in an unpredictable manner *(5–9)*. Investigators found the degree of adsorption varied with the amount of drug present, the amount of blood in the tube, the environmental temperature, and the duration of exposure. There was also evidence that adsorption varied between lots of tubes in that the adsorption observed with one lot did not replicate with a subsequent lot. Because of these observations and studies, most TDM laboratories adopted the use of plain evacuated tubes containing no additives. A few laboratories adopted the use of trace metal (royal blue tops) tubes to reduce confusion among non-laboratory personnel who are involved in the collection process and likely do not appreciate the distinction between the various red- and gold-top tubes.

Over the past few years, manufacturers have introduced plastic tubes containing separator gels reportedly formulated to minimize adsorption. Unfortunately, the literature contains few reports validating the suitability of these tubes; but the studies conducted by Bush et al. *(10)* demonstrate the types of studies useful in validating performance of such tubes. Serum pools containing commonly monitored drugs were prepared and aliquoted into plain tubes (containing no gel) as a control and two types of gel separator tubes. The respective drug concentration was measured immediately, at 4 h and at 24 h. When the results were compared with those obtained initially and with those obtained from the samples in the plain tubes, the investigators found no significant difference in the concentrations of most of the drugs for the samples in contact with the SST II gel separator tubes. Additional studies did reveal small effects using samples collected from patients receiving phenytoin or carbamazepine, two drugs for which adsorption has been shown to be a significant problem. Previous investigations had shown adsorption was more likely if the tube was partially filled or if the sample was allowed to remain in contact with the gel for extended periods of time. The investigators thus performed an extended study in which full tubes of blood were collected from patients receiving one of these two drugs. The tubes were processed and tested immediately. These results were compared with those obtained after the samples had been held at room temperature for 8 and 24 h, and stored at 4°C for 7 days. A third set of experiments were conducted simulating partial filling of tubes by placing 2 ml of serum in each of the types of tubes and the samples stored at 4°C, room temperature, and 32°C for 4, 24, and 48 h. After storage for 7 days at 4°C, the concentration of carbamazepine declined by 10% whereas that of phenytoin declined

by 4%. Loss of analyte was less than 5% for the other conditions tested, well within the analytical precision of the analyses. At the time this chapter was prepared, there were few reports regarding the suitability of other reformulated barrier tubes and the previous study did not test all possible drugs or multiple lots. It is thus left to the individual laboratories to validate the claim that these new formulations do not adsorb the drugs we seek to measure. The studies outlined by Bush et al. are a reasonable approach to such studies.

Chemicals from the stoppers of the tubes, as well as chemical additives used to enhance clotting or prevent adsorption to the tubes themselves, have been found to interfere with analyses. This problem was first recognized in the 1970s when it was recognized that tris(2-butoxyethyl)phosphate (TBEP), a plasticizer, found in some stoppers, interfered with the measurement of propranolol, alprenolol, lidocaine, chlorimipramine imipramine, nortriptyline, meperidine, and quinidine *(11)*. The mechanism of interference was interesting in that it was discovered that upon leaching into the blood sample, TBEP displaced these basic drugs from their binding sites on alpha-1 acid glycoprotein. The free drugs were postulated to then diffuse from the plasma/sera into the erythrocytes effectively reducing the total amount detected in the sera *(12,13)*.

TBEP is no longer an issue, but problems continue to arise from interferences related to new stoppers, contaminants within the glass or plastic, or additives used enhance clotting or reduce adsorption *(14–22)*. Methods using chromatography or mass spectrometry are particularly susceptible to some of these interferences as these materials result in extraneous peaks, co-eluting peaks, and more subtle problems of ion suppression or enhancement. For example, Murthy *(14)* reported a sudden appearance of an extraneous peak in a high-performance liquid chromatography (HPLC)-based method for amiodarone when a brand of evacuated tubes had been replaced by another. Unfortunately, the events that led to this problem are neither unique nor isolated. Someone outside the laboratory made the decision to switch to a different brand of tubes and did not communicate the information to the laboratory performing the testing. The potential for the collection tube to cause interferences was demonstrated by Drake et al. *(15)*. This group tested for potential interferences from sample collection tubes for MALDI TOF mass spectrometry-based analyses by incubating 1 ml of phosphate-buffered saline (pH 7.2) in various sample collection tubes. To assure all surfaces of the tubes were considered and to maximize the ability to detect interferences, the tubes were gently rocked at room temperature for 4 h. The solutions were then processed, extracted, and analyzed. Multiple peaks were observed in the m/z range from 1000 to 3000 for solutions incubated in seven of 11 tube types.

Interferences from collection tubes extend beyond chromatography-based methods to other types of methods *(19–24)*. For example, Sampson et al. *(19)* documented interference from a silica clot activator when using an ion-selective electrode-based method for measuring serum lithium. This particular report demonstrates the complexity of the interferences and the difficulty in detecting and investigating such problems. The problem was identified when a double-blind study of lithium, carbamazepine, and/or valproic acid therapy was initiated and lithium concentrations of approximately 0.1 mmol/L were reported for a patient who was not receiving lithium. The investigators found that the electrode membrane erroneously detected lithium when first exposed

to samples that contained the silica clot activator, but after repeated exposure to the activator, the electrodes did not appropriately detect lithium present in patient samples. In other words, the lithium concentrations were falsely decreased. Most importantly, quality control material prepared using a bovine protein matrix was not affected and would not have detected the problem.

After considerable investigation, Bowen et al. determined that an organosilicone surfactant, Silwet L-720, was the culprit interfering with several immunoassays for thyrosine, cortisol, progesterone, thyroid-binding gobulin, and triiodothyronine *(21–22)*. After contact with collection tubes containing this compound, results for these analytes determined using specific immunoassays were increased by as much as 11–36%. The surfactant was found to desorb antibodies from the solid phase beads used in the immunoassays leading to a reduction in the chemiluminescent signal generated and falsely increasing the apparent hormone concentration. Although the tests involved were hormonal, TDM or toxicology analyses could just have easily been involved.

Other tubes or devices, for example, microfuge tubes and filtration devices, used in the analyses should also be considered. Yen and Hsu *(23)* determined that contaminants from polypropylene microcentrifuge tubes yielded extraneous peaks in an HPLC electrochemical detection-based method for the measurement of antioxidants. In this case, none of the peaks interfered with the analyses as the measurements were made at multiple potentials, but as the authors cautioned, had a single potential been used for detection, interference would have been much more likely. As with several of the studies we have discussed, the extraneous peaks were not consistent across all brands tested and emphasize the lessons above that what may be perceived to be inconsequential changes to materials or methodologies may indeed be monumental.

That we continue to experience such interferences suggests that we need to continue to be diligent to detect such problems. Not only should the problems be reported to the manufacturers involved but should also be reported to the Food and Drug Administration (FDA) by accessing the MedWatch system through their Web site (www.fda.gov).

In the early days of cyclosporine measurements, small clots were found to result from the use of heparin salts and to reduce extraction efficiency. For this reason, EDTA-anticoagulated whole blood has been the sample of choice for cyclosporine and subsequent immunosuppressives found to also partition into the erythrocytes. One characteristic of cyclosporine that was not recognized for some time was its ability to adsorb to some types of plastics *(25–27)*. I first encountered this problem when trying to automate the tedious pipetting associated with the early cyclosporine radioimmunoassays. Unfortunately, this characteristic is still not appreciated by many. Any plastic that comes into contact with these drugs should be tested before use in collection, storage, or analysis. An example of the type of study that should be conducted is seen in that reported by Faynor and Robinson *(28)* in which they determined that cyclosporine did not significantly adsorb to the Vacutainer PLUS evacuated tubes (Becton Dickinson Vacutainer Systems) over a 7-day period.

It may seem to be a fairly obvious statement, but lithium heparin should not be used to collect samples for lithium determinations. One practice to decrease the sample preparation time has been to adopt the use of plasma as a specimen for routine chemistry analyses. EDTA-anticoagulated blood is of course unsuitable, but lithium heparin is a

popular specimen and avoids issues with sodium or potassium measurements. Which heparin salt is present within a green-top tube cannot be easily determined simply by looking at the color of the stopper. One must be able to read the contents on the label, and this may be difficult to do if the label is covered with the patient identification label. More than one laboratory has spent time investigating unexpected, elevated lithium concentrations for patients who had no evidence of toxicity only to find a lithium heparin tube had been inadvertently used for collection.

## 4. PROCESSING AND STORAGE

Serum, plasma, and whole blood are complex matrices. We tend to forget that after collection and subsequent processing, the samples are far from static. As the samples are allowed to sit, carbon dioxide is lost and the pH changes. Enzymes present may lose activity or gain activity. Chemical interactions between various endogenous and exogenous chemicals occur between and amongst each other. Recognizing the potential for these and understanding how they impact the stability of a given drug is important *(29,30)*.

One of the best examples comes from toxicology when cocaine needs to be measured in blood. The metabolism of this drug by circulating cholinesterases continues unless fluoride is used to inhibit the process. Similarly, interactions between two drugs administered may continue after sample collection. For example, it is not unusual for several antibiotics to be used sequentially to treat some infections. One practice involves the use of both an aminoglycoside and a beta-lactam antibiotic. The drugs are not co-administered in the same infusion because it is known that beta-lactams will inactivate aminoglycosides rendering the aminoglycoside ineffective against the microorganism for which it is intended and unrecognizable by the antibodies in the immunoassays used to quantify serum concentrations. The rate at which inactivation occurs depends upon the aminoglycoside involved, the beta-lactam antibiotic involved, as well as the time and temperature of exposure *(31)*. Although administered sequentially to avoid this in vivo, both drugs may be present in the circulation and subsequently in the collection tube when samples are collected. Consequently, the amount of measurable aminoglycoside will decline over time leading to lower and lower apparent concentrations. It is therefore advisable to process, separate, and analyze the samples immediately or to freeze the samples if testing is delayed *(31)*.

Methotrexate has long been considered to be unstable. Limelette et al. *(32)* investigated this question by spiking methotrexate into whole blood collected from healthy donors. The blood was collected on citrate–phosphorus–dextrose. Using an HLPC-based method, they compared whole blood and plasma methotrexate concentrations from these samples over time and various storage conditions. The whole blood stability study was performed using a pool, which was then divided into three sub-pools. The sub-pools were stored at room temperature exposed to light, at room temperature protected from light, and at 4°C protected from light. Aliquots were tested immediately after preparation and at intervals up to 144 h. Visual inspection of the graphic display of the resulting data shows similar changes in the methotrexate concentrations regardless of the storage conditions from initial preparation until 48 h later. Concentrations declined slightly, but the deviation from baseline was less than 10%. After 48 h, there was little change in the levels observed for the light-protected pool stored

at 4°C. Interestingly, of the two pools retained at room temperature, the one protected from light showed the greatest loss of methotrexate with changes of more than 20% occurring by 100 h.

When the investigators tested three plasma pools under the same conditions, they found that the drug concentrations also steadily declined by approximately 17% over the first 3 days of testing before leveling off. Little change was observed over an additional 7 days. The study has the following limitations: only one concentration of methotrexate was tested, the samples were prepared pools not patient samples, and the blood used to prepare the pools did not simulate typical patient samples in that it contained citrate–phosphorus–dextrose. Because the use of methotrexate extends beyond its use as a chemotherapeutic agent for leukemia and other cancers and monitoring is widely performed, it would thus be of great use to clinical laboratories to simplify the collection, processing, and storage of specimens.

## 5. AVOIDING SURPRISES: ASSESSING PRE-ANALYTICAL PROCESSES AND TROUBLESHOOTING

Surprises are fun when hosting birthday parties or when one wins a sweepstakes. They are not enjoyable when encountered in the laboratory. By taking time to include the evaluation of a few pre-analytical characteristics when considering new analytes or methods, one can minimize later surprises. In addition to assessing the effects of hemolysis, lipemia, and icterus remember to evaluate the tubes and devices used in the sample collection, storage, or preparation. The pharmaceutical literature is often a good place to start. Descriptions of the stability (or lack thereof) of a drug in a pharmaceutical solution often provide insight into the stability of the drug in our samples. When considering changes in vendors of established materials, remember to obtain supplies in advance to assess comparability. Make sure your purchasing agent does not have the authority to make changes in sources without approval and validation. Our laboratory has found that the recording of lot of changes facilitates troubleshooting. This is not easy to do, but it is amazing how often a lot of buffer or solvent is found to be the culprit.

Although I have focused on the samples used in TDM, the lessons are applicable to those used in toxicology as well. Because of forensic needs, for example, the need to be able to replicate results with retesting at later dates, the stabilities of these analytes are probably better documented than most therapeutically monitored drugs.

## 6. CONCLUSIONS

As shown by the literature reviewed, the pre-analytical phase of TDM is extremely important. Care must be taken to assure samples are collected at the right time, under the right conditions. Although I have focused on the samples used in TDM, the lessons are applicable to those used in toxicology as well. Because of forensic needs, for example, the need to be able to replicate results with retesting at later dates, the stabilities of these analytes are probably better documented than most therapeutically monitored drugs.

## REFERENCES


1. Howanitz PJ, Steindel SJ. Digoxin therapeutic drug monitoring practices. A College of American Pathologists Q-Probes study of 666 institutions and 18,679 toxic levels. *Arch Pathol Lab Med* 1993;117:684–690.
2. Titus K. For digoxin, simple fix to stubborn problem. *CAP Today*. Available at www.cap.org (accessed 7/12/2006).
3. Bernard DW, Bowman RL, Grimm FA, Wolf BA, Simson MB, Shaw LM. Nighttime dosing assures postdistribution sampling for therapeutic drug monitoring of digoxin. *Clin Chem* 1996;42:45–49.
4. Pohjola-Sintonen S, Kivisto KT, Vuori E, Lapatto-Reiniluoto O, Tiula E, Neuvonen PJ. Identification of drugs ingested in acute poisoning: correlation of patient history with drug analyses. *Ther Drug Monit* 2000;22:749–752.
5. Landt M, Smith CH, Hortin GL. Evaluation of evacuated blood-collection tubes: effects of three types of polymeric separators on therapeutic drug-monitoring specimens. *Clin Chem* 1993;39:1712–1717.
6. Dasgupta A, Dean R, Saldana S, Kinnaman G, McLawhon RW. Absorption of therapeutic drugs by barrier gels in serum separator blood collection tubes. Volume- and time-dependent reduction in total and free drug concentrations. *Am J Clin Pathol* 1994;101:456–461.
7. Dasgupta A, Blackwell W, Bard D. Stability of therapeutic drug measurement in specimens collected in VACUTAINER plastic blood-collection tubes. *Ther Drug Monit* 1996;18:306–309.
8. Dasgupta A, Yared MA, Wells A. Time-dependent absorption of therapeutic drugs by the gel of the Greiner Vacuette blood collection tube. *Ther Drug Monit* 2000;22:427–431.
9. Karppi J, Akerman KK, Parviainen M. Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring (TDM). *Clin Chem Lab Med* 2000;38:313–320.
10. Bush V, Blennerhasset J, Wells A, Dasgupta A. Stability of therapeutic drugs in serum collected in vacutainer serum separator tubes containing a new gel (SST II). *Ther Drug Monit* 2001;23:259–262 (Erratum in: *Ther Drug Monit* 2001;23:738).
11. Chrzanowski F, Niebergall PJ, Mayock R, Taubin J, Sugita E. Interference by butyl rubber stoppers in GLC analysis for theophylline. *J Pharm Sci* 1976;65:735–736.
12. Amitai Y, Kennedy EJ, DeSandre P, Frischer H. Distribution of amitriptyline and nortriptyline in blood: role of alpha-1-glycoprotein. *Ther Drug Monit* 1993;15:267–273.
13. Kennedy E, Frischer H. Distribution of primaquine in human blood: drug-binding to alpha 1-glycoprotein. *J Lab Clin Med* 1990;116:871–878.
14. Murthy VV. Unusual interference from primary collection tube in a high-performance liquid chromatography assay of amiodarone. *J Clin Lab Anal* 1997;11:232–234.
15. Drake SK, Bowen RAR, Bemaley AT, Hortin GL. Potential interferences from blood collection tubes in mass spectrometric analyses of serum polypeptides. *Clin Chem* 2004;50:2398–2401.
16. Wu SL, Wang YJ, Hu J, Leung D. The detection of the organic extractables in a biotech product by liquid chromatography on-line with electrospray mass spectrometry. *PDA J Pharm Sci Technol* 1997;51:229–237.
17. Zhang XK, Dutky RC, Fales HM. Rubber stoppers as sources of contaminants in electrospray analysis of peptides and proteins. *Anal Chem* 1996;68:3288–3289.
18. Gaind VS, Jedrzejczak K. HPLC determination of rubber septum contaminants in the iodinated intravenous contrast agent (sodium iothalamate). *J Anal Toxicol* 1993;17:34–37.
19. Sampson M, Ruddel M, Albright S, Elin RJ. Positive interference in lithium determinations from clot activator in collection container. *Clin Chem* 1997;43:675–679.
20. Bowen RAR, Chan Y, Ruddel ME, Hortin GL, Csako G, Demosky SJ, Remaley AT. Immunoassay interference by a commonly used blood collection tube additive, the organosilicone surfactant silwet L-720. *Clin Chem* 2005;51:1874–1882.
21. Bowen RAR, Chan Y, Cohen J, Rehak NN, Hortin GL. Effect of blood collection tubes on total triiodothyronine and other laboratory assays. *Clin Chem* 2005;51(2):424–433.
22. van den Besselaar AM, van Dam W, Sturk A, Bertina RM. Prothrombin time ratio is reduced by magnesium contamination in evacuated blood collection tubes. *Thromb Haemost* 2001;85:647–650.

23. Yen HC, Hsu YT. Impurities from polypropylene microcentrifuge tubes as a potential source of interference in simultaneous analysis of electrochemical detection. *Clin Chem Lab Med* 2004;42:390–395.
24. Toulon P, Ajzenberg N, Smahi M, Guillin MC. A new plastic collection tube made of polyethylene terephtalate is suitable for monitoring traditional anticoagulant therapy (oral anticoagulant, unfractionated heparin, and low molecular weight heparin). *Thromb Res* 2007; 119:135–43 [Epub ahead of print].
25. Blifeld C, Ettenger RB. Measurement of cyclosporine levels in samples obtained from peripheral sites and indwelling lines. *N Engl J Med* 1987;317:509.
26. Lee YJ, Chung SH, Shim CK. The prevention of cyclosporin A adsorption to Transwell surfaces by human plasma. *Int J Pharm* 2001;224:201–204.
27. Reisman L, Cooper D, Lieberman KV, Martinelli GP. Effect of cyclosporine adherent to tissue culture plates on in vitro immunological studies. *Transplantation* 1989;48:872–874.
28. Faynor SM, Robinson R. Suitability of plastic collection tubes for cyclosporine measurements. *Clin Chem* 1998;44:2220–2221.
29. Chen J, Hsieh Y. Stabilizing drug molecules in biological samples. *Ther Drug Monit* 2005;27:617–624.
30. Hinderling PH, Hartmann D. The pH dependency of the binding of drugs to plasma proteins in man. *Ther Drug Monit* 2005;27:71–85.
31. Hammett-Stabler CA, Johns T. Laboratory guidelines for monitoring of antimicrobial drugs. National Academy of Clinical Biochemistry. *Clin Chem* 1998;44:1129–1140.
32. Limelette N, Ferry M, Branger S, Thuillier A, Fernandex C. In vitro stability study of methotrexate in blood and plasma samples for routine monitoring. *Ther Drug Monit* 2003;25:81–87.

# 5 Effect of Hemolysis, High Bilirubin, Lipemia, Paraproteins, and System Factors on Therapeutic Drug Monitoring

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***Contents***



**Summary**

Among the endogenous interferents affecting assay results, the most common are bilirubin, hemoglobin, lipids, and paraproteins. These interferents may affect therapeutic drug monitoring (TDM), drugs of abuse (DAU) testing, and toxicology assays of any format where the sample is used directly for analysis without any pretreatment of specimen. Immunoassays are commonly used in clinical laboratories where analyte-specific antibody or binding agents are used to estimate the analyte concentration in the specimen. Some enzyme and chemistry assays are also utilized in TDM and DAU analysis. Such assays use various types of signals, the most common being colorimetry, fluorimetry, and chemiluminescence. Assays may be prone to interference depending on the format or label used. Commercial assay kits report the result of such interference in the kit inserts (up to levels of >20 mg/dL bilirubin, >500 mg/dL hemoglobin, and >1000 mg/dL lipids). The interference is caused by the optical, fluorescent, or chemiluminescent properties of these interferents. Thus, bilirubin interferes by its absorption and fluorescence properties, hemoglobin by its absorption, fluorescence and chemiluminescence properties, and lipids interfere mostly from their light-scattering (turbidity) properties. Bilirubin and hemoglobin may also interfere because of side reactions in the assay. Modern auto-analyzers can detect all three interferents and flag the results. Flagged results should be carefully reviewed for the accuracy. Both hypo- and hyper-proteinemia can affect assay results. Paraproteins interfere in many assays by precipitating out during the sample blanking step thus producing false results. Another source of interference may be from probe (sample or reagents) or reaction cuvettes carryover contamination in random-access auto-analyzers. If the validity of test results

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is questioned, the assay should be repeated either by removing the interferent from the sample or by using different method which is known to suffer less from that type of interference.

**Key Words:** Bilirubin; hemoglobin; lipids; interference; assays.

## 1. INTRODUCTION

Most therapeutic drug monitoring (TDM) and drugs of abuse (DAU) testing are performed in clinical laboratories by using automated systems. There are two main technologies used in such determination. The most common one is immunoassay, which has been described in detail in Chapter 3. Immunoassay has the benefits of high sensitivity and specificity and thus has become the major technique in TDM and DAU assays. The second method used in TDM/DAU is enzymatic and/or chemistry methods. In these assays, the specificity of an enzyme is used to generate analyte-specific signals (in direct or indirect reaction), and the concentration is calculated based on generated signal. There are four major sources of "endogenous" interference that derive from specimen condition, especially in serum or plasma (Table 1).

**Table 1**
**Specimen Appearance and Causative Interferents**

| *Specimen appearance* | *Causative Interferent* | *Comment* |
|---|---|---|
| Greenish, icteric | Bilirubin; Reference Range (NR): <1 mg/dL; High levels up to 20 mg/dL may be found in jaundiced patients | Different conjugates of bilirubin; interference caused by photometric or fluorimetric properties of bilirubin. Bilirubin may participate in side reactions interfering with the assay. Additionally, bilirubin may interfere in free drug assays by changing the distribution of drug between free versus complexed compartments |
| Red, hemolyzed | Hemoglobin; NR: <1 mg/dL; Higher concentrations are found in vivo (hemolytic diseases) or in vitro (preanalytic causes) | Hemoglobin has photometric, fluorimetric, and chemiluminescent properties which may interfere in immunoassays using such signals |
| Turbid | Lipids (chylomicrons and VLDL) | Lipids interfere mainly by light scattering and absorption of lipoprotein micelles |
| Nothing specific. Hyper-proteinemia may make the specimen viscous. There may be protein fibers in stored plasma or serum samples | Protein (includes paraproteins) | Both hypo- and hyper-proteinemia may cause assay interference |

VLDL, very-low-density lipoproteins.

## 2. MECHANISM OF INTERFERENCE

Most TDM immunoassays involve analytes of small molecular size, and these assays employ the competition format where the analyte molecules compete with limited number of specific binding sites, e.g., on specific antibodies, with labeled analyte molecules in a reaction (details described in Chapter 3). Signals generated by the bound label are converted into the analyte concentration in the assay. The signals are mostly optical in nature including absorbance, fluorescence, or chemiluminescence. The assays can be homogeneous or heterogeneous. In the former format, the bound label has different properties than the free label, thus the method is simpler, requiring no separation and the difference in signal between bound and free labels is utilized to quantify the analyte. In heterogeneous immunoassays, the bound label is physically separated from the unbound labels and then the signal is measured.

As explained in Table 1, most of the interference from bilirubin, hemoglobin, lipids, or paraproteins are caused by the fluorimetric or photometric properties of the interferents, which interfere with the generation of signal. Thus, such interference is more common in homogeneous than in heterogeneous assays. In certain assay formats, the interference enhances the signal. As in most competitive immunoassays the signal is inversely proportional to the reported specimen analyte concentration, such interference generates false-negative results. In some other assay formats, the increased signal causes false-positive results.

Bilirubin absorbs around 450–460 nm. Hemoglobin begins to absorb from 340 to 560 nm and absorbance peak is observed at 541 nm (oxyhemoglobin). Therefore, hemolysis affects assays that use the absorbance properties of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) *(1)* at 340 nm. Selecting a longer wavelength (>650 nm) for detection may minimize the interference from bilirubin or hemolysis in a turbidimetric immunoassay. But because lipid interferes mostly by light scattering, lipid may still interfere with the method.

Bilirubin and hemoglobin can also interfere in assays through unintended side reactions. Both participate in redox reactions, commonly used in TDM or DAU assays that are not immunoassays. The Package Inserts from most commercial assay kits normally lists the effect of these interferents. Most manufacturers follow the EP7-P protocol from National Committee for Clinical Laboratory Standardization (NCCLS, currently called Clinical Laboratory Standards Institute or CLSI), where the interference is studied at two different steps *(2)*. In the "Screening" protocol, serum pools containing a clinically important concentration of the analyte are spiked with various concentrations of the interferent, and the assay results are compared with a suitable control sample (without spiked interferent). Interference is judged significant when the results of a spiked sample are statistically different from the control sample and the difference between the two means is >10%. The protocol also recommends plotting analyte versus interferent concentrations to find the pattern of interference, if any. If there is more than single clinically relevant analyte concentration, then the interference should be studied with serum pools containing each of those analyte concentrations *(3)*.

Table 2
Clinical Laboratory Standards Institute (CLSI) (NCCLS)-Recommended Interference Levels

| *Interferent* | *Highest Interferent Concentration* | | | |
|---|---|---|---|---|
| | *NCCLS Recommended* | | *Manufacturer Reported* | |
| | *Common Unit (mg/dL)* | *SI Unit* | *Common Unit (mg/dL)* | *SI Unit* |
| Bilirubin | 20 | 342 μmol/L | 30 | 513 μmol/L |
| Hemoglobin | 500 | 5.00 g/L | 2000 | 20.00 g/L |
| Lipid[a] | 1000 | 11.3 mmol/L | 2000 | 22.6 mmol/L |

[a]As triolein.

The CLSI-recommended levels of interfering substances up to which interference should be studied are summarized in Table 2. However, many package inserts report results beyond those levels.

### *2.1. Specimen Types*

Serum and plasma are the most common type of specimen used in TDM. These specimens may be interfered by all four types of interferents covered in this chapter. Additionally, collection tube additives for plasma or whole blood specimens, such as ethylene-diamino-tetra-acetate (EDTA), heparin, citrate, fluoride, and oxalate may chelate metal ions and thus interfere with label enzymes such as alkaline phosphatase and thus generating false-positive or false-negative results.

Whole blood specimens must be used for most of the immunosuppressant drugs. For such samples, hematocrit in addition to the main four sources of interference can also affect the assay results. Thus, false-positive tacrolimus results were reported in a microparticle enzyme immunoassay (MEIA) for patients with low hematocrit values and high imprecision at tacrolimus concentration <9 ng/mL *(4)*. The enzyme-multiplied immunoassay technique (EMIT assay) for tacrolimus was not affected. When the authors divided the study specimens in three groups by hematocrit percentage (<25, 25–35, and >35%), the difference between MEIA and EMIT assays increased as hematocrit percentage decreased. Moreover, false-positive results were reported in 63% of specimens with MEIA where patients did not receive any tacrolimus but only 2.2% of specimens using EMIT. Such false-positive values in the MEIA and EMIT methods ranged up to 3.7 and 1.3 ng/mL, respectively.

Urine is the most commonly used specimen for DAU testing. Urine samples less frequently contain measurable amounts of hemoglobin and bilirubin to cause measurable interference with an assay. Turbidity interference is possible in urine, but the cause is most likely bacterial growth. Preservatives in urine, such as acetic acid, boric acid, or alkali may interfere in some urine assays.

Cerebrospinal fluid (CSF) specimens are rarely used in TDM or DAU. Most common CSF interferent is hemolysis. Interference from turbidity is also possible in such specimens.

## 3. INTERFERENCE OF VARIOUS AGENTS

In this section, common agent, bilirubin, hemoglobin, lipids etc., that may cause interference with various assays when present in high amounts will be discussed.

### *3.1. Bilirubin*

Bilirubin is derived from hemoglobin of aged or damaged red blood cells (RBC). Bilirubin does not have iron and is rather a derivative of the heme group. Some part of serum bilirubin is conjugated as glucuronides ("direct" bilirubin); the unconjugated bilirubin is also referred as indirect bilirubin. In normal adults, bilirubin concentrations in serum are from 0.3 to 1.2 mg/dL (total) and <0.2 mg/dL (conjugated) *(5)*. In different forms of jaundice, total bilirubin may increase to as high as 20 mg/dL, but the ratio of direct versus indirect bilirubin also varies. In obstructive jaundice, the increase in total bilirubin is contributed mainly by direct bilirubin. In hemolytic and neonatal jaundice, the increase is mostly in indirect bilirubin. Both fractions of bilirubin increase in hepatitis.

Elevated bilirubin causes interference, proportional to its concentration. The interference of bilirubin in TDM/DAU assays is mainly caused by bilirubin absorbance at 454 or 461 nm. Thus, it may interfere in colorimetric enzyme-linked immunosorbent assay (ELISA) that use alkaline phosphatase label and *p*-nitro phenol phosphate substrate (measured at 405 nm measured at). However, if the assay is enzymatic or colorimetric, bilirubin may interfere also by reacting chemically to the reagents *(6)*.

In one case study *(7)*, a severely jaundiced 17-year-old male patient (total bilirubin 19.8 mg/dL) with abdominal pain and increased serum transaminase results was suspected of acetaminophen overdose, although the patient himself denied using any medications containing acetaminophen within the previous week. The apparent plasma acetaminophen concentration by an enzyme method was found to be 3.4 mg/dL. In this method, acetaminophen is enzymatically (by arylacylamidase) hydrolyzed to *p*-aminophenol, which is condensed with *o*-cresol in the presence of periodate to form the blue indophenol chromophore. The method was run on the Roche Modular chemistry analyzer, with absorbance measurement at 600 nm (2-point rate) and background correction at 800 nm. To investigate false-positive results from elevated bilirubin, the authors spiked twelve hyperbilirubinemic plasma samples and bilirubin linearity calibrators with various levels of acetaminophen concentrations and measured them in the acetaminophen assay. Plasma specimens with bilirubin (range: 15.9–33.8 mg/dL), but without any acetaminophen spiking (the patients from whom these specimens were collected had no recent acetaminophen exposure), showed false-positive acetaminophen (0.6–1.8 mg/dL) results. The false-positive acetaminophen results plateaued at 2.5–3.0 mg/dL at total bilirubin concentrations of 23–35 mg/dL. The acetaminophen dilution profiles of these samples were non-linear, reaching to undetectable levels (the expected results) after fourfold dilution with the assay diluent. Because the background correction failed to correct the bilirubin interference in this assay, the authors hypothesized that bilirubin, with substantial reducing activity, might have reacted with periodate to produce a product that absorbed more strongly at 600 nm than did unreacted bilirubin. The authors also found that accuracy of spiked (5.0–15.0 mg/dL) acetaminophen results was not affected by high bilirubin (92–97%

recovery), suggesting that the nominal cross-reactivity of bilirubin with the arylacylamidase or periodate-catalyzed reaction was at a competitive disadvantage in the presence of acetaminophen.

Another example of bilirubin interference was noted in the acetaminophen assay but utilizing different assay technology *(8)*. Fifteen serum samples, none containing acetaminophen, but with total bilirubin concentrations between 2.2 and 16.7 mg/dL, when tested in an acetaminophen assay involving the reaction of the analyte with ferric-2,4,6-tripyridyl-s-triazine, demonstrated false-positive results between 0.7 and 13.6 mg/dL. This is critical, because detoxification treatment of acetaminophen is indicated when the serum levels of the drug exceed 5.0 mg/dL. The authors found that the interference could be minimized by using the protein-free ultrafiltrate because bilirubin is mostly bound to proteins, but acetaminophen is not.

Wood et al. *(9)* reported a case where increased bilirubin (22.6 mg/dL), specially consisting of high percentage of conjugated fraction (82%), caused *negative* interference in a fluorescence polarization immunoassay (FPIA) for vancomycin. In their study, the authors first compared 28 plasma samples with total bilirubin <5.9 mg/dL, between two different Abbott's vancomycin assays, by using a TDx analyzer and AxSYM analyzer. Vancomycin, a glycopeptide antibiotic used in treating serious infections, is toxic with plasma concentration >20 μg/mL (trough) and >80 μg/mL (peak). The method used in the TDx analyzer is a homogeneous FPIA, using a polyclonal sheep antibody and fluorescein-labeled antigen. The assay on the AxSYM analyzer also uses the same assay principle but utilizes a different, monoclonal mouse antibody. The vancomycin results from these 28 samples, ranging from 2.0 to 34.5 μg/mL, were in close agreement between the assays performed using two different analyzers (correlation coefficient $r^2 = 0.996$). When the authors analyzed plasma specimens containing abnormal bilirubin, they observed discordant results between the two vancomycin assays. For example, in specimen containing 22.6 mg/dL of total bilirubin, the vancomycin concentration observed by using the TDx analyzer was 2.6 μg/mL but the corresponding value obtained by the AxSYM analyzer was 8.0 μg/mL *(9)*.

Suspecting the elevated bilirubin as the source of discordance between the two vancomycin methods, the authors spiked vancomycin in 10 plasma specimens from jaundiced patients (total bilirubin ranging from 9.5 to 28.2 mg/dL; direct bilirubin ranging from <1.0 to 16.0 mg/dL) not receiving vancomycin and measured the samples using both vancomycin assays. By the Wilcoxon rank-sum test, they found vancomycin recoveries using the assay on the TDx analyzer significantly were lower than with the recoveries using the assay on the AxSYM ($p < 0.001$) analyzer (mean TDx and AxSYM recoveries were 79.7 ± 13.1% and 102.2 ± 6.4%, respectively). The lower recovery of the assay using the TDx analyzer was inversely related to the direct (conjugated) bilirubin concentration in the specimens ($r^2 = 0.54$, $p < 0.005$). No such correlation was found between the recovery and the total bilirubin. The negative interference in the assay using the TDx analyzer was probably caused by direct bilirubin generating falsely increased fluorescence blanks. The authors noted that for the assay method for the TDx analyzer, the package-insert reported interference of <5% for bilirubin concentrations of 15 mg/dL. This suggested a possibility that the package insert data were generated using unconjugated bilirubin, which as the authors' data demonstrated, does not interfere with the assay. The authors concluded that the assay on the AxSYM

analyzer somehow was not affected by high direct bilirubin (because of either the antibody difference, or method difference), whereas the assay on the TDx analyzer demonstrated false-negative results for such samples *(9)*.

### *3.2. Hemoglobin and Blood Substitutes*

Hemoglobin is mainly released from hemolysis of RBC. Hemolysis can occur in vivo, during venipuncture and blood collection or during processing of the sample. Hemoglobin interference depends on its concentration in the sample. Serum appears hemolyzed when the hemoglobin concentration exceeds 20 mg/dL *(10)*. However, icteric serum may contain higher concentration of hemoglobin before hemolysis can be noticed. Hemoglobin interference is caused not only by the spectrophotometric properties of hemoglobin but also by its participation in chemical reaction with sample or reactant components as well *(11)*. The absorbance maxima of the heme moiety in hemoglobin are at 540- to 580-nm wavelengths. However, hemoglobin begins to absorb around 340 nm, absorbance increasing at 400–430 nm as well. The iron atom in the center of the heme group is the source of such absorbances. Of the many variants of hemoglobin, methemoglobin (where the iron is in $3^+$ oxidation state) and cyanmethemoglobin (cyanide complex of hemoglobin) also absorb at 500 and 480 nm, respectively. Methods that use the absorbance properties of NADH or NADH (340 nm) may thus be affected by hemolysis. When hemoglobin is oxidized to methemoglobin, the absorbance at 340 nm decreases.

In renal failure as well as damage to kidney or urinary pathways, the heme or its derivatives may be present in urine, generating hemolytic interference similar to that of serum. Urine may also have interference from myoglobin, the oxygen-binding protein in striated micelles, also containing the heme group. Whereas hemoglobin is a tetramer of the heme and globin complex, myoglobin is a monomer and, consequently, is smaller than hemoglobin by three-fourths. Thus, when there is injury to skeletal or cardiac muscle, myoglobin may be released and then excreted in urine (myoglobinuria). Under such condition, urine samples have a color similar to that of a cola drink or black coffee and show interference similar to that of hemolysis in serum.

If the type of blood for a patient is in short supply, many hospitals now use blood substitutes, which are mostly derivatized or polymerized hemoglobin. The blood substitutes interfere in many analyses in the same way as hemoglobin. Thus, it was demonstrated that Hemolink®, an **O**-raffinose cross-linked hemoglobin blood substitute, showed positive or negative interference in many routine chemistry and immunochemistry assays *(12)*. Another type of blood substitute is polyfluorocarbon, which also has been reported to interfere with immunoassays.

### *3.3. Lipids*

All the lipids in plasma exist as complexed with proteins. Lipoproteins, consisting of various proportions of lipids, range from 10 to 1000 nm in size (the higher the percentage of the lipid, lower is the density of the resulting lipoprotein, and larger is the particle size). Chylomicrons (diameter 70–1000 nm, density $<0.95$ g/mL) are present in plasma after a person ingests a fatty meal. These particles originate in the intestinal epithelial cells and consist mostly of lipids. Chylomicrons are absorbed by

the adipose tissue and liver. Liver secretes lipoprotein particles called very-low-density lipoproteins (VLDL, density <1.006 g/mL), low-density lipoproteins (LDL, density = 1.006–1.063 g/mL), and high-density lipoproteins (HDL, density = 1.063–1.21 g/mL) containing decreasing amounts of lipids in that order. The lipoprotein particles with high lipid contents are micellar and are the main source of assay interference. Unlike bilirubin and hemoglobin, lipids normally do not participate in chemical reactions and mostly cause interference in assays by their turbidity. The micellar particles scatter light, and the amount of light scattered is higher at lower wavelength. Because scattered light does not follow Lambert-Beer law of absorbance, scattering normally reduces absorbance producing false results (positive or negative, depending on the reaction principle) *(13)*. Lipemic interference is most pronounced with spectrophotometric assays, less important with fluorimetric methods, and rarely interferes with chemiluminescent methods. Thus, assays that use turbidimetry for signal are the ones most affected by lipid interference *(14)*. Lipemia may also interfere with assays for fat-soluble analytes, such as steroids and their derivatives. In such cases, interference arises from solvent partitioning and solute exclusion of the analyte between the lipid and the aqueous phases.

Like bilirubin and hemolysis, package inserts do report the extent of lipid interference in a commercial assay. Lipids, however, present a special problem because of lack of readily available standardized materials. Most manufacturers use IntraLipid, a synthetically produced emulsion containing soybean oil and egg phospholipids, for intravenous administration, to spike specimens to simulate lipemic samples. However, samples with IntraLipid do not perfectly mimic lipemic samples *(15)*. Sometimes native lipemic samples have falsely low results in certain assays, but IntraLipid-spiked samples containing same triglyceride concentration do not. This can be understood from the fact that native plasma lipids are very heterogeneous with a wide variety of micellar particle size distribution. Size, charge, and shapes of the particles influence their light-scattering capabilities. Among the plasma lipoproteins, chylomicrons and VLDL particles only scatter light. VLDL exists in three size classes: small (27–35 nm), intermediate (35–60 nm), and large (60–200 nm). Only the latter two sizes of VLDL scatter light. Chylomicron particles vary greatly among individuals, and even in the same individual, depending upon the time that the sample is collected after the meal *(16)*. Thus, even though Kazmierczak and Catrou *(17)* argue that interference studies need to be done with specimens from patients with hyper-lipidemia, or hyperbilirubinemia, the subject to subject variation makes it impossible to guarantee that an assay will not be subjected to interference from these interferents. Therefore, studies performed using IntraLipid-spiked lipemic specimens may not necessarily represent lipemic samples from a patient. These results, at best, may be taken as a guideline, rather than final. Assay results from lipemic samples must be interpreted with caution.

### *3.4. Proteins and Paraproteins*

Interference from proteins is possible both in hypo- and in hyper-proteinemia (normal serum protein concentration is about 6–8 g/dL, and in plasma fibrinogen adds to total protein by about 0.2–0.4 g/dL). Such interference is most apparent where the specimen is used in assay without pretreatment, e.g., in immunoassays. Although the

calibrator matrix for an immunoassay is often pooled serum or plasma, or protein-containing buffered solutions, specimens vary in plasma protein concentration from subject to subject and even in a same subject over time or during the day. Plasma protein concentrations change as people age or in various physiological states, such as pregnancy or in various disease states. Often, such kinds of effects are included in the vaguely defined "matrix effect" in an immunoassay. Ezan et al. *(18)* attempted to estimate such effect on TDM assays in plasma samples. The authors spiked a seven-amino-acid peptide experimental drug for the treatment of chronic diarrhea in pooled plasma to generate the calibration curve in their experimental immunoassay for the drug. When they compared the recovery of the spiked drug at two different concentrations in plasma from 25 different subjects, the recoveries ranged from 70 to 152% at the low concentration spike and from 79 to 114% at the high concentration spike. The authors suggested that the differences arose from differences in plasma proteins.

Plasma specimens, which have been refrigerated for prolonged periods or which have undergone freeze-thaw cycles, demonstrate another facet of protein interference. Fibrins precipitate under such conditions. These fibrin clots may block auto-analyzer sample probes, generating incorrect results. Such samples should be centrifuged to remove any precipitates before assay. However, most modern auto-analyzers include clot-detection and alert systems to flag results suspected to be subject of clot interference.

Manufacturers of commercial assays normally report the effects of hypo- (up to 3 g/dL) and hyper-proteinemia (up to 12.5 g/dL) in the package inserts. Hyper-proteinemia may increase the viscosity of the specimen, thus interfering with accurate sampling for the assay.

Paraproteins circulate because of multiple myeloma or similar diseases. The concentrations of a specific class and idiotype of immunoglobulins (Ig) are greatly increased. There have been many literature examples of paraproteins interfering with all kinds of clinical chemistry assays, including immunoassays. In most cases, the mechanism of paraprotein interference in colorimetric or turbidimetric assays is their precipitation when the specimen is treated with the first reagent in acidic or alkaline reaction conditions. Such condition causes turbidity. This occurs especially in methods using sample blanking, because turbidity causes larger blanking, mostly generating false-negative results.

Hullin reported a case study, where a 77-year-old man whose plasma samples had 500 mg/dL of paraprotein (IgMκ monoclonal component) ingested 100 tablets of acetaminophen before 18 h *(19)*. Serum acetaminophen concentration, using a commercial enzyme assay kit, was 5.3 mg/dL (toxic >5–20 mg/dL, depending on ingestion time before sampling). But the sample-blanking absorbance (absorbance measured after the addition of sample to the enzyme reagent, but before adding the chromogen) was very high (0.145, compared with <0.01 for normal sera). Suspecting this acetaminophen result, the authors assayed the sample by a high-performance liquid chromatography (HPLC) method for acetaminophen, the result being 8.6 mg/dL. The sample showed non-linear dilution in the enzymatic assay. Presence of paraprotein in the sample was indicated by the formation of flocculent precipitate when a drop of serum was added to water (the Sia water test) *(20)*.

## 4. FALSE RESULTS CAUSED BY SYSTEMS ISSUES

Today most of TDM and DAU assays are performed on automated systems, where the system pipette probe automatically picks up the required volume of a specimen, dispenses it into a reaction chamber (cuvette), adds reagents, separates and washes the bound label (if required), measures the signal, and converts it into reported results. Unless the system uses disposable pipette tips, the pipette probe is washed between successive specimens. On the contrary, if the system can do analyses in random access, the reagent pipette is also washed between two different reagent pipetting and dispensation procedures. The reaction cuvettes, if not disposable, are also washed between successive tests. In most cases, such washing is highly effective to prevent cross contamination of specimens or reagents. Furthermore, most system manufacturers test their system for such "carryover" and, if necessary, add extra washes or special washing solutions to prevent carryover. However, one must consider the possibility of specimen or reagent carryover as a potential source of discordant results on automated random access systems.

## 5. HOW TO DETECT AND CORRECT INTERFERENCES

The best way to detect false-positive results caused by the interferents described in this chapter is to observe the appearance of the discordant sample. At the interferent concentrations that may cause significant interference, hemolysis, icterus (caused by increased bilirubin), and turbidity (caused by lipids) are easily detectable visually. However, in practice the collection tubes have so many labels and barcode stickers outside, it is often difficult to inspect the specimen within. Many automated analyzers can measure the degree of hemolysis, icterus and turbidity in the sample, and post alert in the results. The degree of interference, if any, is noted by an "index." The index for lipids is generated mostly by spiking specimens with IntraLipid. For example, Table 3 presents the representative system flag indices corresponding to the specimen's approximate interferent (hemoglobin, bilirubin, and triglyceride) concentrations in an auto-analyzer.

There have been several reports about the accuracy of such indexes. Sonntag and Glick *(10)* found that the Hitachi serum index for hemolysis correlated well with the hemoglobin concentration. Dahlin found a linear relationship between serum indices and interferent concentrations: hemoglobin, $R = 0.9976$ (range 0.0–9.9 g/dL), and bilirubin, $R = 0.9851$ (range $<$1.0–10.6 mg/dL) *(21)*. However, because of the heterogenic nature of serum lipids (as described earlier), lipid indexes often do not correlate with the actual triglyceride levels of the specimen, which is often measured by Fossati's method of forming Trinder chromophore from the glycerol that is generated by triglyceride hydrolysis *(22)*. Thus, the lipemic index of an auto-analyzer, which is measured by blank absorbance at 700 nm (scores 1–6 to correspond to IntraLipid concentrations of 0–3000 mg/dL) correlated poorly with the triglyceride concentrations in 1115 patients *(23)*.

Sample blanking is a very effective way to minimize interferences from bilirubin, lipid, and hemoglobin in homogeneous assays. In this method, applicable ideally for two reagent methods, the sample is treated with the first reagent, followed by signal measurement—the "sample blank." Reagent 2 is then added and final signal reading is

**Table 3**
**System Flags Commonly Used for Bilirubin, Hemoglobin, and Lipid Interference in Autoanalyzers**

| | | *Interferent Concentration* | |
|---|---|---|---|
| *Interferent* | *System Flag Index* | *Common Unit (mg/dL)* | *SI Unit* |
| Bilirubin | + | 1.7 | 29 μmol/L |
| | ++ | 6.6 | 113 μmol/L |
| | +++ | 16.0 | 274 μmol/L |
| | ++++ | ≥30 | 513 μmol/L |
| Hemoglobin | + | 50 | 0.5 g/L |
| | ++ | 150 | 1.5 g/L |
| | +++ | 250 | 2.5 g/L |
| | ++++ | ≥500 | 5.0 g/L |
| Lipids (triglyceride, as triolein) | + | 24 | 0.27 mmol/L |
| | ++ | 65 | 0.73 mmol/L |
| | +++ | 280 | 3.16 mmol/L |
| | ++++ | ≥650 | 7.35 mmol/L |

taken. The difference between the two readings is the actual signal arising from analyte reaction and is converted to equivalent analyte concentration. Thus, when sample blanking was introduced to the turbidimetric determinations of immunoglobulin A, G, or M, there was significant improvement in interferences observed *(24,25)*.

Modification of reagents such that assay signal can be generated at wavelengths farther away from where the interferents absorb (or fluoresce) is another way to reduce these interferences. An example of this approach is reformulations of triglyceride and uric acid reagents on an auto-analyzer, which moved the absorbance measurement wavelength from 520/600 to 660/800 nm (primary/secondary wavelengths) to reduce interferences *(26)*.

## 6. REMOVAL OF INTERFERING SUBSTANCES

If the methods described above to correct the interference do not work or cannot be applied, then interference can be resolved by removing the interferents and re-assaying the treated specimen. Because bilirubin in plasma is mostly bound to plasma proteins, wherever possible, free drug measurement or assay with protein-free ultrafiltrate (e.g., digoxin) can avoid bilirubin interference. An example is the removal of bilirubin interference in the enzymatic acetaminophen assay as described earlier *(8)*. Some commercial assays (TDx Digoxin from Abbott Diagnostics, Abbott Park, IL, USA) routinely use sulfo-salicylic acid precipitation of proteins to make protein-free specimens, which are then assayed in the analyzer.

Hemoglobin and blood substitutes, however, are soluble and cannot be removed this way. A report in 1997 described a synthetic solid phase anionic polyelectrolyte, Hemoglobind®, which, when used with a hemolytic sample could effectively remove most hemoglobin or hemoglobin derivatives *(27)*. The authors reported the removal of hemoglobin of 91% at 1000 mg/dL, 86% at 1500 mg/dL, and 84% at 2000 mg/dL of

hemoglobin. In this simple protocol, 250 μL of the solid agent is added to the 500 μL of the hemolyzed specimen, centrifuged at 3000 × g for 10 min, and the supernatant is used for the assay. However, the method could remove only up to 0.6 g/dL Hemolink.

Because the interfering triglyceride particles, chylomicron and VLDL, have lower density than the serum, they can be removed by ultra-centrifugation (they remain in the supernatant and the remaining serum in bottom layers).

## 7. CONCLUSION

Increased levels of bilirubin, hemoglobin, and lipids interfere in assays through their spectrophotometric, fluorimetric, or chemiluminescence properties, or through side reactions. Protein interference can occur in both hypo- or hyper-proteinemia because of alteration in matrix composition. Such interference occurs mostly in immunoassays. In addition to watching for interference from these four sources, one must consider the potential of sample or reagent carryover in probes and cuvettes in the auto-analyzers. Assay developers undertake steps such as sample blanking to minimize such interferences. Auto-analyzers may include measurements of icterus, hemolysis, and turbidity to flag results that may be affected by such interferents. Sample blanking and robust assay design can be used to minimize these interferences, including matrix effect arising from protein and other non-specific constituent in the specimen. When suspected, the interferents may be removed from the specimen by specific agents, ultrafiltration, or centrifugation, before reanalysis. Alternatively, the specimen may be analyzed by a different method that is known to be free from such interference.

## REFERENCES

1. Fonseca-Wolheim FD. Hemoglobin interference in the bichromatic spectrophotometry of NAD(P)H at 340/380 nm. *Eur J Clin Chem Clin Biochem* 1993;31:595–601.
2. NCCLS Recommendation (EP7-P), Interference testing in Clinical Chemistry, 1986 (Vol. 6, No. 13), pp 259–371.
3. Miller JM, Valdes R Jr. Methods for calculating crossreactivity in immunoassays. *J Clin Immunoassay* 1992;15:97–101.
4. Armedariz Y, Garcia S, Lopez R, et al. Hematocrit influences immunoassay performance for the measurement of tacrolimus in whole blood. *Ther Drug Monit* 2005;27:766–769.
5. Tietz NW. *Clinical Guide to Laboratory Tests*. 3rd Ed. Philadelphia, PA: WB Saunders Company; 1995:88–91.
6. Perlstein MT, Thibert RJ, Watkins RJ, Zak B. Spectrophotometric study of bilirubin and hemoglobin interactions in several hydrogen peroxide generating procedures. *Clin Chem* 1977;23:1133 [Abstract].
7. Bertholf RL, Johannsen LM, Bazooband A, Mansouri V. False-positive acetaminophen results in a hyperbilirubinemic patient. *Clin Chem* 2003;49:695–698.
8. Kellmeyer K, Yates C, Parker S, Hilligoss D. Bilirubin interference with kit determination of acetaminophen. *Clin Chem* 1982;28:554–555.
9. Wood FL, Earl JW, Nath C, Coakley JC. Falsely low vancomycin results using the Abbott TDx. *Ann Clin Biochem* 2000;37:411–413.
10. Sonntag O, Glick MR. Serum-index und interferogram-ein neuer weg zur prufung und darstellung von interferengen durch serumchromogene. *Lab Med* 1989;13:77–82.
11. Wenk RE. Mechanism of interference by hemolysis in immunoassays and requirements for sample quality. *Clin Chem* 1998;44:2554.
12. Chance JJ, Norris EJ, Kroll MH. Mechanism of interference of a polymerized hemoglobin blood substitute in an alkaline phosphatase method. *Clin Chem* 2000;46:1331–1337.

13. Kroll MH. Evaluating interference caused by lipemia. *Clin Chem* 2004;50:1968–1969.
14. Weber TH, Kaoyho KI, Tanner P. Endogenous interference in immunoassays in clinical chemistry. *Scand J Clin Lab Invest Suppl* 1990;201:77–82.
15. Bornhorst JA, Roberts RF, Roberts WL. Assay-specific differences in lipemic interference in native and Intralipid-supplemented samples. *Clin Chem* 2004;50:2197–201.
16. Park Y, Grellner, Harris WS, Miles JM. A new method for the study of chylomicron kinetics in vivo. *Am J Physiol Endocinol Metab* 2000;279:E1258–263.
17. Kazmierczak SC, Catrou PG. Analytical interference, more than just a laboratory problem. *Am J Clin Pathol* 2000;113:9–11.
18. Ezan E, Emmanuel A, Valente D, Grognet J-M. Effect of Variability of plasma interferentes on the accuracy of drug immunoassays. *Ther Drug Monit* 1997;19:212–218.
19. Hullin DA. An IgM paraprotein causing a falsely low result in an enzymatic assay for acetaminophen. *Clin Chem* 1999;45:155–156.
20. Laurell CB, Waldenstrom J. Sera with exceptional appearance and the euglobulin reaction as screen test. *Acta Med Scand Suppl* 1961;367:97–100.
21. Dahlin J, Omar A, Ng HT, et al. An evaluation of automated serum indexing on the Roche Modular Serum Work Area. *Clin Chem* 2006;52:A104 [Abstract].
22. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 1982;28:2077.
23. Viljoen A, Cockrill G, Martin SC. The ability of the lipemic index to predict assay interference. *Clin Chem* 2006:52: A8 [Abstract].
24. ADVIA 1650 IgA, IgG, and IgM Method Sheets.
25. Dai J, Higgins T, Aistrope K, Peters C, Levine R, Datta P. Evaluation of the Bayer new immunoglobulin methods on the ADVIA® 1650 chemistry auto-analyzer. *Clin Chem* 2006:52:A154 [Abstract].
26. Murphy L, Leamy A, O'Sullivan A, et al. Evaluation of new Olympus low interference triglyceride and uric acid assays on the Olympus AU400™, AU640/600™, and AU2700/5400™analyzers. *Clin Chem* 2006:52:A160 [Abstract].
27. Balion CM, Champagne PA, Ali ACY. Evaluation of hemoglobind for removal of O-raffinose cross-linked hemoglobin (Hemolink) from serum. *Clin Chem* 1997;43:1796–1798.

# 6 Digoxin

## *So Many Interferences and How to Eliminate Them*

*Amitava Dasgupta, PhD*

***Contents***



**Summary**

Digoxin is a cardioactive drug with a narrow therapeutic range. Both endogenous and exogenous compounds interfere with the determination of digoxin in serum by using immunoassays. Endogenous compounds are termed as digoxin-like immunoreactive substances (DLIS) and elevated DLIS concentrations are encountered in volume-expanded patients such as those with uremia, essential hypertension, liver disease, and preeclampsia. DLIS cross-reacts with anti-digoxin antibodies and may falsely elevate (positive interference) or falsely lower (negative interference) serum digoxin concentrations, thus causing problems in interpreting results. Exogenous compounds that interfere with digoxin assays are various Chinese medicines such as Chan Su, Lu-Shen Wan, and oleander-containing herbal preparations. Therapy with spironolactone, canrenone, or potassium canrenoate may also interfere with digoxin immunoassays. However, endogenous DLIS as well as components of various Chinese medicines are strongly protein bound whereas digoxin is weakly protein bound (25%). Taking advantage of the differences in protein

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binding, interference of both endogenous DLIS and Chinese medicines in serum digoxin measurement can be mostly eliminated by monitoring digoxin concentrations in the protein-free ultrafiltrates. Monitoring free digoxin also eliminates spironolactone interference (if used in moderate dosage) in serum digoxin measurement using immunoassay.

**Key Words:** Digoxin; DLIS; Chinese medicines; spironolactone; free digoxin.

## 1. THERAPEUTIC DRUG MONITORING OF DIGOXIN

Digitalis glycosides have been in use in medicine over 200 years. The main pharmacological effects include a dose-dependent increase in myocardial contractility and a negative chronotropic action. Digitalis also increases the refractory period and decreases impulse velocity in certain myocardial tissue [such as the atrioventricular (AV) node]. The electrophysiological properties of digitalis are reflected in the ECG by shortening of the QT interval. Both digoxin and digitoxin have narrow therapeutic index; thus, therapeutic drug monitoring is essential for achieving optimal efficacy as well as to avoid toxicity. The therapeutic range of digoxin usually is 0.8–2.0 ng/mL, but there is a substantial overlap between therapeutic and toxic concentrations. Moreover, mild-to-moderate renal failure may also significantly increase the risk with digoxin therapy *(1)*. Digoxin toxicity may occur with a lower digoxin level, if hypokalemia, hypomagnesemia, or hypothyroidism coexists. Likewise, the concomitant use of drugs such as quinidine, verapamil, spironolactone, flecainide, and amiodarone can increase serum digoxin levels and increase the risk of digoxin toxicity. A recent clinical trial indicated that a beneficial effect of digoxin was observed at serum concentrations from 0.5 to 0.9 ng/mL whereas serum concentrations at or over 1.2 ng/mL appeared harmful *(2)*.

Although digoxin concentration in serum or plasma can be detected accurately by sophisticated analytical techniques such as high-performance liquid chromatography (HPLC) combined with tandem mass spectrometry, in clinical laboratories digoxin immunoassays are the preferred method because of automation and rapid turnaround time. Immunoassays are subjected to interference by endogenous digoxin-like immunoreactive substances (DLIS) because of structural similarity with digoxin. Moreover, several Chinese medicines such as Chan Su, Lu-Shen-Wan (LSW), and oleander-containing herbs may interfere with serum digoxin measurements by immunoassays because of structural similarity of components of these alternative medicines with digoxin. Spironolactone and anti-digoxin antibodies used in treating patients overdosed with digoxin also interfere with digoxin measurement using immunoassays *(3)*.

## 2. DISCOVERY OF ENDOGENOUS DLIS

Digoxin is a cardiac glycoside, and after the discovery of endorphins, the endogenous equivalent of opiates, there was a hypothesis for the presence of an endogenous equivalent of cardiac glycosides. It was further hypothesized that anti-digoxin antibody may be able to detect the presence of DLIS in body fluids. Gruber et al. *(4)* first demonstrated the presence of endogenous DLIS in 1980, in volume-expanded dogs. Then Craver and Valdes reported an unexpected increase in serum digoxin concentration in a renal failure patient who was taking digoxin. Apparent serum digoxin level was still

present after discontinuation of digoxin therapy *(5)*. Balzan et al. *(6)* also confirmed the presence of DLIS in human plasma and urine. DLIS were found in various human body fluids and tissues including cord blood, placenta, amniotic fluid, bile meconium, cerebrospinal fluid, and saliva *(7,8)*. DLIS cross-react with anti-digoxin antibodies as well as inhibit Na, K-ATPase.

### *2.1. Detection of DLIS in Human Body Fluids*

DLIS can be detected in serum and other body fluids by using commercial immunoassays for digoxin, taking advantage of the cross-reactivity of DLIS with anti-digoxin antibody. Apparent digoxin concentrations as detected by radioimmunoassay (RIA) digoxin assays had been reported in the 1980s by several investigators in patients not receiving digoxin *(9,10)*. Some of those RIA digoxin assays were later discontinued because of high interference with DLIS. Early reports also indicated cross-reactivity of DLIS with the fluorescence polarization immunoassay (FPIA) marketed by the Abbott Laboratories (Abbott Park, IL) *(11)*. Many investigators used commercially available digoxin assays for detecting DLIS in body fluid. However, other approaches have also been reported. Panesar *(12)* used bufalin as an antigen and developed polyclonal antisera for detecting DLIS. Lin et al. developed a polyclonal antibody-based ouabain enzyme immunoassay for detecting DLIS. The authors also developed a Fab fragment of the anti-digoxin antibody-based enzyme immunoassay for this purpose. The authors concluded that a polyclonal antibody-based ouabain assay was more efficient to detect DLIS in human blood *(13)*. More specific high-performance liquid chromatographic techniques using reverse phase columns had also been used for detecting DLIS in biological fluid *(14,15)*. However, these techniques are time consuming and technically more difficult than automated immunoassays.

### *2.2. Criteria for DLIS*

DLIS can be divided into two groups. One class of DLIS interferes only with digoxin immunoassays because of their cross-reactivity with anti-digoxin antibody, and the other class of compounds inhibits or binds with Na, K-ATPase. These compounds may also cross-react with anti-digoxin antibody. Because of the ability of DLIS to inhibit Na, K-ATPase, it was hypothesized that DLIS is a natriuretic hormone. Several studies have been reported where investigators took advantage of digoxin-like immunoreactivity and Na, K-ATPase-binding ability of DLIS to purify these compounds from intact cells or isolated receptors. These investigators also studied the biochemical and or physiochemical parameters of isolated DLIS *(16–18)*.

### *2.3. DLIS Concentrations: Healthy Individuals versus Disease*

#### 2.3.1. Healthy Individuals

DLIS concentration in sera of subjects not taking any digoxin depends on the particular immunoassay used. The FPIA marketed by the Abbott Laboratories has significant cross-reactivity with DLIS. However, even with the FPIA, the concentrations of DLIS in healthy individuals are usually below the detection limit of the instrument ($<0.20$ ng/mL) in most cases. One of the factors that may contribute to

significant interference of the FPIA with DLIS is the use of a rabbit polyclonal antibody against digoxin in the assay design of FPIA. Newer digoxin immunoassays, which utilize a more specific monoclonal antibody against digoxin, are subjected to significantly less interference with DLIS.

### 2.3.2. Volume Expansion

Volume expansion is a major cause of elevated DLIS in blood. Elevated concentrations of DLIS have been reported in uremia, essential hypertension, hypertension of water volume expansion, liver disease, preeclampsia, liver and kidney transplant, congestive heart failure, premature babies, and other conditions *(19–24)*.

### 2.3.3. Critically Ill Patients

Howarth et al. reported elevated DLIS in plasma of intensive care unit patients. Although some patients showed either hepatic or renal dysfunction, another 42 patients who showed elevated DLIS had neither hepatic nor renal dysfunction. The authors used an FPIA for measuring DLIS. The DLIS concentrations ranged from 0.0 to 1.69 nmol/L in 16 patients with coexisting hepatic and renal dysfunction, whereas 38 patients with hepatic dysfunction but normal renal function showed a range of DLIS concentration of 0.0–0.77 nmol/L. Four patients with renal dysfunction only had DLIS concentrations between 0.0 and 0.34 nmol/L, and the remaining 42 patients had DLIS concentrations ranging from 0.0 to 36 nmol/L *(25)*.

Berendes et al. reported that different types of endogenous glycosides are elevated in significant proportions in critically ill patients. The authors used FPIA for digoxin and digitoxin for measuring DLIS in patients not treated with cardiac glycosides. Of the 401 critically ill patients, 343 (85.5%) did not show any measurable concentration of DLIS but the remaining 58 patients (14.5%) had measurable DLIS. Of these 58 patients, 18 patients showed significant digoxin levels ($0.54 \pm 0.36$ ng/mL) and 34 patients showed measurable digitoxin levels ($2.28 \pm 1.7$ ng/mL). Interestingly, mean endogenous ouabain concentrations were ninefold increased in DLIS-positive patients and only threefold increases were observed in DLIS-negative patients. The mortality of DLIS-positive patients was 12% whereas mortality in DLIS-negative patients was only 3.2% *(26)*.

### 2.3.4. Pediatric Population

Concentration of DLIS may be significantly increased in cord blood as well as in sera of neonates. Chicella et al. measured DLIS concentrations in 80 pediatric patients never exposed to digoxin by using both FPIA and microparticle enzyme immunoassay (MEIA). Both digoxin assays are marketed by the Abbott Laboratories. The authors reported that 48% of the specimens showed measurable DLIS using the MEIA and 79% of the specimens showed measurable DLIS using FPIA, whereas values obtained by the FPIA were higher than the corresponding values obtained by the MEIA. The highest apparent digoxin concentration was 0.38 ng/mL, and a poor correlation was noted between patient age, serum creatinine, total bilirubin, and DLIS concentration *(27)*. Ijiri et al. reported that although DLIS concentrations were elevated in neonates with jaundice ($0.58 \pm 0.13$ ng/mL before phototherapy

and 0.33 ± 0.06 ng/mL after phototherapy) compared with that in neonates without jaundice (0.34 ± 0.04 ng/mL), a fluorescent compound related to bilirubin increased the blank intensity measurement in the FPIA. This compound was not related to DLIS *(28)*.

Concentrations of DLIS in cord blood may be significantly elevated compared with that in maternal blood. In one study, the mean DLIS concentration in umbilical cord plasma was 0.55 ng/mL whereas the average DLIS concentration in maternal plasma was 0.23 ng/mL (measured using FPIA). Moreover, dehydroepiandrosterone sulfate in maternal plasma and progesterone in maternal and umbilical cord plasma may be measured as digoxin by the FPIA *(29)*.

### *2.4. DLIS Concentrations and Therapeutic Range of Digoxin*

The issue of interference of DLIS in serum digoxin measurement depends on the choice of immunoassay. If the FPIA (Abbott Laboratories) assay is used for the determination of serum digoxin concentrations, the interference of DLIS may be significant in volume-expanded patients. Miller et al. *(30)* reported a DLIS concentration of 0.88 ng/mL in a patient who never took digoxin. A DLIS concentration of 1.15 ng/mL in another patient with liver failure had also been reported *(31)*. Logoglu et al. used an RIA (double antibody RIA) for detecting DLIS in sera of patients with normal and preeclamptic pregnancies. The mean DLIS concentration in the normotensive group ($n = 14$) was 0.29 ng/mL, whereas the mean was 0.31 ng/mL in the preeclamptic group ($n = 17$). The authors concluded that there was no statistical difference between DLIS concentrations in these two groups *(32)*. Doolittle et al. *(33)* described a case where a residual level of 1.0 ng/mL of digoxin was observed for 11 days in a patient despite no digoxin being administered. Garbagnati measured DLIS concentrations in children (age: 5–16 years) using a FPIA digoxin assay. The authors observed measurable DLIS concentrations in 50% of the children (range 0.03–0.35 ng/mL) *(34)*.

Lusic et al. reported comparable plasma and cerebrospinal fluid levels (CSF) of DLIS in 40 patients diagnosed with aneurysmal subarachnoid hemorrhage. On the first day, DLIS concentrations were detected in sera of 34 patients (range: 0–0.86 ng/mL,) and CSF of 32 patients (range: 0–1.01 ng/mL). On the seventh day post hemorrhage, DLIS were present in plasma of 37 patients (range: 0–1.52 ng/mL) and in CSF of 38 patients (range: 0–1.67 ng/mL). The authors used an FPIA (Digoxin II, Abbott Laboratories) for their study *(35)*.

### *2.5. Decreased DLIS in Bipolar Disease*

Although most reports in literature described increased DLIS concentration, Grider et al. reported decreased concentrations of DLIS in patients with manic bipolar disorder compared with that in normal controls. The authors used an RIA for measuring DLIS concentrations. The mean DLIS concentration in the control group was 296.6 pg/mL whereas the mean DLIS concentration in the bipolar disorder group was 143.6 pg/mL *(36)*. Conditions that cause abnormality or change in DLIS concentrations are summarized in Table 1.

**Table 1**
**Conditions that Cause Abnormality or Change in Digoxin-Like Immunoreactive Substances (DLIS)**

| *Disease* | *Method for Measurement* |
|---|---|
| Essential hypertension ↑ | RIA, Ouabain binding |
| Hypertension/volume expansion ↑ | Rubidium uptake |
| Uremic syndrome ↑ | RIA, FPIA, ACA^ |
| Liver disease, liver failure ↑ | EMIT, FPIA |
| Transplant recipients ↑ | FPIA, RIA, ACA |
| Premature babies/new born ↑ | FPIA |
| Pregnancy and preeclampsia ↑ | RIA, FPIA, Na, K-ATP inhibition |
| Congestive heart failure ↑ | FPIA |
| Hypertrophic cardiomyopathy ↑ | FPIA |
| Myocardial infarction ↑ | FIA, mass spectrometry |
| Intensive care unit patients ↑ | FPIA |
| Diabetes ↑ | FPIA |
| Mucocutaneous lymph node syndrome ↑ | RIA |
| Aneurysmal subarachnoid hemorrhage ↑ | FPIA |
| Postmortem blood ↑ | FPIA |
| Bipolar disorder (decreased DLIS) ↓ | RIA |

RIA, radioimmunoassay; FPIA, fluorescence polarization immunoassay; ACA, affinity-mediated immunoassay; FIA, fluoroimmunoassay.

## 3. POSITIVE AND NEGATIVE INTERFERENCE OF DLIS IN SERUM DIGOXIN MEASUREMENT: IMPACT ON TDM OF DIGOXIN

Positive interference of DLIS in the FPIA digoxin assay (Abbott Laboratories) is very well documented in literature. Many investigators used this assay to measure DLIS levels in various patients not receiving digoxin. Avendano et al. reported 89% false-positive digoxin values in blood drawn from peripheral veins of neonates and a striking 100% false-positive digoxin levels in the corresponding cord blood when FPIA (Digoxin II) was used for the measurement. The authors also observed 60% false-positive values in patients with severe hepatic disease and concluded that digoxin levels must be interpreted very carefully in these patients *(37)*. Frisolone et al. studied apparent serum digoxin levels in patients with liver disease using FPIA (Digoxin II, TDx analyzer), RIA (Gamma goat I 125), and a fluorometric assay (Stratus, Dade). These patients did not receive digoxin or spironolactone. The authors observed measurable apparent digoxin concentrations in 57% of the patients using the RIA (range: 0.2–0.6 ng/mL), 55% in patients using the FPIA (range: 0.2–1.56 ng/mL), and only 28% patients with the fluorometric assay (range: 0.2–0.38 ng/mL) and concluded that FPIA and RIA digoxin assays were more susceptible to DLIS interference *(10)*. Datta et al. studied potential interference of DLIS with different digoxin assays and concluded that chemiluminescent assay (CLIA on ACS:180 analyzer, Bayer Diagnostics) and the fluoroimmunoassay (Stratus, Baxter Corporation) showed almost no interference from DLIS compared with an RIA (Magic, Ciba-Corning) *(38)*.

Miller et al. studied analytical performance of the CLIA digoxin assay on the ACS:180 analyzer (Ciba-Corning currently marketed by Bayer Diagnostics) by comparing this assay with the FPIA (Digoxin II), Stratus II digoxin assay, and an RIA digoxin assay (Magic RIA, Ciba-Corning). The authors detected no DLIS in sera using CLIA, but measurable concentrations of DLIS were observed with the FPIA, Stratus II digoxin assay, and the RIA method. The authors also compared digoxin levels in 121 sera from 49 patients and observed comparable values with all digoxin assays. However, 11 patients showed discrepant digoxin values ($>2S_{y,x}$ from the regression line). These patients had renal disease or hepatic disease. The discrepant digoxin values were always lower with the CLIA compared with other digoxin assays. The authors concluded that the CLIA digoxin assay on the ACS:180 analyzer had improved specificity for digoxin *(30)*.

Way et al. evaluated Vitros digoxin assay (Johnson and Johnson) for interference from DLIS. The Vitros digoxin assay is an enzymatic heterogeneous competitive immunoassay that uses dry slide technology. The authors compared this assay with the Online digoxin assay (Roche), which is a homogenous microparticle immunoassay based on the aggregation of digoxin-coated microparticles in the presence of anti-digoxin antibody. Digoxin in the specimen partly inhibits aggregation, and thus, the rate of aggregation (as measured by light scattering) is inversely proportional to digoxin concentration. The authors also used an MEIA (Abbott Laboratories) that utilizes digoxin–alkaline phosphatase conjugate and 4-methyllumbelliferyl as a substrate. The authors compared three digoxin assays using 26 adult patients receiving digoxin and observed mean digoxin concentrations of $1.30 \pm 0.69$ ng/mL (SD) by Roche assay, $1.34 \pm 0.58$ ng/mL by the Abbott assay, and $1.46 \pm 0.68$ ng/mL by the Vitros digoxin assay. To study the potential interference of DLIS (suspected cause of such discrepancy) in these assays, the authors added known amounts of digoxin to serum samples prepared from newborns (high DLIS) and adults (no DLIS). The samples from newborns showed a mean digoxin level of 0.41 ng/mL by the Roche method and 0.7 ng/mL by the Vitros. The specimens from adults showed a mean value of 0.7 ng/mL by the Roche method and 0.8 ng/mL by the Vitros. The authors concluded that the positive bias in the Vitros assay compared with Roche OnLine assay was probably because of DLIS *(39)*.

Bonagura et al. *(40)* reported high specificity of the Roche OnLine assay for digoxin, which had no cross-reactivity with DLIS and negligible cross-reactivity with noncardioactive metabolites of digoxin. Marzullo et al. *(41)* reported that the EMIT 2000 digoxin immunoassay and the Roche OnLine digoxin immunoassay were least affected by DLIS compared with other digoxin assays. Saccoia et al. also confirmed improved specificity of the EMIT 2000 digoxin assay and very low cross-reactivity from DLIS compared with the FPIA digoxin assay and concluded that the EMIT 2000 had adequate specificity, sensitivity, precision, and accuracy for routine monitoring of digoxin in clinical laboratories *(42)*. More recently marketed digoxin immunoassays such as a turbidimetric assay on ADVIA 1650 analyzer and an enzyme-linked immunosorbent digoxin assay on the ADVIA IMS 800 I system (both marketed by Bayer diagnostics) are virtually free from DLIS interference *(43,44)*. This may be related to the use of specific monoclonal antibodies targeted against digoxin in this new assay compared with rabbit polyclonal antibody targeted against digoxin in the FPIA.

Although most investigators reported positive interference of DLIS with serum digoxin measurement, negative interference (falsely lower digoxin values) of DLIS in the MEIA for digoxin has been reported *(45)*. This may result in digoxin toxicity because a clinician may increase a digoxin dose based on a falsely low digoxin concentration because of elevated DLIS *(46)*.

### *3.1. Elimination of DLIS Interference in Digoxin Immunoassay by Ultrafiltration*

Although several monoclonal antibody-based digoxin immunoassays are virtually free from DLIS interference, ultrafiltration technique can be used to completely eliminate DLIS interference in serum digoxin measurement by immunoassays. Valdes and Graves *(47)* reported strong serum protein binding of DLIS. Therefore, DLIS is usually absent in the protein-free ultrafiltrate. Taking advantage of high protein binding of DLIS and poor protein binding of digoxin (25%), both positive and negative interference of DLIS in serum digoxin measurement, can be completely eliminated by measuring digoxin concentration in the protein-free ultrafiltrate *(48,49)*. Protein-free ultrafiltrate of digoxin can be easily prepared by centrifuging the specimen at 1500–2000 $\times g$ in a Centrifree Micropartition System (Ultrafiltration device, Amicon, distributed by Millipore Corporation; Molecular weight cutoff 30,000 Da) for 20–30 min at room temperature. Digoxin is only 25% protein bound and approximately 75% of digoxin is found in the ultrafiltrate. Immunoassay kits used for monitoring total digoxin concentration have adequate sensitivity to measure free digoxin concentration in the protein-free ultrafiltrate. It is also possible to calculate true total digoxin concentration and the extent of DLIS interference in digoxin measurement by measuring albumin, total and free digoxin concentrations, and then using mathematical equations *(50)*.

## 4. IS DLIS A NATRIURETIC HORMONE?

Serum DLIS concentrations are elevated in volume-expanded patients. DLIS can also inhibit Na, K-ATPase. In addition, it has been associated with natriuresis, thus raising the possibility that DLIS play a role in water and sodium homeostasis. Garbagnati demonstrated that children with elevated concentrations of DLIS showed significantly lower natremia, higher urinary and fractional excretion of sodium, and increased systolic blood pressure compared with children with no measurable DLIS concentration *(34)*. Ebara et al. *(51)* prepared DLIS from cord blood of healthy full-term infants by acetone/hydrochloric acid extraction followed by purification with a gel-filtration column and demonstrated natriuretic activity of DLIS in a rat model. Goodline reported a case where the blood pressure of a pregnant woman with preeclampsia was reduced significantly after intravenous treatment with the Fab fragment of anti-digoxin antibody. This was probably because of binding of free DLIS with Fab *(52)*. However, there are other reports in the literature that dispute the link between elevated DLIS and natriuresis. Scott et al. *(53)* did not find any difference in circulating levels of DLIS in normotensive and hypertensive rabbits despite marked alteration in dietary sodium intake. Trachtman et al. *(54)* concluded that a rise in DLIS concentration does not lead to an increase in blood pressure.

### *4.1. Structure of DLIS*

Instead of being a single compound, DLIS may be a class of compounds. Several investigators identified nonesterified fatty acids, phospholipids, and lysophospholipids as DLIS *(55,56)*. When the first anti-digoxin antibody was introduced, it was recognized that most steroids cross-react to some extent with these antibodies. Several investigators reported progesterone, 17-OH progesterone, cortisol, and glycodihydroxy and glycotrihydroxy bile salts as DLIS *(57)*. Shaikh et al. *(58)* reported that DLIS has a molecular weight of 780 Da comprising one 390-Da aglycone and several sugar moieties. De Angelis et al. *(59)* characterized DLIS as a single peak by HPLC from human serum with similar retention time as digoxin and concluded that the structure of DLIS was similar to digoxin. Qazzaz et al. reported that subtle structural differences exist between DLIS and digoxin at or near the lactone ring as well as in the nature of sugar. Moreover, deglycosylated congeners of DLIS also exist in human serum *(60)*. Bagrov et al. *(61)* characterized DLIS as marinobufagenin with a molecular weight of 400 Da. Qazzaz et al. *(62)* also reported de novo biosynthesis of digoxin-like immunoreactive factors.

## 5. EFFECT OF FAB FRAGMENT OF ANTIDIGOXIN ANTIBODY ON DIGOXIN IMMUNOASSAYS

The Fab fragment of antidigoxin antibody is commercially available as Digibind and DigiFab. Digibind has been available in the USA since 1986 (Glaxo Wellcome Inc.), and more recently in 2001, the Food and Drug Administration of the USA approved DigiFab for treating potentially life-threatening digoxin toxicity or overdose. Digibind is produced by immunizing sheep with digoxin followed by the purification of the Fab fragment from blood, whereas DigiFab is prepared by injecting sheep with digoxindicarboxymethylamine followed by purification of fab fragment. The molecular weight of DigiFab (46,000 Da) is similar to that of Digibind (46,200 Da). The approximate dose of Fab fragment is 80 times the digoxin body burden (in mg), or if neither the dose ingested nor the plasma digoxin concentration is known, then 380 mg of Fab fragment should be given. The half-life of the Fab fragment in humans is 12–20 h, but this may be prolonged in patients with renal failure *(63)*. The Fab fragment is also effective in treating digitoxin overdose.

The concentration of digoxin in myocardium is substantially higher than the corresponding digoxin concentration in serum. The Fab binds free digoxin in serum because of its high affinity for digoxin and effectively removes pharmacologically active free digoxin from serum. Therefore, the equilibrium between free and bound digoxin in serum is disturbed and digoxin bound to myocardium is released back in serum and subsequently binds with the Fab fragments. In this process, toxicity because of digoxin can be reversed.

The Fab fragment is known to interfere with serum digoxin measurements using immunoassays, and the magnitude of interference depends on the assay design and the specificity of the antibody used. The MEIA of digoxin (on the AxSYM analyzer) as well as the Stratus digoxin assay show digoxin values that are higher than measured free digoxin concentration in the presence of Fab fragment *(64)*. McMillin et al. studied the effect of Digibind and DigiFab on 13 different digoxin immunoassays. Positive

interference in the presence and absence of digoxin was observed with Digibind and DigiFab, although the magnitude of interference was somewhat less with DigiFab. The magnitude of interference varied significantly with each method whereas IMMULITE, Vitros, Dimension, and Access digoxin methods showed highest interference. The magnitudes of interference were in the order of Elecsys, TinaQuant, Integra, EMIT, and Centaur methods whereas minimal interferences were observed with FPIA, MEIA, Synchron, and CEDIA methods *(65)*.

### *5.1. Elimination of Fab Interference by Ultrafiltration*

The molecular weight of the Fab fragment (46,000 or 46,200 Da) is much higher than the cutoff of the Amicon Centrifree filters (30,000) used in the preparation of protein-free ultrafiltrate for measuring free digoxin concentrations. Therefore, Fab fragment is absent in the protein-free ultrafiltrate, and monitoring free digoxin concentration is not subjected to the interference by the Fab fragment. Jortani et al. reported that analysis of serum ultrafiltrate for digoxin concentration remains the most accurate approach in monitoring unbound digoxin in the presence of the Fab fragment. Moreover, no matrix bias was observed in measuring digoxin concentrations in protein-free ultrafiltrates using immunoassays *(66)*. McMillin et al. commented that patients treated with Digibind can be monitored reasonably by using either MEIA (on AxSYM analyzer) or the Stratus. Another alternative is to measure free digoxin concentration in the protein-free ultrafiltrate. The immunoassays for direct measurement of digoxin in serum in the presence of Digibind however will overestimate free digoxin concentration *(65)*.

## 6. INTERFERENCE OF SPIRONOLACTONE, POTASSIUM CANRENOATE, AND CANRENONE IN DIGOXIN ASSAYS

Spironolactone, a competitive aldosterone antagonist, has been used clinically in the therapy of hypertension and congestive heart failure for a long time. Spironolactone is rapidly and extensively metabolized, and its metabolite canrenone is also pharmacologically active. Spironolactone and canrenone have structural similarity with digoxin (Fig. 1). Although not in formulary in the USA, potassium canrenoate is used in Europe and other countries. Potassium canrenoate is also metabolized to canrenone.

Because spironolactone and digoxin may be used concurrently in the patient management, interference of spironolactone and canrenone in therapeutic monitoring of digoxin is troublesome. Positive interference of spironolactone and its active metabolite canrenone in the RIA for digoxin has been reported as early as 1974 *(67)*. Potassium canrenoate also showed positive interference with serum digoxin monitoring by both RIA and enzyme immunoassay *(68,69)*. Morris et al. *(70)* reported positive interference of spironolactone in digoxin measurement using the FPIA. Later, other authors verified the interference of spironolactone and canrenone in the FPIA and other commonly used immunoassays for digoxin *(71,72)*. Okazaki et al. also reported falsely elevated digoxin levels in patients receiving digoxin and potassium canrenoate. The authors reported two cases where cross-reactivity of the assay system caused clinical problem and recommended use of the OPUS digoxin assay, which showed minimum cross-reactivity *(73)*.

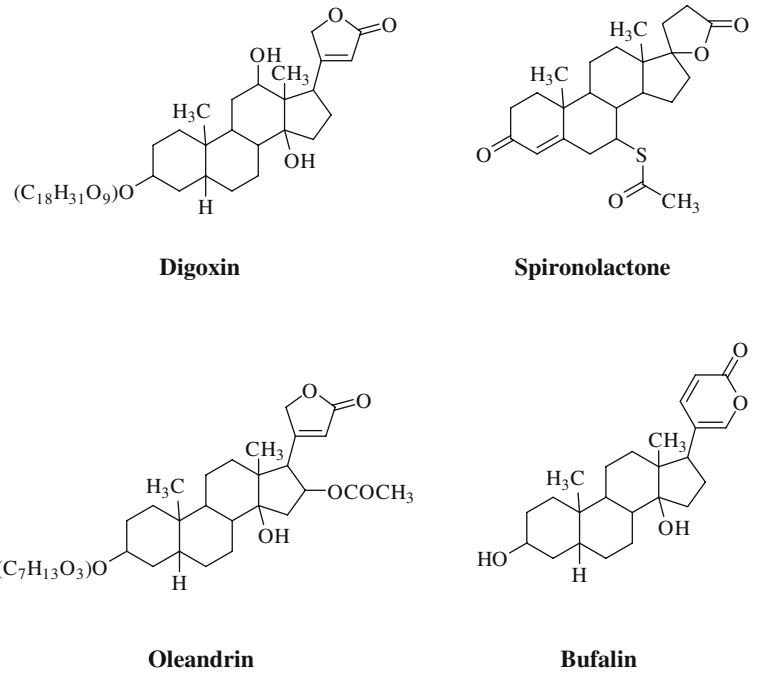


**Fig. 1.** Chemical Structure of Digoxin, Spironolactone, Oleandrin and Bufalin.

Steimer et al. reported negative interference of canrenone in digoxin measurement. Canrenone and spironolactone caused falsely low digoxin values because of negative interference in serum digoxin measurement when a MEIA for digoxin was used. Misleading sub-therapeutic concentrations of digoxin as measured on several occasions led to falsely guided digoxin dosing resulting in serious digoxin toxicity in the patients *(74)*.

Interference of spironolactone, potassium canrenoate, and their common metabolite canrenone may be positive or negative in serum digoxin measurement using immunoassays. Spironolactone and its metabolite canrenone can falsely elevate serum digoxin levels if measured using FPIA, ACA, or Elecsys (positive interference) or falsely lower digoxin levels if measured by MEIA, Imx, and Dimension (negative interference). The magnitude of interference is more significant with potassium canrenoate where concentration of its metabolite canrenone can be significantly higher. In one report, authors observed a 42% decline in expected value of serum digoxin in the presence of 3125 ng/mL of canrenoate using MEIA, 78% decline in using Dimension and 51% decrease using IMx. A positive bias was observed with the ACA (0.7 ng/mL), TDx (0.62 ng/mL), and Elessys (0.58 ng/mL). EMIT 2000, and the Vitros digoxin assay, is free from such interference *(75)*.

Our experience is that Bayer's Chemiluminescent digoxin assay is also free from such interference. Moreover, interference of spironolactone and its metabolite canrenone can be mostly eliminated by ultrafiltration because both compounds are strongly protein bound. However, in the case of therapy with K-canrenoate (not used in the USA), complete elimination of this interference in certain digoxin assays cannot be

achieved because of higher concentrations of K-canrenoate and higher concentrations of its metabolite canrenone observed in plasma *(76)*. Howard et al. *(77)* demonstrated that low-dose spironolactone (up to 25 mg per day) as used for oral therapy does not cause clinically significant negative interference in the MEIA digoxin assay on the AxSYM analyzer by comparing results with the EMIT assay which is free from spironolactone interference. However, with a higher spironolactone dose, such as 200 mg per day, a significant interference may be observed with the MEIA *(78)*.

## 7. INTERFERENCE OF COMPLEMENTARY AND ALTERNATIVE MEDICINES IN DIGOXIN MEASUREMENT

Herbal medicines without prescriptions are readily available in the USA from stores. Chinese medicines are an important component of herbal medicines available today. In developing countries, as much as 80% of the indigenous populations depend on local traditional system of medicines. Within the European market, herbal medicines represent an important pharmaceutical market. Several Chinese medicines interfere with serum digoxin measurements by immunoassays.

### *7.1. Chan Su*

Traditional Chinese medicines are readily available without prescription in local Chinese herbal stores. One such Chinese medicine is Chan Su, which is prepared from the dried white secretion of the auricular glands and the skin glands of Chinese toads (*Bufo melanostictus* Schneider or *Bufo bufo gargarzinas* Gantor). Chan Su is also a major component of traditional Chinese medicines Liu-Shen-Wan and Kyushin *(79, 80)*. These medicines are used for the treatment of such disorders as tonsillitis, sore throat, and palpitation. Traditional use of Chan Su given in small doses also includes stimulation of myocardial contraction, anti-inflammatory effect, and analgesia *(81)*. The cardiotonic effect of Chan Su is due to its major bufadienolides such as bufalin (Fig. 1), cinobufagin, and resibufogenin *(82)*. Bufalin is known to block vasodilatation and increases vasoconstriction, vascular resistance, and blood pressure by inhibiting Na, K-ATPase *(83)*.

Fushimi and Amino *(84)* reported a serum digoxin concentration of 0.51 nmol/L (0.4 ng/mL) in a healthy volunteer after ingestion of Kyushin tablets containing Chan Su as the major component. Panesar *(85)* reported an apparent digoxin concentration of 1124 pmol/L (0.88 ng/mL) in a healthy volunteer who ingested LSW pills. The author used the FPIA and TDx analyzer for the study. Chan Su is the major component of LSW pills. An apparent digoxin concentration of 4.9 ng/mL (FPIA) was reported in one woman who died from ingestion of Chinese herbal tea containing Chan Su *(86)*. Therefore, FPIA digoxin assay can be used to indirectly show the presence of Chan Su in serum in a suspected overdose, although a definite diagnosis is not possible without verification of the presence of bufalin in serum using a direct analytical technique such as HPLC combined with tandem mass spectrometry. In a recent article, Panesar commented that the digoxin measured by the FPIA in 2004 showed less sensitivity to the FPIA the authors used in 2003 to demonstrate apparent digoxin concentrations in volunteers after taking Chan Su-containing Chinese medicines *(87)*.

Although the FPIA demonstrated the highest reported cross-reactivity with Chan Su, the Beckman digoxin assay (Synchron LX system) and Roche assay (Tian-Quant) are also affected by Chan Su. However, the magnitude of interference was almost 50% less compared with that of the FPIA. This may be related to the use of a more specific monoclonal antibody against digoxin in the assay design of both Tina-Quant and the Beckman assay although the FPIA utilizes a rabbit polyclonal antibody against digoxin *(88)*.

Chan Su extracts also falsely increased serum digoxin concentration in vitro when FPIA (Digoxin II, TDx analyzer Abbott Laboratories) was used for serum digoxin measurement. In contrast, serum digoxin levels were falsely lowered (negative interference) when the MEIA (Abbott Laboratories) and AxSYM analyzer were used. However, the components of Chan Su responsible for digoxin-like immunoreactivity are significantly bound to serum proteins (>90%) and are virtually absent in the protein-free ultrafiltrate. Therefore, measuring free digoxin concentration in the protein-free ultrafiltrate maybe used to eliminate the interference of Chan Su in serum digoxin measurements. A chemiluminescent digoxin assay marketed by the Bayer Diagnostics is also virtually free from interference of Chan Su *(89)*.

### *7.2. Oleander poisoning and Oleander-Containing Herbs*

The oleanders are evergreen ornamental shrubs with various colors of flowers that belong to the Dogbane family and grow in the Southern parts of the USA from Florida to California, Australia, India, Sri Lanka, China, and other parts of the world. All parts of the oleander plant are toxic. Human exposure to oleander includes accidental exposure, ingestion by children, administration in food or drink, medicinal preparations from oleander (herbal products), and criminal poisoning *(90–93)*. Despite toxicity, oleander is used in folk medicines *(94)*. The fatality rate from oleander toxicity is around 10% in Sri Lanka whereas approximately 40% of patients require specialized management in a tertiary care hospital. Deliberate ingestion of oleander seeds is also a popular method of suicide in Sri Lanka *(95)*. The toxic effect of oleander can occur with exposure from a small amount of the plant. Boiling or drying the plant does not inactivate the toxins. Death from drinking a herbal tea containing oleander has been reported *(96)*. Oleander toxicity has been studied in a Tunisian toxicology intensive care unit from 1983 to 1998 in connection with plant poisoning and use of herbal medicines. The authors reported that 7% of all poisoning from the use of herbal medicines was due to oleander *(97)*.

Early reports indicated that cardiac glycosides present in oleander cross-react with digoxin RIA *(98)*. Cheung et al. *(99)* reported detection of poisoning by plant origin (including oleander) using the digoxin immunoassay on the TDx analyzer. Jortani et al. *(100)* reported rapid detection of oleandrin and oleandrigenin using FPIA, fluorometric enzyme assay on Stratus analyzer, RIA, ACS:180, and On-Line digoxin assays. Osterloh et al. reported an apparent digoxin level of 5.8 ng/mL after suicidal ingestion of oleander tea in a patient with no history of taking any cardioactive drug. The person eventually died from oleander toxicity *(101)*. Eddleston et al. reported a mean apparent serum digoxin concentration of 1.49 nmol/L (1.16 ng/mL) in patients who were poisoned with oleander but eventually discharged from the hospital. Severe toxicity from oleander resulted in a mean apparent serum digoxin concentration of 2.83 nmol/L (2.21 ng/mL) as measured using the FPIA digoxin assay *(102)*. In our

experience, the FPIA has the highest cross-reactivity with oleander extract as well as oleandrin, an active component of oleander extract. The Beckman digoxin assay on Synchron LX as well as the turbidimetric assay on the ADVIA 1650 analyzer (Bayer Diagnostics) also showed significant interference with oleander, although the magnitude of interference was approximately 65% less with both the Beckman assay and the turbidimetric assay. The CLIA, marketed by Bayer Diagnostics, is virtually free from interference of oleander *(103)*. Although FPIA digoxin assay can be used for indirect detection of apparent digoxin concentration in a suspected oleander poisoning, for a definite diagnosis, the presence of oleandrin, the toxic glycoside in oleander in blood, should be confirmed by HPLC and mass spectrometry (HPLC/MS), a direct analytical technique for detection of oleandrin in blood *(104)*. However, this technique is complex and cannot be used routinely in a small hospital.

Oleandrin is strongly bound to serum protein and is absent in the protein-free ultrafiltrate. Therefore, monitoring free digoxin in the protein-free ultrafiltrate may eliminate some interference of oleander in serum digoxin assay provided that the oleandrin concentration is low to moderate. For total elimination of interference, a specific analytical technique such as HPLC combined with mass spectrometry should be used for measurement of digoxin concentration.

### *7.3. Uzara Roots and Digoxin Immunoassays*

Thurmann et al. reported that glycosides from Uzara roots may interfere with serum digoxin measurement by immunoassays. The authors investigated digoxin and digitoxin concentrations after four healthy volunteers ingested 1.5 ml (approximately 22 drops) of Uzara. Maximum digoxin concentrations of 1.4–6.34 μg/L (1.1–4.9 ng/mL) were observed 6 h post dosing *(105)*. However, Uzara root is not usually found in herbal stores in the USA but is a popular remedy in Germany.

### *7.4. Siberian Ginseng, Asian Ginseng and Ashwagandha*

There is one case report of interference of Siberian ginseng in serum digoxin measurement. A 74-year-old man had a steady serum digoxin level of 0.9–2.2 ng/mL for 10 years. His serum digoxin increased to 5.2 ng/mL on one occasion after taking Siberian ginseng. Although the level was toxic, the patient did not experience any sign or symptoms of digoxin toxicity. The patient stopped taking Siberian ginseng, and his digoxin level returned to normal *(106)*. However, in our experience, Siberian ginseng only has a very modest interference with the FPIA and most digoxin assays we tested had no effect at all *(107)*. Therefore, in the case report, it is possible that the patient ingested some other herbal remedy mislabeled as Siberian ginseng. Mislabeling of Chinese herbs has been previously reported. Asian ginseng, which is prepared from a different herb than Siberian ginseng, also showed modest interference with the FPIA but other digoxin immunoassays were not affected at all *(107)*.

More recently, Ashwagandha (loosely also called Indian ginseng) has become available on the U.S. market. Although labeled as "ginseng products," this herbal supplement is prepared from entirely different plants. Ashwagandha (*Withania somnifera*) has been used in Ayurvedic medicine for over 3000 years as an aphrodisiac, liver tonic, anti-inflammatory agent, and astringent. The major biochemical constituents

**Table 2**
**Interference of Herbal Products in Digoxin Immunoassays**

| *Herbal Product* | *Interference* | *Comments* |
|---|---|---|
| Chan Su | High | Chan Su has active components such as bufalin that cross-react with digoxin immunoassays. Most assays are affected. FPIA showed high interference |
| Uzara root (diuretic) | Moderate–High | Additive effect with digoxin also interferes with digoxin immunoassays |
| Siberian ginseng | Low–Moderate | May falsely increase digoxin level measured by FPIA and falsely lower digoxin level using MEIA. Other digoxin assays such as Roche, Beckman, Bayer show no interference |
| DanShen | Low | May interfere with FPIA assay for digoxin. Other immunoassays not affected |

FPIA, fluorescence polarization immunoassay; MEIA, microparticle enzyme immunoassay.

of Ashwagandha are steroidal alkaloids and steroidal lactones in a class of compounds termed as "withanolides," which have structural similarity with digoxin. Although Ashwagandha showed a very modest interference with the FPIA, other digoxin assays such as the Tina-quant (Roche) assay and the Beckman assay on the Synchron LX analyzer are totally free from interference of Indian ginseng *(108)*.

### *7.5. DanShen*

DanShen is another Chinese herb prepared from the root of the Chinese medicinal plant *Salvia miltiorrhiza*. This drug has been used in China for many years in the treatment of various cardiovascular diseases including angina pectoris and is readily available in the USA through Chinese herbal stores. More than 20 diterpene quinones known as tanshinones have been isolated from DanShen *(109)*. These compounds have structural similarity with digoxin. Feeding DanShen to mice caused digoxin-like immunoreactivity in sera *(110)*. However, the extent of DLIS activity was less remarkable than observed in mice after feeding with Chan Su and interference of DanShen in serum digoxin measurement using the FPIA should be considered as low to modest, and other digoxin assays such as Tina-Quant (Roche) and Beckman assay (on Synchron LX analyzer) are completely free from the interference of DanShen *(88)*. The digoxin-like immunoreactive components of DanShen are strongly protein bound, and monitoring free digoxin eliminates interference of DanShen in digoxin measurement using FPIA *(111)*. The effects of different complementary and alternative medicines in serum digoxin measurements using immunoassays are summarized in Table 2.

## 8. CONCLUSIONS

Both endogenous and exogenous DLIS can cause significant interference in serum digoxin measurement. DLIS causes low to moderate false increases in serum digoxin value in most digoxin immunoassays. However, FPIA (Digoxin II) showed significant

interference from DLIS. Negative interference of DLIS in the MEIA digoxin assay may also be problematic because the clinician may increase the digoxin dose based on falsely low serum digoxin concentrations. Both positive and negative interferences in serum digoxin measurement can be eliminated by monitoring free digoxin concentration.

Interference in digoxin assays because of ingestion of Chinese medicines can cause more confusion. Most patients do not inform their physicians when they use alternative medicines. Present studies indicate that components of those Chinese medicines causing DLIS activity are strongly protein bound. Monitoring free digoxin may eliminate such interferences because of certain herbal supplements.

## REFERENCES


1. Rea TD, Siscovick DS, Psaty BM, Pearce RM, et al. Digoxin therapy and risk of primary cardiac arrest in patients with congestive heart failure: effect of mild-moderate renal impairment. *J Clin Epidemol* 2003; 56; 646–650.
2. Adams KF, Patterson JH, Gattis WA, O'Connor CM, et al. Relationship of serum digoxin concentrations to mortality and morbidity in women in the digitalis investigation group trial: a retrospective study. *J Am Coll Cardiol* 2005; 46: 497–504.
3. Mandy M, Manchon M, Meley R, Eynard JC, Grafmeyer D. Digoxin assay: results of 10 years of intra laboratory controls. *Ann Biol Clin (Paris)* 2005; 63: 345–349 [Article in French].
4. Gruber KA, Whitaker JM, Buckalew VM. Endogenous digitalis-like substances in plasma of volume expanded dogs. *Nature* 1980; 287: 743–745.
5. Craver JL, Valdes R. Anomalous serum digoxin concentration in uremia. *Ann Intern Med* 1983; 98: 483–484.
6. Balzan S, Clerico A, Grazia del Chicca M, Montall U, Ghione S. Digoxin-like immunoreactivity in normal human plasma and urine as detected by a solid phase radioimmunoassay. *Clin Chem* 1984; 30: 450–451.
7. Krivoy N, Lalkin A, Jakobi P. Digoxin-like immunoreactivity detected in cerebrospinal fluid of humans with fever. *Clin Chem* 1990; 36: 703–704.
8. Jacobi P, Krivoy N, Schwartz K, Ben Aryeth L, Laufer D. Digoxin-like immunoreactivity in plasma and saliva of pregnant women. *Clin Chem* 1991; 37: 135–136 [letter].
9. Schrader BJ, Maddux MS, Veremis SA, Mozes MF, et al. Digoxin-like immunoreactivity in renal transplant patients. *J Clin Pharmacol* 1991; 12: 1126–1131.
10. Frisolone J, Sylvia LM, Gelwan J, Pal S, Pellechia C. False-positive serum digoxin concentrations determined by three digoxin assays in patients with liver disease. *Clin Pharm* 1988; 6: 444–449.
11. Dasgupta A, Nakamuar A, Doria L, Dennen D. Comparison of free digoxin and total digoxin: extent of interference from digoxin like immunoreactive substances (DLIS) in a fluorescence polarization assay. *Clin Chem* 1989; 35: 323–324.
12. Panesar NS. Bufalin radioimmunoassays; in search of the endogenous digitalis-like substances. *J Immunoassay* 1994; 4: 371–391.
13. Lin MH, Liao CP, Lee JS, Chin YW, et al. Detection of endogenous digitalis-like immunoreactive factors in human blood. *Proc Natl Sci Counc Repub China B* 1998; 3: 129–135.
14. Dasgupta A, Yeo K, Malik S, Sandu P, et al. Two novel endogenous digoxin-like immunoreactive substances isolated from human plasma ultrafiltrate. *Biochem Biophys Res Comm* 1987; 148: 623–628.
15. Qazzaz HM, Valdes R. Simultaneous isolation of endogenous digoxin-like immunoreactive factor, ouabain-like factor, and deglycosylated congeners from mammalian tissue. *Arch Biochem Biophys* 1996 328: 193–200.
16. Stone JA, Soldin SJ. An update on digoxin. *Clin Chem* 1989; 35: 1326–1331.
17. Vinge E, Ekman R. Partial characterization of endogenous digoxin like substances in human urine. *Ther Drug Monit* 1988; 10: 8–15.

18. Shilo L, Werber MM, Dolev S, Shapiro M, Shenkman L. Digoxin-like immunoreactivity: occurrence in three molecular form and partial characterization. *J Clin Endocrinol Metab* 1987; 64: 1257–1260.
19. Nanji AA, Greenway DC. Falsely raised plasma digoxin concentrations in liver disease. *Br Med J* 1985; 290: 432–435.
20. Gault MH, Vasdev SC, Longerich LL. Endogenous digoxin-like substances in patients with combined hepatic and renal failure. *Ann Intern Med* 1984; 101: 567–568 [letter].
21. Graves SW. The possible role of digitalis-like factors in pregnancy induced hypertension. *Hypertension* 1987; 10 (Suppl.): I84–86.
22. Cloix JF. Endogenous digitalis like compounds: a tentative update of chemical and biological studies. *Hypertension* 1987; 10 (Suppl.): I67–70.
23. Shilo L, Adwani A, Solomon G, Shenkman L. Endogenous digoxin-like immunoreactivity in congestive heart failure. *Br Med J* 1987; 295: 415–416.
24. Ahmad S, Kenny M, Scribner BH. Hypertension and a digoxin like substance in the plasma of dialysis patients: possible marker for a natriuretic hormone. *Clin Physiol Biochem* 1986; 4: 210–216.
25. Howarth DM, Sampson DC, Hawker FH, Young A. Digoxin-like immunoreactive substances in the plasma of intensive care unit patients: relationship to organ dysfunction. *Anaesth Intensive care* 1990; 18: 45–52.
26. Berendes E, Cullen P, van Aken H, Zidek W, et al. Endogenous glycosides in critically ill patients. *Crit Care Med* 2003; 31: 1331–1337.
27. Chicella M, Branim B, Lee KR, Phelps SJ. Comparison of microparticle enzyme and fluorescence polarization immunoassays in pediatric patients not receiving digoxin. *Ther Drug Monit* 1998; 20: 347–351.
28. Ijiri Y, Hayahi T, Ogihara T, Ohi K, et al. Increased digitalis-like immunoreactive substances in neonatal plasma measured using fluorescence polarization immunoassay. *J Clin Pharm Ther* 2004; 29: 565–571.
29. Ijiri Y, Hayashi T, Kamegai H, Ohi K, et al. Digitalis-like immunoreactive substances in maternal and umbilical cord plasma: a comparative sensitivity study of fluorescence polarization immunoassay and microparticle enzyme immunoassay. *Ther Drug Monit* 2003; 25: 234–239.
30. Miller JJ, Straub RW, Valdes R. Analytical performance of a monoclonal digoxin assay with increased specificity on the ACS:180. *Ther Drug Monit* 1996; 18: 65–72.
31. Crossey MJ, Dasgupta A. Effects of digoxin-like immunoreactive substances and digoxin FAB antibodies on the new digoxin microparticle enzyme immunoassay. *Ther Drug Monit* 1997; 19: 185–190.
32. Logoglu G, Erdogan S, Ozgunen FT, Dogan A, et al. Endogenous digoxin-immunoreactive substance in normal and preeclamptic pregnancies. *Int J Gynaecol Obstet* 1993; 43: 137–143.
33. Doolittle MH, Lincoln K, Graves SW. Unexplained increase in serum digoxin: a case report. *Clin Chem* 1994; 40: 487–492.
34. Garbagnati E. Serum digoxin-like immunoreactive factor in children and its relation to sodium metabolism. *Acta Paediatr* 1998; 87: 500–504.
35. Lusic I, Ljutic D, Maskovic J, Jankovic S. Plasma and cerebrospinal fluid endogenous digoxin-like immunoreactivity in patients with aneurysmal subarachnoid haemorrhage. *Acta Neurochir (Wien)* 1999; 141: 691–697.
36. Grider G, El-Mallakh RS, Huff MO, Buss TJ, et al. Endogenous digoxin-like immunoreactive factor (DLIF) serum concentrations are decreased in manic bipolar patients compared to normal controls. *J Affect Disord* 1999; 54: 261–267.
37. Avendano C, Alvarez JS, Sacristan JA, Adin J, Alsar MJ. Interference of digoxin-like immunoreactive substances with TDx digoxin II assay in different patients. *Ther Drug Monit* 1991; 13: 523–527.
38. Datta P, Hinz V, Klee G. Comparison of four digoxin immunoassays with respect to interference from digoxin-like immunoreactive factors. *Clin Biochem* 1996; 29: 541–547.
39. Way BA, Wilhite TR, Miller R, Smith CH, Landt M. Vitros digoxin immunoassay evaluated for interference by digoxin-like immunoreactive factors. *Clin Chem* 1998; 44: 1339–1440.
40. Bonagura E, Law T, Rifai N. Assessment of the immunoreactivity of digoxin metabolites and the cross-reactivity with digoxin-like immunoreactive factors in the Roche-TDM online digoxin assay. *Ther Drug Monit* 1995; 17: 532–537.

41. Marzullo C, Bourderont D, Dorr R. Interference of digoxin-like immunoreactive substances with four recent reagents for digoxin determination. *Ann Biol Chem (Paris)* 1996; 54: 91–96.
42. Saccoia NC, Hackett LP, Morris RG, Ilett KF. Enzyme-multiplied immunoassay (EMIT 2000) digoxin assay compared with fluorescence polarization immunoassay and amerlex 125I-radioimmunoassay at two Australian center. *Ther Drug Monit* 1996; 18: 672–676.
43. Datta P, Dasgupta A. Interference of endogenous digoxin-like immunoreactive factors in serum digoxin measurement is minimized in a new turbidimetric digoxin immunoassay on ADVIA 1650 analyzer. *Ther Drug Monit* 2004; 26: 85–89.
44. Dasgupta A, Kang E, Datta P. New enzyme-linked immunosorbent digoxin assay on the ADVIA IMS 800i system is virtually free from interference of endogenous digoxin-like immunoreactive factor. *Ther Drug Monit* 2005; 27: 139–143.
45. Valdes R, Jortani SA, Gheorghiade M. Standards of laboratory practice: cardiac drug monitoring. National Academy of Clinical Biochemistry. *Clin Chem* 1998; 44: 1096–1109.
46. Dasgupta A, Trejo O. Suppression of total digoxin concentration by digoxin-like immunoreactive substances in the MEIA digoxin assay: elimination of interference by monitoring free digoxin concentrations. *Am J Clin Pathol* 1999; 111: 406–410.
47. Valdes R, Graves SW. Protein binding of endogenous digoxin-like immunoreactive factors in human serum and its variation with clinical condition. *J Clin Endocrinol Metab* 1985; 60: 1135–1143.
48. Christenson RH, Studenberg SD, Beck-Davis SS, Sedor FA. Digoxin-like immunoreactivity eliminated from serum by centrifugal ultrafiltration before fluorescence polarization immunoassay of digoxin. *Clin Chem* 1987; 33: 606–608.
49. Dasgupta A, Saldana S, Heimann P. Monitoring free digoxin instead of total digoxin in patients with congestive heart failure and high concentrations of digoxin like immunoreactive substances. *Clin Chem* 1990; 36: 2121–2123.
50. Dasgupta A, Schammel D, Limmany A, Datta P. Estimating concentration of total digoxin and digoxin-like immunoreactive substances in volume expanded patients being treated with digoxin. *Ther Drug Monit* 1996; 18: 34–39.
51. Ebara H, Suzuki S, Nagashima K, Kuroume T. Natriuretic activity of digoxin-like immunoreactive substance extracted from cord blood. *Life Sci* 1988; 42: 303–309.
52. Goodline RC. Antidigoxin antibodies and eclampsia. *N Engl J Med* 1988; 318: 518–519 [letter].
53. Scott PJ, Little P, Bobik A. Circulating digoxin-like immunoreactivity in renal hypertensive rabbits: lack of modulation by alteration in dietary sodium intake. *Hypertension* 1988; 6: 205–209.
54. Trachtman H, Delpizzo R, Chasalow FI. Endogenous digoxin-like material in normotensive and hypertensive stains of rats. *Life Sci* 1989; 44: 1881–1885.
55. Young A, Giesbrecht E, Soldin J. A study of lipid effects on the digoxin immunoassay and on the binding to and activity of Na/K-ATPase. *Clin Biochem* 1986; 19: 195–200.
56. Dasgupta A, Malik S, Ahmad A, Kenny M. Mass spectrometry studies of a novel digoxin-like substance (DLIS-2) isolated from human plasma ultrafiltrate. *Biochem Biophys Res Comm* 1988; 152: 1435–1440.
57. Longerich L, Johnson R, Brent D, Gault MH. Plasma conjugated androgens in a dialysis-dependent male as immunoreactive digitalis-like factors. *Clin Nephrol* 1990; 34: 72–78.
58. Shaikh IM, Lau BW, Siegfried BA, Valdes R. Isolation of digoxin-like immunoreactive factors from mammalian adrenal cortex. *J Biol Chem* 1991; 266: 13672–13678.
59. De Angelis C, Riscazzi M, Salvini R, Piccoli A, et al. Isolation and characterization of a digoxin-like immunoreactive substance from human urine by affinity chromatography. *Clin Chem* 1997; 43: 1416–1420.
60. Quazzaz H, Goudy S, Valdes R. Deglycosylated products of endogenous digoxin-like immunoreactive factor in mammalian tissue. *J Biol Chem* 1996; 271: 8731–8737.
61. Bagrov A, Fedorova OV, Dmitrieva RI, Howald WN, et al. Characterization of a urinary bufadienolide Na, K-ATPase inhibitor in patient after acute myocardial infarction. *Hypertension* 1998; 31: 1097–1103.
62. Qazzaz HM, Cao Z, Bolanowski DD, Clark BJ, Valdes R. De novo biosynthesis and radiolabeling of mammalian digitalis-like factors. *Clin Chem* 2004; 50: 612–620.

63. Flanagan RJ, Jones AL. Fab antibody fragments: some applications in clinical toxicology. *Drug Saf* 2004; 27: 1115–1133.
64. Rainey P. Digibind and free digoxin. *Clin Chem* 1999; 45: 719–720 [letter].
65. McMillin GA, Qwen W, Lambert TL, De B, et al. Comparable effects of DIGIBIND and DigiFab in thirteen digoxin immunoassays. *Clin Chem* 2002; 48: 1580–1584.
66. Jortani SA, Pinar A, Johnson NA, Valdes R. Validity of unbound digoxin measurements by immunoassays in presence of antidote (Digibind). *Clin Chim Acta* 1999; 283: 159–169.
67. Huffman DH. The effects of spironolactone and canrenone in digoxin radioimmunoassay. *Res Comm Chem Pathol Pharmacol* 1974; 9: 787–790.
68. Lichey J, Rietbrock N, Borner K. The influence of intravenous canrenoate on the determination of digoxin in serum by radio and enzyme immunoassay. *Int J Clin Pharmacol Biopharm* 1979; 17: 61–63.
69. Silber B, Sheiner LB, Powers JL, Winter ME, Sadee W. Spironolactone-associated digoxin radioimmunoassay interference. *Clin Chem* 1979; 25: 48–50.
70. Morris RG, Frewin DB, Taylor WB, Glistak ML, Lehmann DR. The effect of renal and hepatic impairment and of spironolactone on serum digoxin assay. *Eur J Clin Pharmacol* 1988; 34: 233–239.
71. Pleasants RA, Williams DM, Porter RS, Gadsden RH. Reassessment of cross-reactivity of spironolactone metabolites with four digoxin assays. *Ther Drug Monit* 1989; 11: 200–204.
72. Foukaridis GN. Influence of spironolactone and its metabolite canrenone on serum digoxin assays. *Ther Drug Monit* 1990; 12: 82–84.
73. Okazaki M, Tanigawara Y, Kita T, Komada F, Okumura K. Cross-reactivity of TDX and OPUS immunoassay system for serum digoxin determination. *Ther Drug Monit* 1997; 19: 657–662.
74. Steimer W, Muller C, Eber B, Emmanuilidis K. Intoxication due to negative canrenone interference in digoxin drug monitoring. *Lancet* 1999; 354: 1176–1177 [letter].
75. Steimer W, Muller C, Eber B. Digoxin assays: frequent, substantial and potentially dangerous interference by spironolactone, canrenone and other steroids. *Clin Chem* 2002; 48: 507–516.
76. Dasgupta A, Saffer H, Wells A, Datta P. Bidirectional (positive/negative) interference of spironolactone, canrenone and potassium canrenoate on serum digoxin measurement: elimination of interference by measuring free digoxin or using a chemiluminescent assay. *J Clin Lab Anal* 2002; 16: 172–177.
77. Howard G, Barclay M, Florkowski C, Moore G, Roche A. Lack of clinically significant interference by spironolactone with the AxSYM digoxin II assay. *Ther Drug Monit* 2003; 25: 112–113.
78. Steimer W. Lack of critically significant interference by spironolactone with the AxSYM digoxin II assay only applies to low dose therapy with spironolactone. *Ther Drug Monit* 2003; 25: 484–485 [letter].
79. Hong Z, Chan K, Yeung HW. Simultaneous determination of bufadienolides in the traditional Chinese medicine preparations, Liu-Shen-Wan by liquid chromatography. *J Pharm Pharmacol* 1992; 44: 1023–1026.
80. Chan WY, Ng TB, Yeung HW. Examination for toxicity of a Chinese drug, the total glandular secretion product Chan SU in pregnant mice and embryos. *Biol Neonate* 1995; 67: 376–380.
81. Chen KK, Kovarikove A. Pharmacology and toxicology of toad venom. *J Pharm Sci* 1967; 56: 1535–1541.
82. Morishita S, Shoji M, Oguni Y, Ito C, et al. Pharmacological actions of "Kyushin" a drug containing toad venom: cardiotonic and arrhythmogenic effects and excitatory effect on respiration. *Am J Chin Med* 1992; 20: 245–256.
83. Pamnani MB, Chen S, Bryant HJ, Schooley JF. Effect of three sodium-potassium adenosine triphosphate inhibitors. *Hypertension* 1991; 18: 316–324.
84. Fushimi R, Amino N. Digoxin concentration in blood. *Rinsho Byori* 1995; 43: 34–40 [article in Japanese, abstract in English].
85. Panesar NS. Bufalin and unidentified substances in traditional Chinese medicine cross-react in commercial digoxin assay. *Clin Chem* 1992; 38: 2155–2156.
86. Ko R, Greenwald M, Loscutoff S, Au A, et al. Lethal ingestion of Chinese tea containing Chan SU. *West J Med* 1996; 164: 71–75.

87. Panesar NS, Chan KW, Law LK. Changing characteristics of the TDx digoxin II assay in detecting bufadienolides in a traditional Chinese medicine: For better or worse? *Ther Drug Monit* 2005; 27: 677–679.
88. Chow L, Johnson M, Wells A, Dasgupta A. Effect of the traditional Chinese medicine Chan Su, Lu-Shen-Wan, DanShen and Asian ginseng on serum digoxin measurement by Tina-Quant (Roche) and Synchron LX system (Beckman) digoxin immunoassays. *J Clin Lab Anal* 2003; 17: 22–27.
89. Dasgupta A, Biddle D, Wells A, Datta P. Positive and negative interference of Chinese medicine Chan SU in serum digoxin measurement: elimination of interference using a monoclonal chemiluminescent digoxin assay or monitoring free digoxin concentrations. *Am J Clin Pathol* 2000; 114: 174–179.
90. Blum LM, Reiders F. Oleander distribution in a fatality from rectal and oral Nerium oleander extract administration. *J Anal Toxicol* 1987; 82: 121–122.
91. Saravanapavananthan N, Ganeshamoorthy J. Yellow oleander poisoning: a case study of 170 cases. *Forensic Sci Int* 1988; 36: 247–250.
92. Brewster D. Herbal poisoning: a case report of fetal yellow oleander poisoning from the Solomon Island. *Ann Trop Paediatr* 1986; 6: 289–291.
93. Langford S, Boor PJ. Oleander toxicity: an examination of human and animal toxic exposure. *Toxicology* 1999; 109: 1–13.
94. Erdemoglu N, Kupeli E, Yesilada E. Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. *J Ethnopharmacol* 2003; 89: 123–139.
95. Eddleston M, Ariaratnam CA, Meyer PW, Perera G, et al. Epidemic of self poisoning with seeds of yellow oleander tree (Thevetia peruviana) in north Sri Lanka. *Trop Med Int Health* 1999; 4: 266–273.
96. Haynes BE, Bessen HA, Wightman WD. Oleander tea: herbal draught of death. *Ann Emerg Med* 1985; 14: 350–353.
97. Hamouda C, Amamou M, Thabet H, Yacoub M, et al. Plant poisonings from herbal medication admitted to a Tunisian toxicological intensive care unit, 1983-1998. *Vet Human Toxicol* 2000; 42: 137–141.
98. Osterloh J, Harold S, Pond S. Oleander interference in the digoxin radioimmunoassay in a fatal ingestion. *JAMA* 1982; 247: 1596–1597.
99. Cheung K, Hinds JA, Duffy P. Detection of poisoning by plant origin cardiac glycosides with the Abbott TDx analyzer. *Clin Chem* 1989; 35: 295–297.
100. Jortani S, Helm A, Valdes R. Inhibition of Na,K-ATPase by oleandrin and oleandrigenin and their detection by digoxin immunoassays. *Clin Chem* 1996; 42: 1654–1658.
101. Osterloh J. Cross-reactivity of oleander glycosides. *J Anal Toxicol* 1988; 12: 53 [letter].
102. Eddleston M, Ariaratnam CA, Sjostrom L, Jayalath S, et al. Acute yellow oleander (Thevetia peruvica) poisoning: cardiac arrhythmias, electrolyte disturbances, and serum cardiac glycoside concentrations on presentation to hospital. *Heart* 2000; 83: 310–306.
103. Dasgupta A, Datta P. Rapid detection of oleander poisoning by using digoxin immunoassays: comparison of five assays. *Ther Drug Monit* 2004; 26: 658–663.
104. Tracqui A, Kintz P, Branche F, Ludes B. Confirmation of oleander poisoning by HPLC/MS. *Int J Legal Med* 1998; 111: 32–34.
105. Thurmann PA, Neff A, Fleisch J. Interference of Uzara glycosides in assays of digitalis glycosides. *Int J Clin Pharmacol Ther* 1004; 42: 281–284.
106. McRae S. Elevated serum digoxin levels in a patient taking digoxin and Siberian ginseng. *Can Med Assoc J* 1996; 155: 293–295.
107. Dasgupta A, Wu S, Actor J, Olsen M, Wells A, Datta P. Effect of Asian and Siberian ginseng on serum digoxin measurement by five digoxin immunoassays: significant variation in digoxin-like immunoreactivity among commercial ginsengs. *Am J Clin Pathol* 2003; 119: 298–303.
108. Dasgupta A, Reyer M. Effect of Brazilian, Indian, Siberian, Asian and North American ginseng on serum digoxin measurement by immunoassays and binding of digoxin-like immunoreactive components of ginseng with Fab fragment of antidigoxin antibody (Digibind). *Am J Clin Pathol* 2005; 124: 229–236.
109. Lee AR, Wu WL, Chang WL, Lin HC, King ML. Isolation and bioactivity of new tanshinones. *J Nat Prod* 1987; 50: 157–160.

110. Dasgupta A, Actor JK, Olsen M, Wells A, Datta P. In vivo digoxin-like immunoreactivity in mice and interference of Chinese medicine Danshen in serum digoxin measurement: elimination of interference by using a chemiluminescent assay. *Clinica Chimica Acta* 2002; 317: 231–234.
111. Wahed A, Dasgupta A. Positive and negative in vitro interference of Chinese medicine Danshen in serum digoxin measurement: elimination of interference by monitoring free digoxin concentrations. *Am J Clin Pathol* 2001; 116: 403–408.

# 7 Interferences with Measurement of Anticonvulsants

*William Clarke, PhD*

***Contents***



**Summary**

Anticonvulsant drugs are the frontline therapy for patients with epilepsy. These drugs fit the "profile" of drugs that should be monitored therapeutically where there is a well-defined relationship between blood concentration and pharmacodynamic effects but a lack of good correlation between dose and the serum blood concentration. For many anticonvulsant drugs, a lack of seizure control can occur when blood concentrations are either above or below the optimum therapeutic interval. There are certain clinical situations where anticonvulsant drugs should be monitored, including establishing baseline effective concentrations, evaluating causes for toxicity or lack of efficacy, evaluating non-compliance versus loss of efficacy, minimizing side effects, and evaluating serum levels when the therapeutic regimen is changed. There are a wide variety of assays available for measuring blood concentrations of anticonvulsants. For chromatographic methods (including those coupled to mass spectrometry), potential interferences come from serum components and other drugs with extraction, or co-eluting substances during chromatography. Antibody-based methods for measurement of drug concentrations allow for an automated, simple analyses of drugs; however, a different source for assay interference must be considered, because cross-reactivity of drugs (or metabolites) with similar structural epitopes must be considered.

**Key Words:** Anticonvulsants; phenytoin; carbamazepine; valproic acid; Phenobarbital.

## 1. INTRODUCTION

Epilepsy is a disease characterized by unprovoked patient seizures. Seizures are classified as partial seizures (localized within the brain) and generalized seizures (involving both hemispheres of the brain). Partial seizures are further split into simple and complex categories; simple seizures are those that do not impair consciousness, whereas the complex ones do impair consciousness. Generalized seizures are split into

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a number of categories, including absence (patient becomes vacant and unresponsive), myoclonic (brief muscle contraction), clonic (myoclonus regularly repeating at a rate of 2–3 per second), tonic-clonic (initial spasm, followed by rhythmic convulsions), and atonic (loss of muscle tone, causing the patient to fall to the ground). Lastly, there is a condition called status epilepticus that refers to continuous seizure activity with no recovery between successive seizures, and this can be life threatening requiring emergency medical attention.

According to the Epilepsy Foundation, it is estimated that there are approximately 2.7 million people affected by epilepsy in the USA, with approximately 200,000 new cases of epilepsy and seizures reported every year, and the World Health Organization (WHO) estimates that as many as 50 million people worldwide may be affected by epilepsy. The cause of epilepsy can be attributed to structural or metabolic abnormalities in the brain stemming from acute injury such as stroke or trauma, infectious disease, and neurosurgical complications. Alternatively, it can be caused by an inherited condition such as ring chromosome 20 syndrome. In some cases, epilepsy is classified as idiopathic, meaning that it is of unknown origin.

The frontline therapy for epilepsy is pharmacotherapy. The first drug for epilepsy, bromide, was introduced in 1857, followed by drugs such as phenobarbital, phenytoin, primidone, ethosuximide, carbamazepine, valproic acid, clobazam, and clonazepam. These drugs are the first generation of antiepileptic drugs. The next generation of anticonvulsants began to be introduced in 1990, after a gap of almost 20 years between development and introduction. These include drugs such as vigabatrin, lamotrigine, gabapentin, topiramate, tiagabine, levetiracetam, oxcarbazepine, and zonisamide. Some of these newer anticonvulsant drugs are also utilized to manage patients with chronic neuropathic pain.

## 2. MONITORING OF ANTICONVULSANTS

Monitoring of serum drug concentrations for anticonvulsants was proposed and pioneered by Charles Pippenger and Harvey Kupferburg in the early 1970s *(1–3)*. These initial papers dealt with developing methods to monitor phenytoin, primidone, phenobarbital, as well as carbamazepine, using gas–liquid chromatography (GC) techniques. The rationale for these initial studies was that patients could not be managed solely by dosing anticonvulsant drugs based on milligram per kilogram of body weight. Feldman and Pippenger *(4)* made the observation that several patients undergoing treatment for epilepsy remained seizure free despite levels well below the "optimal therapeutic interval" established at that time. In addition, anticonvulsant drugs fit the "profile" of drugs that should be monitored in therapeutic settings because there is a well-defined relationship between blood concentration and pharmacodynamic effects but a lack of good correlation between dose and the blood concentration in a patient. It should be noted that for many anticonvulsant drugs, lack of seizure control can occur when blood concentrations are either above or below the optimum therapeutic interval. Historically, specialists in the field of epilepsy have minimized the utility of therapeutic drug monitoring (TDM). However, as the field of TDM in epilepsy has evolved, it has been established that there are specific clinical situations where it can be useful in treatment of epilepsy. These include establishing baseline effective concentrations, evaluating causes for toxicity or lack of efficacy, evaluating non-compliance versus

loss of efficacy, minimization of side effects, and evaluation of serum levels when the therapeutic regimen is changed.

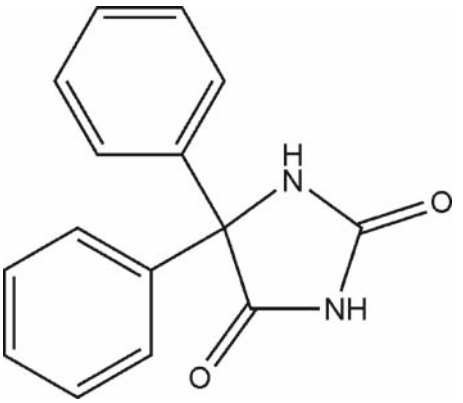
Since then, assays have been developed for many of the currently available anticonvulsant drugs. These include assays based on GC, high-performance liquid chromatography (HPLC), mass spectrometry, and a wide variety of immunochemical assays. For chromatographic methods (including those coupled to mass spectrometry), potential interferences come from serum components and other drugs with extraction, or co-eluting substances during chromatography. The use of antibodies to measure drug levels allows for an automated, simple analysis of drugs; however, it introduces an additional source for assay interference, because cross-reactivity of drugs (or metabolites) with similar structural epitopes must be considered.

## 3. INTERFERENCES WITH COMMONLY MEASURED ANTICONVULSANTS

Immunoassays are frequently used in clinical laboratories for monitoring concentrations of phenytoin, carbamazepine, valproic acid, and phenobarbital in serum or plasma. Immunoassays are subject to interference from drug metabolites and other endogenous factors. These issues are addressed in the sections to follow. Monitoring free concentrations of phenytoin, carbamazepine, and valproic acid are discussed in Chapter 2.

### *3.1. Phenytoin*

Phenytoin (diphenylhydantoin) (Fig. 1) was first introduced as an anticonvulsant agent in 1938, and it is one of the most widely used anticonvulsant drugs. The proposed mechanism of action for phenytoin is to reduce electrical conductance among brain cells, which moderates the runaway brain activity present in seizures. This could be achieved by (a) altering ion fluxes associated with depolarization and/or repolarization, (b) altering membrane stability, (c) influencing calcium uptake in presynaptic terminals, (d) influencing the sodium-potassium ATP-dependent ionic membrane pump or a combination of any of those factors. Side effects occurring at blood concentrations



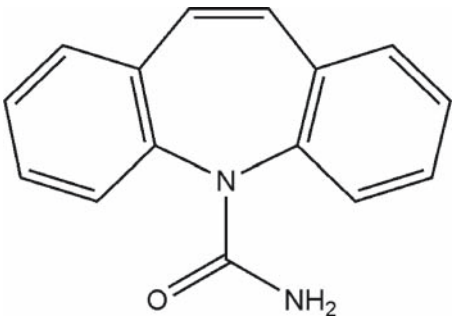

Phenytoin

**Fig. 1.** Phenytoin.

above the optimum therapeutic interval include sedation, ataxia, and paradoxical seizures. Phenytoin is a low-cost drug with a long history of safe use and as a result it is often a first line of defense for seizure patients. It is important to measure the levels of phenytoin in cases where seizures are not controlled to determine whether blood levels are less than therapeutic or whether the seizures are paradoxical from toxic levels of phenytoin. In addition, phenytoin is highly protein bound (~90%), so in cases where toxicity is suspected but total serum phenytoin is within the optimal therapeutic interval, it becomes important to measure free phenytoin levels.

Early methods for measurement of phenytoin were developed using gas chromatography (GC) *(3)* and then HPLC on reversed phase columns to assay a panel of anticonvulsant drugs *(5,6)*. In chromatographic approaches, samples are generally mixed with an internal standard, followed by liquid–liquid extraction and then chromatographic analysis. As previously stated, potential interferences in chromatographic methods stem from effects of serum components and other drugs with extraction, or co-eluting substances during chromatography; however, no interferences of this type were reported for phenytoin. A separate approach to measurement of phenytoin involved development of immunoassays based on antibodies directed toward phenytoin. These assays included the enzyme-labeled immunoassays such as enzyme-multiplied immunoassay (EMIT) and cloned enzyme donor immunoassay (CEDIA), and later fluorescence polarization (FPIA), turbidimetric, and chemiluminescent immunoassays.

One of the primary potential interferences in immunochemical measurements of phenytoin (and free phenytoin) is cross-reactivity of the antibodies with the major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) (Fig. 2) and its glucuronide conjugate *(7–12)*. HPPH is the primary metabolite of phenytoin, and it is readily conjugated to glucuronide (HPPG), which is cleared renally. It is estimated that 60–90% of the administered dose of phenytoin can be recovered in the urine as HPPG *(9)*. This cross-reactivity becomes particularly important in patient with renal insufficiency; as renal clearance of HPPG decreases, the metabolite concentration builds up, and the potential for assay interference increases. Initial studies examining this issue indicated that HPPH and HPPG cross-reactivity in this patient population was not a problem for EMIT-based immunoassays but that the FPIA immunoassay (using TDx analyzer) from Abbott Laboratories, Abbott Park, IL, USA, was affected with respect to both total and free phenytoin measurements *(10)*. In addition, this




Carbamazepine

**Fig. 2.** 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH).

same study discussed two important points: (a) it mentioned that earlier problems of cross-reactivity with an EMIT-based assay were solved by switching to a monoclonal antibody with lower affinity for the metabolites (suggesting that this would solve the same problem in other assays), and (b) this study postulated from their data that there were additional cross-reactants in the specimens because HPPH and HPPG concentrations could not entirely account for the bias that they were observing with respect to HPLC measurements.

These points are important because a follow-up study by the same group *(9)* addressed these same points but come to quite different but interesting conclusions. With respect to monoclonal antibodies, it was noted that the TDx assay had been modified (TDx Phenytoin II assay) to utilize a monoclonal antibody reagent. Later, this was discontinued because of cross-reactivity with the drug oxaprozin (In addition, it was noted that oxaprozin seemed to increase measured free phenytoin concentrations by displacement of phenytoin from binding proteins, as well as cross-reactivity with the antibody reagent). As the authors examined their hypothesis that there were additional cross-reactants other than HPPH and HPPG, they found that a different phenomenon was occurring. They were able to demonstrate instead a concentration-dependent cross-reactivity, where the reaction of HPPH and HPPG with the immunoassay reagents was enhanced by increasing concentrations of phenytoin *(9)*. More recent studies have examined cross-reactivity of these metabolites with a chemiluminescent immunoassay on the IMMULITE 2000 from DPC *(8)* and a turbidimetric immunoassay on the Bayer ADVIA 1650 *(7)*. These studies showed no cross-reactivity of the ADVIA assay with HPPH, and a concentration-dependent cross-reactivity of the IMMULITE assay with HPPH and phenytoin-*N*-glucuronide (an analog in place of HPPG).

Another drug that must be considered for cross-reactivity with phenytoin assays is fosphenytoin (5,5-diphenyl-3-[(phosphonooxy)methyl]-2,4-imidazolidinedione disodium salt). In some cases such as treating patients with status epilepticus, or administration of a loading dose for epileptic patients unable to take oral anticonvulsants, it is desirable to give the drugs through intravenous (IV) or intramuscular (IM) routes of administration. However, phenytoin is poorly soluble in aqueous solution, and it may crystallize in commonly used IV fluids or at the site of IM injection. Fosphenytoin is a phosphate ester derivative of phenytoin that functions as a water-soluble prodrug of phenytoin. This allows the drug to be administered through IV or IM routes. It is rapidly converted to phenytoin after administration (half-life $<15$ min) and provides the anticonvulsant benefits of phenytoin while avoiding complications associated with parenteral administration of phenytoin. Fosphenytoin is not typically monitored clinically because of its short half-life and lack of pharmacological activity.

Cross-reactivity of fosphenytoin with phenytoin immunoassays has been reviewed in the scientific literature for multiple analytical platforms *(8,13–15)* (Table 1). Significant cross-reactivity of fosphenytoin in various degrees was found on the TDx phenytoin *(14)* and phenytoin II *(8,13,15)*, AxSym phenytoin II *(13,15)*, ACS:180 *(13,15)*, Vitros *(15)*, IMMULITE *(8)*, and EMIT 2000 assays *(14)*. The only phenytoin assay that seemed unaffected by the presence of fosphenytoin was the ACA assay from Dade Behring, Newark, DE, USA *(8,15)*. Based on this cross-reactivity, it is recommended that specimens for determination of phenytoin concentrations should not be obtained for patients on fosphenytoin until at least 2 h after IV infusion or 4 h after IM

**Table 1**
**Cross-Reactivity of Fosphenytoin with Phenytoin Immunoassays**

| *Assay* | *Fosphenytoin Cross-Reactivity (%)*[a] | *Fosphenytoin Cross-Reactivity (%)*[b] | *Reference* |
|---|---|---|---|
| TDx | 32–42 | 59–75 | 13 |
| TDx-II | >250 | >250 | 13 |
| Axsym-II | 17–29 | 30–78 | 13 |
| ACS:180 | 48–52 | 50–51 | 13 |
| ACA Star | 8–14 | 2–8 | 15 |
| TDX-II | 518 | Not reported | 15 |
| Vitros | 6–7 | 2–5 | 15 |
| TDx | 22–120 | 32–451 | 14 |
| EMIT 2000 | 7–13 | –20 to 50 | 14 |
| IMMULITE 2000 | 64 | Not reported | 8 |

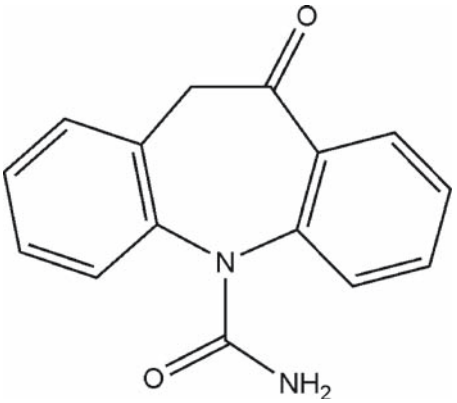
[a] Cross-reactivity in the absence of phenytoin.
[b] Cross-reactivity in the presence of phenytoin.

injection. Also, incubating 1 ml specimen with 10 μl of alkaline phosphatase enzyme (Sigma Chemical Company, St. Louis, MO, USA) converts any fosphenytoin present in the specimen to phenytoin within 5 min at room temperature. This procedure eliminates interference of fosphenytoin in phenytoin immunoassays. The authors observed complete conversion of fosphenytoin to phenytoin by alkaline phosphatase in heparin, ethylenediaminetetraacetic acid (EDTA), and citrated plasma *(16)*.

Roberts et al. *(15)* studied in detail falsely elevated phenytoin values when measured by immunoassays compared with HPLC in patients with renal failure. The authors observed falsely increased phenytoin results up to 20 times higher than the HPLC results using AxSYM, TDx Phenytoin II (Abbott Laboratories, Abbott Park, IL), ACS:180 (Bayer Diagnostics, Tarrytown, NY), and Vitros assays. The ACA star results were comparable to HPLC values. Interestingly, no fosphenytoin was detected in any of these specimens by using HPLC. For example, in the renal failure patient 3 on the 9th day of the hospital stay (300 mg of fosphenytoin dosage), the phenytoin concentration as measured using the HPLC was 5.3 μg/mL. The corresponding phenytoin concentrations measured by immunoassays were 6.3 (ACA Star), 22.0 (ACS:180), 12.7 (AxSYM), and 28.0 μg/mL (TDxII) respectively. On the basis of their study with several patients, the authors proposed the presence of a novel metabolite of fosphenytoin, which has a very high cross-reactivity with antibodies, used in several immunoassays for phenytoin *(15)*. Later, Annesley et al. identified a unique immunoreactive oxymethylglucuronide metabolite derived from fosphenytoin in sera of uremic patients and explained the mechanism of falsely elevated phenytoin in these patients with uremia receiving fosphenytoin *(17)*.

### *3.2. Carbamazepine*

Carbamazepine (Fig. 3) is an anticonvulsant drug that is structurally similar to tricyclic antidepressants and is used in treatment of generalized tonic-clonic, partial, and partial-complex seizures. It was approved for treatment of epileptic patients in



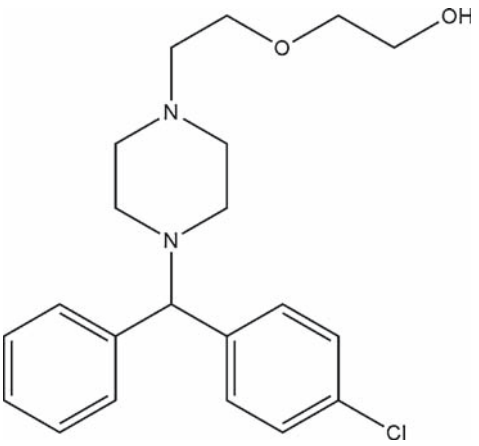

Oxcarbazepine

**Fig. 3.** Carbamazepine.

the USA in 1974 (approved for children over 6 years of age in 1979). Along with phenytoin, carbamazepine is one of the most widely used anticonvulsant drugs. It is also often used in combination therapy with tricyclic antidepressant drugs and can be used in the treatment of neuropathic pain. Like many other anticonvulsant drugs, the pharmacodynamic effects of carbamazepine are better correlated with serum or plasma concentrations rather than drug dosage. The proposed mechanism of action for carbamazepine is that of stabilizing the inactive state of voltage-gated sodium channels in the brain. The result is that brain cells are less excitable, and seizure activity is reduced. Side effects from drug levels exceeding the optimum therapeutic level include loss of coordination, drowsiness, and arrhythmia.

Early methods for monitoring of carbamazepine included GC, HPLC, or GC coupled with mass spectrometry (GC-MS) (18–20). However, most clinical laboratories currently use immunochemical methods for measuring concentrations of carbamazepine in blood *(21)*. In chromatographic based methods, no significant interferences have been reported in the scientific literature with carbamazepine metabolites, but cross-reactivity of carbamazepine metabolites and structurally similar compounds may pose a problem in carbamazepine immunoassays. These include carbamazepine 10, 11-epoxide, oxcarbazepine and its metabolites, as well as hydroxyzine and its metabolites.

The most important metabolite of carbamazepine is carbamazepine 10, 11-epoxide (Fig. 4), which possesses similar pharmacodynamic activity to its parent drug. Carbamazepine is extensively metabolized by the cytochrome P450 enzyme system (CYP3A4 and CYP2C8) to form the epoxide metabolite *(22)*. At steady state, pre-dose concentrations of carbamazepine epoxide should be approximately 20–25% of the parent drug concentration. However, when other drugs are coadministered, the concentration of carbamazepine epoxide can reach much higher levels at steady state because of drug–drug interactions. Quetiapine increases concentrations of epoxide (epoxide to carbamazepine ratio may increase three- to fourfold) and levels of epoxide returned to normal after discontinuation of quetiapine. Quetiapine may inhibit epoxide hydrolase that




Hydroxyzine

**Fig. 4.** Carbamazepine 10, 11-epoxide.

transforms carbamazepine-10, 11-epoxide to carbamazepine 10, 11-*trans*-diol as well as glucuronidation of *trans*-diol *(23)*. Valproic acid also inhibits the glucuronidation of carbamazepine 10, 11-*trans*-diol and probably inhibits the conversion of carbamazepine 10, 11-epoxide to this *trans*-diol thus increasing carbamazepine 10, 11-epoxide concentrations relative to carbamazepine dose in patients receiving both carbamazepine and valproic acid compared with in patients receiving carbamazepine alone *(24,25)*. Valpromide, valnoctamide, and progabide also inhibit epoxide hydrolase thus causing valproic toxicity because of increases in concentrations of carbamazepine 10, 11-epoxide. Inhibition of carbamazepine metabolism and elevation of plasma carbamazepine to potential toxic concentrations can also be due to cotherapy with stiripentol, remacemide, acetazolamide, macrolide antibiotics, isoniazid, metronidazole, verapamil, diltiazem, cimetidine, danazol, or propoxyphene *(26)*.

Phenytoin, phenobarbital, and primidone accelerate metabolism of carbamazepine by inducing cytochrome P450 (CYP) 3A4 and reduce plasma concentrations of carbamazepine to clinically significant levels *(26)*. Serum carbamazepine concentration to dose ratios in patients with carbamazepine polytherapy were decreased while carbamazepine 10, 11 epoxide and *trans* 10-11-dihydroxy-10,11-dihydro-carbamazepine concentrations were increased. The authors concluded that phenytoin has a potent induction effect on carbamazepine epoxidase whereas phenobarbital is a moderate inducer *(27)*. In contrast, Pereira et al. *(28)* reported that lamotrigine did not alter plasma concentrations of carbamazepine significantly. Nevertheless, authors strongly recommended TDM because of narrow therapeutic range of both drugs. There is also no pharmacokinetic interaction between oxcarbazepine and lamotrigine *(29)*.

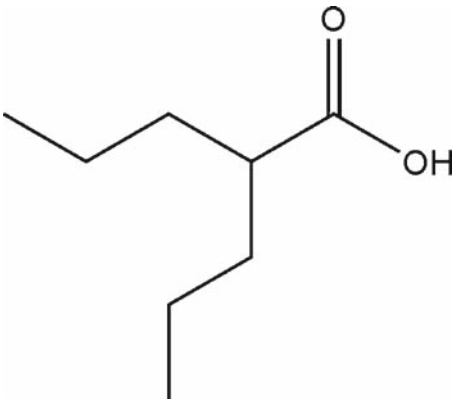
Carbamazepine–indinavir interaction has clinical significance. The indinavir (a protease inhibitor) plasma concentrations in a patient was decreased significantly when carbamazepine was introduced in the drug regime Carbamazepine is a potent inducer of CYP3A enzyme system whereas indinavir is a substrate for that enzyme.

A low-dose carbamazepine (200 mg per day) and the usual dose of indinavir (800 mg q8h) in this patient resulted in carbamazepine concentration within therapeutic range but indinavir concentration was significantly reduced. Authors concluded that concomitant use of carbamazepine and indinavir may cause failure of antiretroviral therapy because of insufficient indinavir plasma concentration, and drugs other than carbamazepine should be considered in prevent this interaction *(30)*.

The cross-reactivity of carbamazepine epoxide in carbamazepine immunoassays has been investigated across numerous analytical platforms (8,21,31–33). The cross-reactivity of carbamazepine 10, 11-epoxide with carbamazepine may vary from 0.0% (Vitros, Rochester, NY, USA) to 93.6% (Dade Behring) with many immunoassays exhibiting low (EMIT; 0.4%, Technicon immuno-1; 1.6% ACS:180; 3.8%, Beckman Synchron; 7.6%) to moderate (Roche Cobas Integra; 10.4%, BDI Opus/plus/magnum; 17.2%, Abbott TDX; 20.8%, Dade ACA; 44.2%) *(34)*. Therefore, authors concluded that Dade Behring's PETINIA assay has significant cross-reactivity with carbamazepine 10, 11-epoxide and provides an estimate of both the parent drug and the metabolite *(34)*. Currently, there is no commercially available immunoassay for measuring carbamazepine 10, 11-epoxide concentration. However, both HPLC and HPLC combined with mass spectrometric methods have been reported in the literature for simultaneous determination of both carbamazepine and its active metabolites (also see Chapter 3).

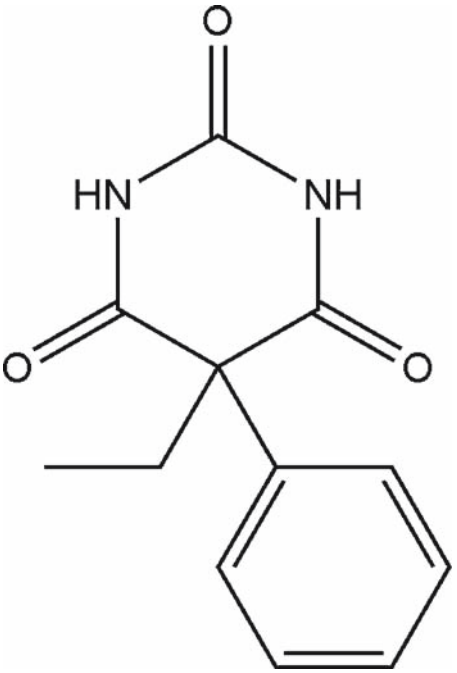
Oxcarbazepine (Fig. 5) is a structurally similar drug to carbamazepine that is used in the treatment of epilepsy. In some cases, both drugs and their metabolites may both be present in patients who are transitioning from one therapeutic regimen to the other. In a study of whether oxcarbazepine or its metabolites cross-reacted with an EMIT carbamazepine assay, it was shown that from a clinical perspective that only the 10-hydroxy-10,11-dihydro-carbamazepine metabolite of oxcarbazepine had any significant cross-reactivity with the assay whereas there was no significant interference from oxcarbazepine *(35)*.

Another study reporting case reports of falsely elevated carbamazepine results associated with the presence of hydroxyzine (Fig. 6)—a benzhydrylpiperazine antihistamine—in the specimen *(36)*. Hydroxyzine is a commonly prescribed first




Valproic Acid

**Fig. 5.** Oxcarbazepine.



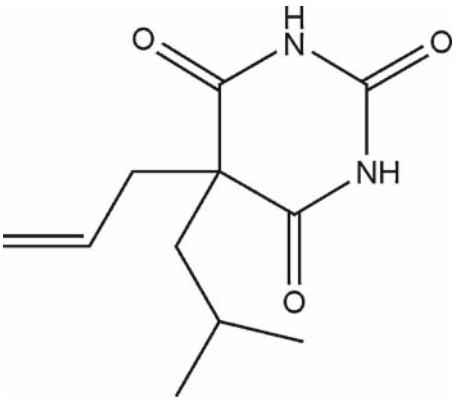

Phenobarbital

**Fig. 6.** Hydroxyzine.

generation antihistamine with sedative properties. Hydroxyzine is also one of the drugs used in the firstline therapy for the treatment of allergic rhinitis and chronic idiopathic urticaria *(37,38)*. Hydroxyzine is structurally unrelated to carbamazepine, but in that report, Parant et al. documented two cases where hydroxyzine in serum caused false-positive carbamazepine levels using the PETINIA (Dade Behring) assay. A 22-year-old female with a hydroxyzine concentration of 1.77 μg/mL and cetirizine concentration of 2.1 μg/mL showed an apparent carbamazepine level of 5.3 μg/mL. Another patient with a hydroxyzine level of 520 ng/mL and cetirizine level of 2.18 μg/mL demonstrated a carbamazepine level of 25.4 μg/mL . The authors of this study also demonstrated cross-reactivity of the PETINIA assay with cetirizine, oxatomide, and other benzhydrylpiperazine drugs. However, EMIT 2000 assay for carbamazepine showed no cross-reactivity *(36)*.

### *3.3. Valproic Acid*

Valproic acid is an 8-carbon branched chain fatty acid (Fig. 7) commonly used in therapy of epileptic patients. This drug is indicated for patients with absence, tonic-clonic, and complex partial seizures. The proposed mechanism of action for valproic acid is interference with the neurotransmitter gamma-aminobutyric acid (GABA). Valproic acid is believed to be a GABA transaminase inhibitor. Valproic acid is highly




Butalbital

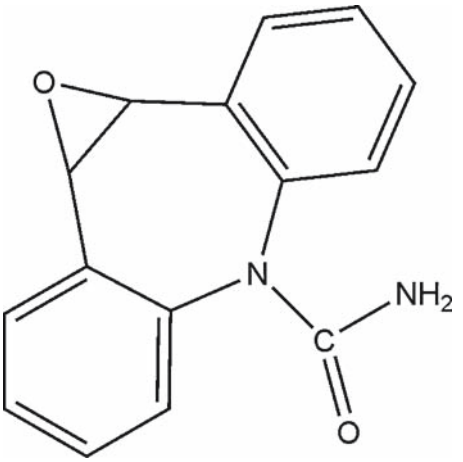
**Fig. 7.** Valproic acid.

metabolized in the liver by either glucuronidation or beta-oxidation. Valproic acid is not enzyme inducer, but it may cause clinically relevant drug interactions by inhibiting the metabolism of selected substrates such as phenobarbital and lamotrigine *(39)*. Side effects of valproic acid include nausea or dyspepsia, sedation, headaches, dizziness, and tremors.

The early method of measuring valproic acid in patient specimens was by gas–liquid chromatography (40–42). However, today it is almost exclusively measured using immunoassay. Two interferences with determination of valproic acid by GC were identified in the mid-1980s. First, Leroux et al. *(40)* demonstrated that there was no interference from hemolyzed, lipemic, or icteric specimens. However, the authors did observe interference when Teflon-lined screw caps were used during the extraction step. It appeared that shavings from the cap would fall into the specimen during the extraction process. This interference was eliminated by using cork stoppers in place of the Teflon-lined caps. A separate study reported interference discovered during proficiency testing sponsored by the Chemical Pathology Quality Assurance Programme Group of the Royal College of Pathologists of Australasia/Australian Association of Clinical Biochemists *(42)*. In the study, the group found octanoic acid contaminant in the control material used for proficiency testing. This was a problem, because the method in question was using octanoic acid as an internal standard. The source of the contamination was not identified, but the problem was solved by choosing a different internal standard. There are no currently documented interferences with immunoassays for measurement of valproic acid.

### *3.4. Phenobarbital*

Phenobarbital (Fig. 8) is a member of the barbiturate family of drugs that was initially introduced as a sedative and hypnotic drug in 1912. A few years later, its utility as an anticonvulsant drug was discovered when it was prescribed as a tranquilizer for epileptic patients. Phenobarbital is indicated for all types of seizures, with the exception of absence seizure. It is the oldest anticonvulsant drug still in use and is the recommended frontline therapy in developing countries; however, it is no longer




10,11-carbamazepine epoxide

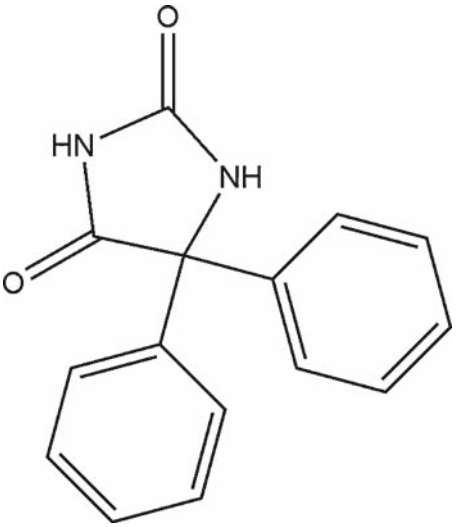
**Fig. 8.** Phenobarbital.

recommend as the first or second choice for seizure control in affluent countries. Phenobarbital is primarily metabolized by CYP2C19 and is a known inducer of most of the enzymes in the CP450 family. When serum levels are above the optimal therapeutic interval, toxic effects include decreased consciousness, slowing of heart rate, shallow breathing, and even edema and renal failure in severe overdose.

Early measurements of phenobarbital in blood were performed using GC *(43)* or HPLC *(5)*. However, as with valproic acid, today, most measurements of phenobarbital in blood are made using commercial immunoassays. One early report demonstrated interference from ethotoin in measurement of phenobarbital using HPLC *(5)*. However, as with most chromatographic methods, interferences can be easily overcome by changing experimental conditions (e.g., changing column type, mobile phase composition). There are few reported cross-reactive interferences for phenobarbital immunoassays. One report demonstrated cross-reactivity for amobarbital, butobarbital, secobarbital, and phenytoin *(44)*. However, these interferences occurred only at concentrations that were beyond toxic levels and at "therapeutic concentrations"; these drugs did not interfere with the assay. There is one case study reported by Nordt *(45)* that demonstrates a possible cross-reactivity of butalbital (Fig. 9) at therapeutic concentrations, but this was not confirmed.

### *3.5. Other Anticonvulsant Drugs*

Although there are other commercial and reference laboratory assays available for anticonvulsant drugs, there have been no significant interference reported for them in the scientific literature. There have recently been two immunoassays released by Seradyn for newer anticonvulsants—zonisamide and topiramate. There are no reports of cross-reactivity or assay interference in the literature for the topiramate assay, but the package insert reports a cross-reactivity of approximately 10% for the metabolite




HPPH

**Fig. 9.** Butalbital.

9-hydroxytopiramate. The package insert for zonisamide reports negligible cross-reactivity for the metabolite *N*-acetyl zonisamide and a small degree (but clinically insignificant) of cross-reactivity with the metabolite 2-sulfamoylacetyl phenol.

## 4. CONCLUSION

Anticonvulsants are a good example of drugs that should be monitored therapeutically, because there is a well-defined relationship between blood concentration and therapeutic effects, but a lack of good correlation between dose and the serum blood concentration. Clinical situations where anticonvulsant drugs should be monitored include establishing baseline effective concentrations, evaluating causes for toxicity or lack of efficacy, evaluating non-compliance versus loss of efficacy, minimization of side effects, and evaluation of serum levels when the therapeutic regimen is changed. Many different methods have been developed for measuring blood concentrations of anticonvulsants. In chromatographic methods (including those coupled to mass spectrometry), potential interferences come from serum components and other drugs with extraction, or co-eluting substances during chromatography. Immunoassays for measurement of drug concentrations allow for an automated, simple analyses of drugs; however, a different source for assay interference must be considered, because cross-reactivity of drugs (or metabolites) with similar structural epitopes must be considered. Important cross-reactivities have been discovered for certain assays including HPPH, HPPG, and fosphenytoin for selected phenytoin assays. In certain carbamazepine immunoassays, there is cross-reactivity with 10,11-carbamazepine epoxide, as well as from benzhydrylpiperazine antihistamine drugs. Minor, but clinically insignificant,

cross-reactivities have been reported for phenobarbital and valproic acid assays. It is important to be aware of such interferences in anticonvulsant assays and to have a sense of how such factors will affect patient care.

## REFERENCES


1. Kupferberg HJ. Quantitative estimation of diphenylhydantoin, primidone and phenobarbital in plasma by gas-liquid chromatography. *Clin Chim Acta* 1970;29:282–288.
2. Kupferberg HJ. GLC determination of carbamazepine in plasma. *J Pharm Sci* 1972; 61:284–286.
3. Pippenger CE, Gillen HW. Gas chromatographic analysis for anticonvulsant drugs in biologic fluids. *Clin Chem* 1969; 15:582–590.
4. Feldman RG, Pippenger CE. The relation of anticonvulsant drug levels to complete seizure control. *J Clin Pharmacol* 1976; 16:51–59.
5. Kabra PM, Stafford BE, Marton LJ. Simultaneous measurement of phenobarbital, phenytoin, primidone, ethosuximide, and carbamazepine in serum by high-pressure liquid chromatography. *Clin Chem* 1977; 23:1284–1288.
6. Turnell DC, Trevor SC, Cooper JD. A rapid procedure for the simultaneous estimation of the anticonvulsant drugs, ethosuximide, phenobarbitone, phenytoin, and carbamazepine in serum using high-pressure liquid chromatography. *Ann Clin Biochem* 1983; 20 Pt 1:37–40.
7. Datta P, Scurlock D, Dasgupta A. Analytic performance evaluation of a new turbidimetric immunoassay for phenytoin on the ADVIA 1650 analyzer: effect of phenytoin metabolite and analogue. *Ther Drug Monit* 2005; 27:305–308.
8. Frank EL, Schwarz EL, Juenke J, Annesley TM, Roberts WL. Performance characteristics of four immunoassays for antiepileptic drugs on the IMMULITE 2000 automated analyzer. *Am J Clin Pathol* 2002;118:124–131.
9. Rainey PM, Rogers KE, Roberts WL. Metabolite and matrix interference in phenytoin immunoassays. *Clin Chem* 1996; 42:1645–1653.
10. Roberts WL, Rainey PM. Interference in immunoassay measurements of total and free phenytoin in uremic patients: a reappraisal. *Clin Chem* 1993; 39:1872–1877.
11. Roberts WL, Rainey PM. Phenytoin overview–metabolite interference in some immunoassays could be clinically important. *Arch Pathol Lab Med* 2004; 128:734; author reply: 5.
12. Soldin SJ, Wang E, Verjee Z, Elin RJ. Phenytoin overview–metabolite interference in some immunoassays could be clinically important: results of a College of American Pathologists study. *Arch Pathol Lab Med* 2003; 127:1623–1625.
13. Datta P, Dasgupta A. Cross-reactivity of fosphenytoin in four phenytoin immunoassays. *Clin Chem* 1998;44:696–697.
14. Kugler AR, Annesley TM, Nordblom GD, Koup JR, Olson SC. Cross-reactivity of fosphenytoin in two human plasma phenytoin immunoassays. *Clin Chem* 1998;44:1474–1480.
15. Roberts WL, De BK, Coleman JP, Annesley TM. Falsely increased immunoassay measurements of total and unbound phenytoin in critically ill uremic patients receiving fosphenytoin. *Clin Chem* 1999; 45:829–837.
16. Dasgupta A, Warner B, Datta P. Use of alkaline phosphatase to correct underestimation of fosphenytoin concentrations in serum measured by phenytoin immunoassays. *Am J Clin Pathol* 1999; 111: 557–562.
17. Annesley T, Kurzyniec S, Nordblom G, et al. Glucuronidation of prodrug reactive site: isolation and characterization of oxymethylglucuronide metabolite of fosphenytoin. *Clin Chem* 2001:46: 910–918.
18. Hallbach J, Vogel H, Guder WG. Determination of lamotrigine, carbamazepine and carbamazepine epoxide in human serum by gas chromatography mass spectrometry. *Eur J Clin Chem Clin Biochem* 1997; 35:755–759.
19. Jurgens U, May T, Hillenkotter K, Rambeck B. Systematic comparison of three basic methods of sample pretreatment for high-performance liquid chromatographic analysis of antiepileptic drugs using gas chromatography as a reference method. *Ther Drug Monit* 1984; 6:334–343.
20. Lensmeyer GL. Isothermal gas chromatographic method for the rapid determination of carbamazepine (“tegretol”) as its TMS derivative. *Clin Toxicol* 1977; 11:443–454.

21. Shen S, Elin RJ, Soldin SJ. Characterization of cross reactivity by carbamazepine 10,11-epoxide with carbamazepine assays. *Clin Biochem* 2001; 34:157–158.
22. Bertilsson L, Tomson T. Clinical pharmacokinetics and pharmacological effects of carbamazepine and carbamazepine-10,11-epoxide. An update. *Clin Pharmacokinet* 1986;11:177–198.
23. Fitzgerald BJ, Okos AJ. Elevation of carbamazepine-10, 11-epoxide by quetiapine. *Pharmacotherapy* 2002; 22:1500–1503.
24. Bernus I, Dickinson RG, Hooper WD, Eadie MJ. The mechanism of the carbamazepine-valproate interaction in humans. *Br J Clin Pharmacol* 1997; 44:21–27.
25. Robbins DK, Wedlund PJ, Kuhn R, Baumann RJ, Levy RH, Chang SL. Inhibition of epoxide hydrolase by valproic acid in epileptic patients receiving carbamazepine. *Br J Clin Pharmacol* 1990; 29:759–762.
26. Spina E, Pisani F, Perucca E. Clinically significant pharmacokinetic drug interactions with carbamazepine: an update. *Clin Pharmacokinetic* 1996; 31:198–214.
27. Liu H, Delgado MR. Interactions of phenobarbital and phenytoin with carbamazepine and its metabolites' concentrations, concentration ratios, and level/dose ratios in epileptic children. *Epilepsia* 1995; 36:249–254.
28. Pereira LR, Velasco TR, Ceiki-Sakamota A, de Carvalho D. Evaluation of the drug interaction between carbamazepine and lamotrigine in the treatment of refractory epilepsy patients. *Rev Neurol* 2006; 43:74–77 [Article in Spanish].
29. Theis JG, Sidhu J, Palmer J, Job S, Bullman J, Ascher J. Lack of pharmacokinetic interaction between oxcarbazepine and lamotrigine. *Neuropsychopharmacology* 2005; 30:2269–2274.
30. Hugen P, Burger DM, Brinkman K, ter-Hofstede HJ, Schuurman R, Koopmans PP, Hekester YA. Carbamazepine-indinavir interaction causes antiretroviral therapy failure. *Ann Pharmacother* 2000; 34:465–470.
31. Dasgupta A, Datta P. Analytic performance evaluation of a new turbidimetric immunoassay for carbamazepine on the ADVIA 1650 analyzer: effect of carbamazepine 10,11-epoxide. *Ther Drug Monit* 2005; 27:31–34.
32. Parant F, Bossu H, Gagnieu MC, Lardet G, Moulsma M. Cross-reactivity assessment of carbamazepine-10,11-epoxide, oxcarbazepine, and 10-hydroxy-carbazepine in two automated carbamazepine immunoassays: PETINIA and EMIT 2000. *Ther Drug Monit* 2003; 25:41–45.
33. Wilson JF, Tsanaclis LM, Williams J, Tedstone JE, Richens A. Evaluation of assay techniques for the measurement of antiepileptic drugs in serum: a study based on external quality assurance measurements. *Ther Drug Monit* 1989; 11:185–195.
34. Hermida J, Tutor CJ. How suitable are currently used carbamazepine immunoassays for quantifying carbamazepine 10, 11-epoxide in serum samples? *Ther Drug Monit* 2003; 25:384–388.
35. Kumps A, Mardens Y. Cross-reactivity assessment of oxcarbazepine and its metabolites in the EMIT assay of carbamazepine plasma levels. *Ther Drug Monit* 1986; 8:95–97.
36. Parant F, Moulsma M, Gagnieu MC, Lardet G. Hydroxyzine and metabolites as a source of interference in carbamazepine particle-enhanced turbidimetric inhibition immunoassay (PETINIA). *Ther Drug Monit* 2005; 27:457–462.
37. Jauregui Presa I. H1 antihistamines: a review. *Allergol Immunol Clin* 2004; 14:300–312.
38. Morgan MW, Khan D, Nathan RA. Treatment for allergic rhinitis and chronic idiopathic urticaria: focus on oral antihistamines. *Ann Pharmacother* 2005; 39:2056–2064.
39. Perucca E. Clinically relevant drug interactions with antiepileptic drugs. *Br J Clin Pharmacol* 2006; 61:246–255.
40. Leroux M, Budnik D, Hall K, Irvine-Meek J, Otten N, Seshia S. Comparison of gas-liquid chromatography and EMIT assay for serum valproic acid. *Clin Biochem* 1981; 14:87–90.
41. Loscher W. Rapid determination of valproate sodium in serum by gas-liquid chromatography. *Epilepsia* 1977; 18:225–227.
42. Webb T. Identification of interference affecting the gas-liquid chromatography analysis of valproic acid in quality control material. *Pathology* 1986; 18:307–309.
43. Flanagan RJ, Withers G. A rapid micro-method for the screening and measurement of barbiturates and related compounds in plasma by gas-liquid chromatography. *J Clin Pathol* 1972; 25:899–904.

44. Ammann H, Vinet B. Accuracy, precision, and interferences of three modified EMIT procedures for determining serum phenobarbital, urine morphine, and urine cocaine metabolite with a Cobas-Fara. *Clin Chem* 1991; 37:2139–2141.
45. Nordt SP. Butalbital cross-reactivity to an Emit assay for phenobarbital. *Ann Pharmacother* 1997; 31:254–255.

# 8 Pitfalls in Measuring Antidepressant Drugs

*Uttam Garg, PhD*

***Contents***



**Summary**

Depressions being a major problem, antidepressants are one of the most frequently used drugs in the clinical practice. Antidepressants are commonly measured in the laboratory for the purpose of therapeutic drug monitoring. Widely used antidepressants include tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). Owing to narrow therapeutic range and higher toxicity, TCAs are a common cause of morbidity and mortality. In emergency situations, the assays of TCAs on urgent basis are needed. Although a number of assays are available for TCA, immunoassays are suitable for screening purpose in case of a suspected overdose. However, immunoassays for TCA also suffer from many limitations including cross-reactivity of active metabolites and are not suitable for therapeutic drug monitoring. Limitations of various assays for estimation of antidepressants and some tips to deal with these limitations are described.

**Key Words:** Tricyclic antidepressants; SSRIs; immunoassays; interference.

## 1. INTRODUCTION

Depression affects approximately 10% of men and 20% of women during their lifetime and 3% of the population at any given time *(1)*. It is estimated that 10–15% of the prescriptions written in the USA are for major depression. The patients with depression are at higher risk of suicide and development of cardiovascular disease and myocardial infarction. The World Health Organization predicts that by 2020 depression will be the second (first being heart disease) leading cause of premature death or disability in adults. Therefore, financial and socioeconomic consequences of

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depression are very high, and need for antidepressants have been at an all time high. A large number of antidepressants including tricyclic antidepressants (TCAs), monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRIs), and atypical agents are available for treatment of depression *(2,3)*. Although therapeutic ranges for some of these antidepressants is not very well established, need for therapeutic drug monitoring for most of them has been well documented. The antidepressants for which therapeutic drug monitoring is well documented are discussed below in section 2.

## 2. TRICYCLIC ANTIDEPRESSANTS

TCAs, including amitriptyline, doxepin, nortriptyline, imipramine, desipramine, protriptyline, trimipramine, and clomipramine, were introduced in the 1950s and 1960s. As the name indicates, TCAs have three-ring structure. Although the exact mechanism of their action is not well understood, they are known to inhibit reuptake of norepinephrine and/or serotonin resulting in increased concentration of these neurotransmitters in the synapse. Despite rapid affect on neurotransmitters uptake, their clinical effect may not be seen for weeks after initiation of therapy, indicating that the mechanism of action of TCAs is more complicated than simply increasing the concentrations of neurotransmitters. This is further substantiated by the fact that not all the compounds that inhibit neurotransmitter uptake are antidepressants (e.g., cocaine and amphetamines). In addition to their use as antidepressants, TCAs are used in the treatment of obsessive-compulsive disorder, attention-deficit hyperactivity disorder, school phobia, and separation anxiety in the pediatric population. In adults, they are also used in treatment of neuralgic pain, chronic pain, and migraine prophylaxis, amongst many others *(2,4)*.

Although effective in the treatment of a number of disorders, TCAs are associated with high morbidity and mortality because of their side effects *(5,6)*. These side effects include hypotension, dizziness, and sedation by blocking $\alpha_1$ adrenoreceptors; weight gain and sedation through $H_1$ histamine receptors; and dry mouth, blurred vision, constipation, and urinary retention through $M_1$ muscarinic receptors. TCAs are known to lower thresholds for seizures. TCAs were the third leading cause of toxic exposures in 2004 after analgesics and sedatives *(7)*.

### *2.1. Pharmacokinetics and Metabolism of TCAs*

Most of TCAs are well absorbed and reach peak plasma concentrations within 2–12 h. Owing to their lipophilic properties, they have a very large volume of distribution. Many of TCAs are tertiary amines and are metabolized by the cytochrome P450 isoenzyme system to secondary amines, which are also active. Some TCAs are metabolized to hydroxylated metabolites that may also be active and are further metabolized by glucuronidation. Some pharmacokinetics properties of TCAs are summarized in Table 1. When interpreting data, it is important to keep in mind that these values, at best, are approximate, and there is a considerable inter-individual variability. Some of the factors responsible for these variations are discussed below in section 2.2.

**Table 1**
**Pharmacokinetic Properties of tricyclic antidepressants (TCAs)**

| *Drug* | *Active metabolite* | *Average Half-Life (hours)* | *Vd (L/kg)* | *Oral Bioavail-ability* | *Average Protein Binding* | *Therapeutic Range (ng/mL)* |
|---|---|---|---|---|---|---|
| Amitriptyline | Nortriptyline | 21 | 15 | 50 | 95 | 120–250[a] |
| Desipramine | NA | 20 | 42 | 40 | 80 | 75–300 |
| Doxepin | Nordoxepin | 17 | 20 | 27 | 90 | 150–250[a] |
| Imipramine | Desipramine | 12 | 18 | 40 | 90 | 150–250[a] |
| Nortriptyline | NA | 30 | 18 | 50 | 92 | 50–150 |
| Protriptyline | NA | 80 | 13 | 75 | 95 | 70–250 |
| Trimipramine | NA | 27 | 32 | 50 | 90 | 100–250 |

Vd = Volume of distribution.
NA, no significantly active metabolite.
[a] It is total concentration of the antidepressant and its active metabolite. TCA concentrations >500 ng/mL should be considered potentially toxic.

### *2.2. Therapeutic Drug Monitoring of TCAs*

Studies have shown that specific changes in ECG such as prolonged QRS interval >100 ms and a terminal 40 ms right axis deviation >120° are reliable predictors of serious cardiovascular and neurological toxicity of TCAs *(8)*. It should be noted that studies have also shown that TCA plasma levels may not correlate very well with clinical outcome and toxicity, particularly in overdose situations. On the basis of these findings, some clinicians question the value of therapeutic drug monitoring for TCAs. Despite these arguments, the measurement of TCA levels is considered unequivocally useful for the following reasons:

When taken orally, TCAs are well absorbed but may undergo considerable first-pass metabolism and thus have considerable variability in bioavailability. Their bioavailability and absorption is also variable as these drugs, because of their anticholinergic properties, slow down gastrointestinal activity and gastric emptying. Owing to high lipophilicity, these drugs bind to plasma proteins and tissues, resulting in a high volume of distribution. A number of tertiary TCAs are demethylated by the cytochrome P-450 enzyme system into their respective secondary amine active metabolites. For example, tertiary amine TCAs such as amitriptyline, imipramine, and doxepin are metabolized to their respective active secondary amines nortriptyline, desipramine, and nordoxepin. Tertiary and secondary amine TCAs have approximately similar activity. Some TCAs are hydroxylated into active metabolites, although these metabolites are not generally measured in clinical laboratories. Some pharmacokinetic properties of TCAs are summarized in Table 1.

Metabolism of TCAs varies significantly with age. Preskorn et al. *(9)* examined steady state concentrations of imipramine and its metabolite desipramine in hospitalized children. The concentrations of imipramine and desipramine varied 12- and 72-fold, respectively. Race also plays an important role in the metabolism of TCAs. African Americans and Japanese usually demonstrate higher concentrations of TCAs compared with Caucasians. Asians and Hispanics respond to lower doses of TCAs because of hypersensitivity receptors *(10)*.

Alcoholics have significantly higher clearance of TCAs compared with subjects who do not abuse alcohol. Half-lives for imipramine and desipramine in alcoholics were 10.9 and 16.5 hours compared with 19.6 and 22.5 h in healthy controls, respectively. Unbound fractions of drug in plasma were decreased in the alcoholic group for both imipramine and desipramine. Taken together, these findings suggest that an alcoholic person who has recently undergone a detoxification program may require higher doses and frequent levels of TCAs *(11)*.

Tamayo investigated the incidence of potentially toxic serum levels (>400 ng/mL) in 196 patients on a standard dosage regimen (75–225 mg/day) of several TCAs: imipramine, amitriptyline, nortriptyline, maprotiline, and clomipramine *(12)*. The serum concentrations of drugs in these patients ranged from 403 to 1776 ng/mL. Despite the detection of higher than therapeutic and even toxic concentrations of TCAs, only 23% of the patients showed clinical symptoms of toxicity. The factors that appeared to contribute to higher levels included interactions of TCAs with neuroleptic agents, age, and administration of doses above 2.5 mg/kg/day. In 64% of the patients, the clinical criteria suggested the need for a reduction in the dose. Although there was a lack of very good correlation between therapeutic and toxic levels and clinical efficacy as well as toxicity, the study points out the need to avoid such high concentrations in light of adequate antidepressant response. This is feasible through therapeutic drug monitoring.

Muller et al. *(13)* compared doses of TCAs given to patients based on clinical judgment and serum levels. In their study, although serum levels of TCAs were analyzed in both groups, the feedback and dose recommendations were only provided for the later group. The outcome was measured weekly using the Hamilton Depression Rating Scale and the Clinical Global Impressions Scale. The study concluded that treating depression with TCAs could be optimized by early therapeutic drug monitoring, which is superior to the clinical judgment.

TCAs are metabolized by the cytochrome P450 enzyme system particularly CYP2D6 which is highly polymorphic. Owing to significant variability in CYP2D6, among various populations, the need for therapeutic drug monitoring of TCAs is even stronger. It is estimated that 5–10% of Caucasians have CYP2D6 gene deletion and 3–8% have gene duplication. The rates of gene duplication are higher in other ethnic groups: 20% in Saudi Arabians *(14)* and 29% in Ethiopians *(15)*. Dose recommendations and therapeutic drug monitoring of antidepressants based on genotypes are thus proposed. In addition to genetics factors, there are a number of acquired factors including co-medications, diet, smoking habit, impaired organ functions such as renal and liver, and compliance which influence blood levels of TCAs, thus justify the need for therapeutic drug monitoring. Cytochrome CYP2D6-inducing drugs such as carbamazepine, phenobarbital, phenytoin, and rifampin increase clearance of TCAs. In contrast, the drugs that inhibit CYP2D6 decrease clearance of TCAs. These drugs include amiodarone, bupropion, celecoxib, chlorpromazine, cimetidine, citalopram, doxorubicin, haloperidol, quinidine, ranitidine, ritonavir, terbinafine, ticlopidine, and histamine H1 receptor antagonists *(16,17)*.

In addition, when a patient fails to respond to TCA therapy, it is important to document that a specific level of the drug in serum is achieved. Similarly, measurement of blood concentrations makes the basis of overdose and toxicity management. Also, therapeutic drug monitoring provides a basis for optimal dosage and to attain a

particular level on which the patient responds clinically in case there are changes in the patient's metabolism due an illness or drug–drug interactions. The other factors that justify TDM for TCAs include compliance, variation in bioavailability between different brands, patient's age, race, and change in lifestyle such as weight loss/gain, smoking, and exercise.

Laboratory practice guidelines for TDM for antidepressants including TCAs are provided by the National Academy of Clinical Biochemistry *(16)*. TDM of the TCAs should be initiated once steady state is achieved, which takes about average of 5 days. For routine monitoring, samples should be collected during the terminal elimination phase, 1–14 h after the last dose for once-daily dosing and 4–6 h after the last dose for divided daily dosing. When patients are treated with the tertiary amines, because of their substantial contribution to pharmacological activity, the secondary amine metabolites should also be measured. The secondary amines are further metabolized to hydroxy metabolites, which should be monitored only in specific cases of renal impairment where these metabolites may accumulate and thus contribute significantly in drug toxicity.

## 3. LABORATORY ANALYSIS OF TCAs

It is important to stress the need to avoid serum or plasma separator tubes when collecting samples for analysis of TCAs. Nyberg and Martensson *(18)* studied several types of blood collection tubes for stability of amitriptyline, imipramine, clomipramine, and their mono-demethylated metabolites collected in these tubes. Ethylenediaminetetraacetic acid (EDTA) tubes were most suitable and serum separator gel tubes were unsuitable because of loss of more than 40% of drug concentrations on storage. The losses were not caused by redistribution between blood cells and plasma but occurred mainly because of sample contact with serum or plasma separator gel or the caps of the tubes. Dasgupta et al. *(19)* studied Greiner serum separator gel tubes for stability of TCAs and many other drugs, and concluded that these tubes are not suitable for blood collection for analysis of TCAs.

Qualitative, semiquantitative, and quantitative methods are available for the analyses of TCAs. There are no reliable spot tests available for TCAs. Common methods of analysis for TCAs include immunoassays, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC). Immunoassays provide a rapid method for determination of TCAs. Owing to their faster turnaround time and unavailability of specific methods on an emergency basis, immunoassays provide rapid clinically useful information, particularly in overdose situations. Two commonly used formats are individual assays for a particular TCA and "total TCAs." The individual immunoassays employ monoclonal antibodies whereas "total TCAs" assays utilize polyclonal antibodies. The problem with these assays is the considerable cross-reactivity of the tertiary and secondary amines, for example, imipramine and amitriptyline cross-react with each other in their individual assays. Similarly, at therapeutic concentration, desipramine cross-reacts with nortriptyline assay and vice versa. Many structurally related drugs, including clomipramine, cyclobenzaprine, doxepin, and chlorpromazine also cross-react with these assays. Although these assays may provide reliable results in patients treated with monotherapy, these assays are not suitable in patients receiving any drug, which may cross-react

with the assays. Owing to common interferences in immunoassays, both positive and negative data should be interpreted according to manufacturer guidelines and in context to clinical information.

TLC can reliably detect TCAs. One of the common TLC systems is Toxi-Lab (Varian Inc., Palo Alto, CA). Gas chromatographic methods coupled with flame ionization detector (FID), nitrogen phosphorus detector (NPD), or mass spectrometer (MS) are widely used for screening and quantification of TCAs. Owing to their lipophilic characters, TCAs are good candidates for liquid–liquid extraction, although numbers of solid-phase extraction procedures have also been reported. Columns are typically fused silica capillary columns with bonded non-polar to intermediate polarity methyl silicone liquid phases (0–50% phenyl). Although sample derivatization is not necessary, it improves chromatographic separation. Trifluoroacetyl and heptafluorobutyryl are commonly used derivatives. However, these derivatives are not very stable. Way et al. *(20)*, using stable isotope dilution GC-MS, found that 4-carbethoxyhexafluorobutyryl chloride derivatives are more stable than trifluoroacetyl derivatives.

HPLC is another widely used method for the analysis of TCAs. Problems posed in GC by polar secondary amines and hydroxy metabolites are easily overcome by HPLC. As reported on CAP proficiency-testing surveys, HPLC with UV detection is the most common method for quantitative analysis of TCAs. The columns predominantly used in HPLC are C18, C8, phenyl, and CN, and permit simultaneous determination of tertiary and secondary amines. Common mobile phases are phosphate buffers with or without ion-pairing agents. HPLC methods with normal phase silica and aqueous base mobile phase with fluorescence or electrochemical detection methods are also available *(21)*. Occasionally, HPLC will not separate all the drugs of interest. Application of HPLC-mass spectrometry in TCA analysis is relatively new and can overcome such problems. A sensitive and specific HPLC-MS-MS method has been described for the rapid identification and quantitation of seven TCAs: amitriptyline, nortriptyline, doxepin, dosulepin, dibenzepin, opipramol, and melitracen. The method uses direct injection and on-line removal of proteins and other large biomolecules with total analysis time of 12 min *(22)*.

### *3.1. Interferences in TCA Assays*

Rapid toxicological screening by immunoassays is a common practice in the clinical laboratories. False-positive results because of cross-reacting compounds in drug assays may lead to misdiagnosis and mismanagement of a patient. The issue becomes even more serious when such false-positive result is found in a child and child neglect or child abuse is suspected. Most of these false-positive results are due to cross-reactivity in TCA immunoassays with structurally similar drugs. The drugs which are shown to interfere with TCA immunoassays are shown in Fig. 1.

Carbamazepine, structurally close to TCAs, is known to interfere in various TCA immunoassays. Several cases of false-positive TCAs because of carbamazepine have been reported *(23–26)*. Fleischman et al. *(23)* report a case of a 16-year-old girl who was found unresponsive and had a remote history of seizures. The patient's serum screen for TCAs was positive. ECG showed no evidence of QRS or axis deviation. The validity of the TCA value was questioned and carbamazepine level was measured and

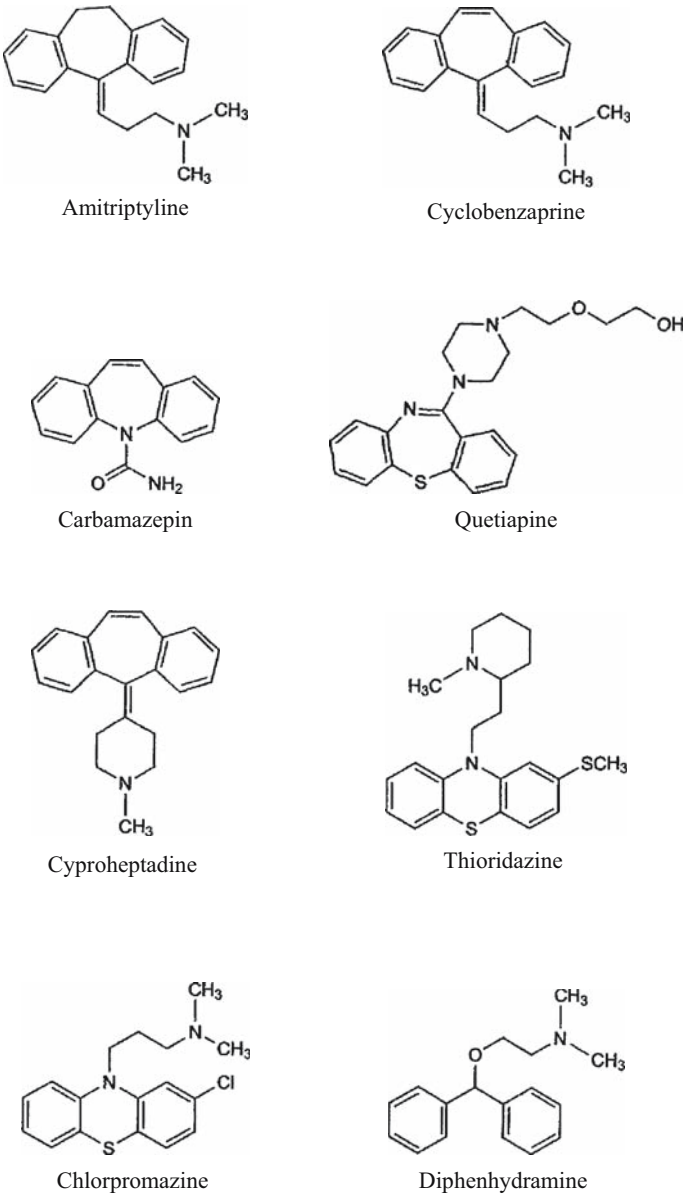


**Fig. 1.** Chemical structure of amitriptyline and some drugs that interference with tricyclic antidepressants immunoassays.

the value was found to be 17 μg/mL. Further investigation showed that the positive TCA screening result was due to carbamazepine in the specimen. Chattergoon et al. reported two patients with history of ingestion of carbamazepine who tested positive for TCAs using the fluorescence polarization immunoassay (FPIA) screening assay. The apparent TCA concentrations were 80 and 130 ng/mL, respectively *(25)*. The HPLC analysis failed to detect any TCAs in the serum. Tomaszewski et al. *(26)* reported three

false-positive cases of TCAs, on the Triage Panel Immunoassay System, because of interference from iminostilbene (a carbamazepine metabolite).

Quetiapine, an antipsychotic drug, has been found to interfere in TCA immunoassays. Sloan et al. *(27)* reported a case where a 34-year-old patient tested positive for TCAs by immunoassay. The patient was not prescribed any TCAs, and he denied their use. The patient was receiving quetiapine. The possibility of quetiapine interference in TCA assay was raised because it is structurally similar to TCAs. Various concentrations ranging from 1 to 10 μg/mL were tested for interference in Diagnostic Reagent Inc. (Fremont, CA) (now called Microgenics) immunoassay on the Hitachi 911 analyzer. Cross-reactivity was found to be 4.3%. The authors also tested olanzapine, another antipsychotic, and did not find any significant cross-reactivity with the assay. Hendrickson and Morocco *(28)* investigated three common TCA immunoassays (Microgenics, Syva Rapid Test, and Biosite Triage) using quetiapine solution. They also used urine samples from a patient overdosed with quetiapine as well as a patient with therapeutic concentration of the drug. Syva and Microgenics immunoassays, but not Triage immunoassay, tested positive in both the overdose and the therapeutic samples. Syva and Microgenics immunoassays were positive at urine levels of 100 and 10 μg/mL, respectively, whereas the Triage immunoassay was negative in solutions up to 1000 μg/mL. The study concluded that quetiapine might cause false-positive results in certain TCA immunoassays in both therapeutic and overdose situations and significant variations exist between different immunoassays so far quetiapine cross-reactivity is concerned. Schussler et al. *(29)* showed false-positive results by quetiapine in Abbott Laboratories' fluorescence polarization immunoassay on TDx/TDxFlx. Caravati investigated Abbott's FPIA, and Syva as well as STAD-ACA qualitative TCA immunoassays for cross-reactivity with quetiapine using spiked plasma samples, and found all these assays cross-react with quetiapine in a concentration-dependent manner *(30)*.

Several reports of interference of phenothiazepine in TCA immunoassays have been published. Ryder and Glick *(31)* reported a case where a patient who ingested thioridazine and flurazepam tested positive for TCAs by immunoassays. Investigation showed that false-positive TCA result was due to thioridazine. It is important to note that even therapeutic concentration of thioridazine (125 ng/mL) produces a false-positive serum TCA screen result. Maynard and Soni *(32)* reported false elevations of imipramine and desipramine in HPLC (cyanopropyl column) caused by thioridazine. High therapeutic (200–300 ng/mL) or toxic concentrations of chlorpromazine produce false-positive result in the enzyme-multiplied immunoassay technique (EMIT). In another study, false-positive results were obtained with high concentrations of thioridazine (4000 nM), chlorpromazine (300 nM), and trimeprazine (5000 nM) *(33)*.

Sorisky and Watson *(34)* reported a case where a 21-year-old female who ingested 2 g of diphenhydramine tested positive for TCAs using EMIT. Unlike certain phenothiazines that have tricyclic structure, diphenhydramine is an ethanolamine.

Wians et al. reported a case of a 14-year-old girl who ingested approximately 120 mg of cyproheptadine, an antihistamine and serotonin antagonist with anticholinergic and sedative properties. The patient tested positive for TCAs by EMIT *(35)*. In vitro studies indicated that a cyproheptadine concentration of 400 μg/L may cause false-positive TCA results. However, serum obtained from a volunteer who was given a 12-mg dose of cyproheptadine for 3 days tested negative for TCAs. Yuan et al. *(36)* report

a pediatric case of false-positive TCAs because of cyproheptadine and found that the false-positive test was mainly because of cyproheptadine metabolite. Cyproheptadine has tricyclic structure very similar to tricyclic structure of TCAs (Fig. 1).

Cyclobenzaprine and its major metabolite nor-cyclobenzaprine differ from amitriptyline and nortriptyline only by the presence of a double bond in the cycloheptane ring and are known to interfere with immunoassays and HPLC. Wong et al. *(37)* reported positive interferences by cyclobenzaprine in both the Syva EMIT assay and the HPLC. In an HPLC, cyclobenzaprine co-eluted with amitriptyline and nor-cyclobenzaprine eluted very close to nortriptyline. These interferences could be overcome by GC-MS after derivatization with trifluoroacetic anhydride, as these compounds had distinguishable mass spectra. A review on cyclobenzaprine interference in TCA assay has been published *(38)*.

## 4. TIPS IN DEALING WITH INTERFERENCES IN TCAS

Over time, several methods have been developed to eliminate or reduce the inferences in TCA assays. Dasgupta et al. *(39)* proposed a mathematical model for the estimation of TCA concentration in the presence of carbamazepine using the FPIA. Using sera from 30 patients who were receiving carbamazepine but no TCAs, and negative sera spiked with carbamazepine and its metabolite, they determined apparent TCA concentrations. In sera of patients, the carbamazepine concentrations ranged from 1.4 to 20.9 μg/mL and apparent TCAs concentrations ranged from 31.8 to 130.1 ng/mL. From the known carbamazepine concentrations and apparent TCA concentrations, they developed mathematical equation for estimation of TCAs in the presence of carbamazepine. They tested the equation by spiking carbamazepine-containing patients' samples with known concentrations of TCAs. There was a good agreement between the calculated and the targeted TCA concentrations. The differences between the predicted and observed values were <10%. This mathematical modeling was feasible because TCAs, even at very high concentrations, do not show interference with the carbamazepine FPIA. The carbamazepine showed significantly higher interference than its metabolite carbamazepine 10, 11-epoxide. Therefore, the authors cautioned the use of the equation in patients who may accumulate higher concentration of carbamazepine 10, 11-epoxide, e.g., renal patients. Also, the equation may not be valid at high concentrations of carbamazepine. The highest carbamazepine concentration from a patient sample was 20.9 μg/mL. When a carbamazepine concentration of 40 μg/mL was tested, the difference between observed and expected concentration was 18%.

Adamczyk et al. *(40)* described a method for removal of phenothiazine interference in TCA assays. The method involved alkalinization of phenothiazine containing serum sample followed by treatment with isoamyl alcohol to dissociate the analyte from serum proteins. The analyte was extracted with decane and transferred to an acidic buffer (0.1 M Gly-Gly, pH 3) containing chloramines-T. This results in selective oxidation of the phenothiazine sulfur atom in an acidic buffer system. The aqueous layer was analyzed for TCA by using the FPIA and TDx analyzer. This method allowed accurate quantification of the TCAs in the presence of 1000 ng/mL chlorpromazine or desmethyl chlorpromazine.

Cyclobenzaprine, a commonly used skeletal muscle relaxant, interferes with immunoassays and may co-elute with amitriptyline in HPLC. However, amitriptyline and

cyclobenzaprine can be distinguished using HPLC with diode array detector, as these drugs have different UV spectra. Puopolo and Flood *(41)* used dual wavelength (214 and 254 nm) spectrometry for detection of cyclobenzaprine interference in TCA HPLC.

In TLC, cyclobenzaprine may co-migrate with amitriptyline but can be distinguished by difference in fluorescence at stage III. Amitriptyline gives pink fluorescence, whereas cyclobenzaprine has orange fluorescence. Sometimes it is hard to distinguish between pink and orange color on TLC. Looking for amitriptyline and cyclobenzaprine metabolites is very helpful in distinguishing the presence of these drugs on TLC.

On capillary GC, cyclobenzaprine and amitriptyline are generally well separated. However, in an overdose situation, when peak size is too large and peak shape is not symmetrical or column performance is not optimal, the two drugs can co-elute and retention times may shift, causing confusion. In that case, diluting and reanalyzing the sample generally resolves the issue. Upon GC-MS, cyclobenzaprine and amitriptyline show certain similarity in mass spectra (Fig. 2). In both drugs, m/z 202 and 215 ions are prominent, and these ions ratios can be used to differentiate these drugs.

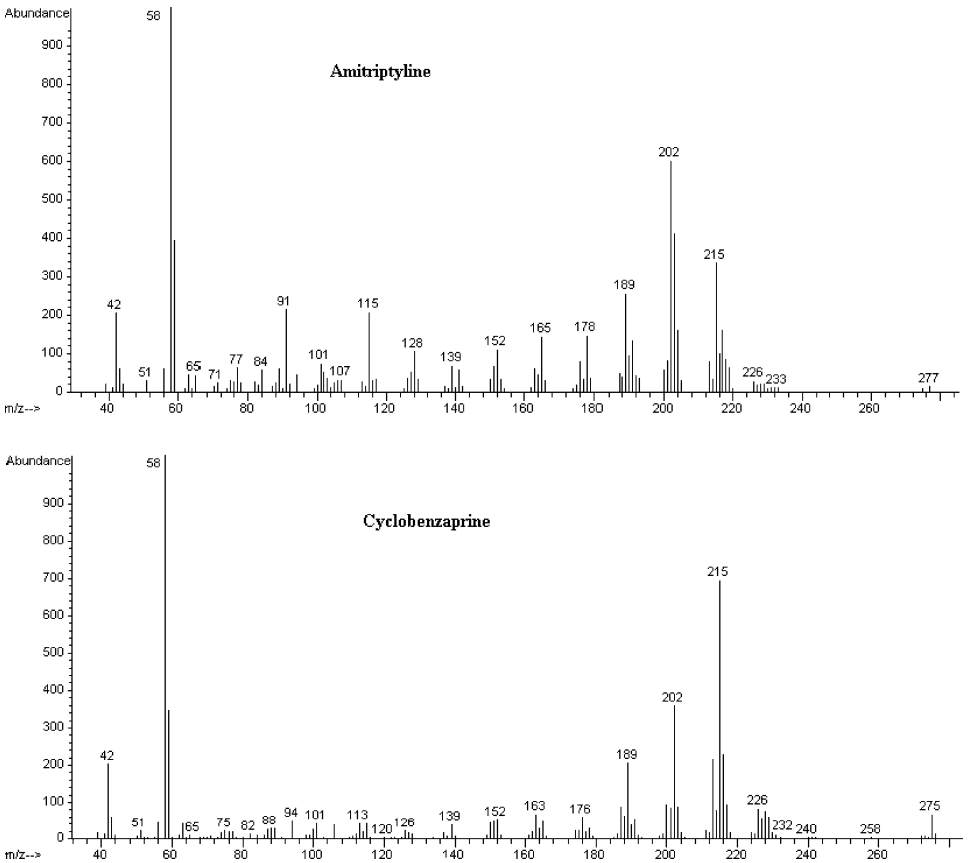


**Fig. 2.** Electron impact ionization mass spectra of amitriptyline and cyclobenzaprine. They share number of common ions and cause confusion in identification, particularly if there is baseline noise. Careful examination of ions (e.g., ratio of 202/215) may help distinguishing the spectra. To show other ions, abundance of ion 58 has been truncated.

A method to eliminate adsorption loss of TCAs during solvent extraction and evaporation has been described. The authors reported that the loss can be as high as 50%, and addition of as little as 0.05% diethylamine to the extract before evaporation completely eliminates the adsorption loss of amitriptyline, nortriptyline, imipramine-desipramine, doxepin, and nordoxepin *(42)*.

## 5. NON-TRICYCLIC ANTIDEPRESSANTS

After discovery of TCAs and monoamine oxidase inhibitors, collectively called first-generation antidepressants, many other classes of antidepressants were discovered. These include amoxapine and maprotiline that affect reuptake of monoamines similar to secondary amine TCAs. Trazodone is a weak inhibitor of serotonin reuptake but has little effect on norepinephrine uptake. Bupropion inhibits reuptake of norepinephrine and dopamine. In addition to its use as an antidepressant, bupropion is used as an aid to stop smoking. Venlafaxine and mirtazapine are other non-TCAs with novel properties of inhibiting both norepinephrine and serotonin. Their side effects profile is lower than that of TCAs *(43)*.

The other class of antidepressants called SSRIs has become the most widely prescribed group of antidepressants in the USA. In addition to inhibiting serotonin uptake, these drugs interact with serotonin receptors to cause pharmacological response. Advantages of SSRIs over TCAs include their lack of adrenergic, antihistaminic and anticholinergic effects, better tolerability, and superior safety profile. SSRIs are also used in the treatment of obsessive-compulsive disorder, panic disorder, bulimia, and many other conditions. Drug interactions include any drug that increases serotonin concentrations including monoamine oxidase inhibitors, tramadol, sibutramine, meperidine, sumatriptan, lithium, St. John's wort, ginkgo biloba, and atypical antipsychotic agents. Overdose situations or drug–drug interactions leading to an increase in serotonin may cause serotonin syndrome. The syndrome is associated with changes in mental status, agitation, myoclonus, diaphoresis, shivering, tremor, diarrhea, incoordination, and fever *(2)*. SSRIs that are commonly used and measured in the laboratory include citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline. Some pharmacological properties of these antidepressants are summarized in Table 2.

## 6. METHODS FOR DETERMINATION OF NON-TCAS

There is no reliable spot test for commonly used non-TCAs. Also, currently there are no widely used immunoassays for non-TCAs. Chromatography techniques are most common, and well-established techniques are available for measurements as either a single drug or a group. Most of these antidepressants are well detected during comprehensive drug screening performed by using GC or HPLC.

Like TCAs, GC is widely used for screening and quantitation of non-TCAs. The methods involve either liquid–liquid or solid-phase extraction. Single-step extractions are generally successful, but methods describing multiple extractions or back-extractions have also been described. The method may involve assay of single drug or multiple drugs. A GC-MS method, involving acid hydrolysis, for simultaneous determination of citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline in human urine samples has been described *(44)*. A capillary GC-MS method, using selected ion

Table 2
Pharmacokinetic Properties of Other (Nontricyclic) Antidepressants

| *Drug* | *Active Metabolite* | *Average Half-Life (hours)* | *Vd (L/kg)* | *Oral Bioavailability* | *Average Protein Binding* | *Therapeutic Range (ng/mL)* | *Toxic Level (ng/mL)* |
|---|---|---|---|---|---|---|---|
| Amoxapine | 8-hydroxyamoxapine | 10 | 1 | 90 | 90 | 200–400[b] | >600[b] |
| Bupropion | Hydroxybupropion[a] | 15 | 45 | 90 | 85 | 25–100 | >400 |
| Citalopram | Norcitalopram[a] | 30 | 14 | 80 | 50 | 40–100 | >250 |
| Fluoxetine | Norfluoxetine | 60 | 50 | 100 | 94 | 300–1000[b] | >2000[b] |
| Fluvoxamine | NA | 23 | 25 | 95 | 77 | 20–400 | Not known |
| Maprotiline | NA | 33 | 24 | 100 | 88 | 150–300 | >1000 |
| Mirtazapine | Normirtazapine | 30 | 12 | 90 | 85 | 4–40 | Not known |
| Paroxetine | NA | 22 | 15 | 90 | 95 | 20–200 | >800 |
| Sertraline | Norsertraline[a] | 28 | 20 | 90 | 98 | 30–200 | >500 |
| Trazodone | NA | 9 | 1 | 80 | 90 | 800–1600 | >5000 |
| Venlafaxine | *o*-Desmethyl venlafaxine | 5 | 7 | 90 | 27 | 250–500[b] | >1000 |

Vd = Volume of distribution.
NA, no significantly active metabolite.
[a] Significantly less active than parent drug.
[b] Total concentration of parent and active metabolite.

monitoring, for the simultaneous determination of five antidepressant drugs fluoxetine, fluvoxamine, citalopram, sertraline, and paroxetine has been published *(45)*.

Owing to heat labiality, certain antidepressants such as trazodone and nefazodone are not suitable for measurement using GC but can be easily analyzed using HPLC. HPLC is a commonly used method for determination of non-TCAs *(46–49)*. An HPLC method for the simultaneous determination of citalopram, fluoxetine, paroxetine, and their metabolites has been described *(48)*. The method involves solid-phase extraction and reversed-phase HPLC with fluorescence and UV detection. The limits of quantitation were 0.025 μg/L for citalopram and paroxetine, and 0.10 μg/L for fluoxetine and nor-fluoxetine, respectively. Another reversed-phase HPLC method for simultaneous determination of bicyclic, tricyclic and tetracyclic, and their metabolites is available *(49)*.

When non-TCAs are measured using chromatographic techniques, their assays are relatively free from interferences.

## 7. CONCLUSION

Even with the discovery of many new antidepressants, TCAs are still widely used and are a common cause of morbidity and mortality. In overdose situations, comprehensive drug-screening methods commonly involve screening of TCAs by using immunoassays. There are a number of drugs that interfere with immunoassays for TCAs and can cause false-positive results and may lead to misdiagnosis and mismanagement of a patient. Knowledge of the drugs that interfere with TCA assays and methods to eliminate these interferences may be very useful in correct diagnosis. Chromatographic methods, although cumbersome and not readily available, are relatively free from interferences. The role of therapeutic drug monitoring in analysis of TCAs and many non-TCAs is unequivocal.

## REFERENCES

1. Wong ML, Licinio J. Research and treatment approaches to depression. *Nat Rev Neurosci* 2001; 2:343–351.
2. Ables AZ, Baughman OL, 3rd. Antidepressants: update on new agents and indications. *Am Fam Physician* 2003; 67:547–554.
3. Pacher P, Kecskemeti V. Trends in the development of new antidepressants. Is there a light at the end of the tunnel? *Curr Med Chem* 2004; 11:925–943.
4. Sindrup SH, Otto M, Finnerup NB, Jensen TS. Antidepressants in the treatment of neuropathic pain. *Basic Clin Pharmacol Toxicol* 2005; 96:399–409.
5. Bateman DN. Tricyclic antidepressant poisoning: central nervous system effects and management. *Toxicol Rev* 2005; 24:181–186.
6. Thanacoody HK, Thomas SH. Tricyclic antidepressant poisoning: cardiovascular toxicity. *Toxicol Rev* 2005; 24:205–214.
7. Watson WA, Litovitz TL, Rodgers GC, Jr., Klein-Schwartz W, Reid N, Youniss J, et al. 2004 Annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. *Am J Emerg Med* 2005; 23:589–666.
8. Liebelt EL, Ulrich A, Francis PD, Woolf A. Serial electrocardiogram changes in acute tricyclic antidepressant overdoses. *Crit Care Med* 1997; 25:1721–1726.
9. Preskorn SH, Bupp SJ, Weller EB, Weller RA. Plasma levels of imipramine and metabolites in 68 hospitalized children. *J Am Acad Child Adolesc Psychiatr* 1989; 28:373–375.

10. Ziegler VE, Biggs JT. Tricyclic plasma levels. Effect of age, race, sex, and smoking. *JAMA* 1977; 238:2167–2169.
11. Ciraulo DA, Barnhill JG, Jaffe JH. Clinical pharmacokinetics of imipramine and desipramine in alcoholics and normal volunteers. *Clin Pharmacol Ther* 1988; 43:509–518.
12. Tamayo M, Fernandez de Gatta MM, Gutierrez JR, Garcia MJ, Dominguez-Gil A. High levels of tricyclic antidepressants in conventional therapy: determinant factors. *Int J Clin Pharmacol Ther Toxicol* 1988; 26:495–499.
13. Muller MJ, Dragicevic A, Fric M, Gaertner I, Grasmader K, Hartter S, et al. Therapeutic drug monitoring of tricyclic antidepressants: How does it work under clinical conditions? *Pharmacopsychiatry* 2003; 36:98–104.
14. McLellan RA, Oscarson M, Seidegard J, Evans DA, Ingelman-Sundberg M. Frequent occurrence of CYP2D6 gene duplication in Saudi Arabians. *Pharmacogenetics* 1997; 7:187–191.
15. Aklillu E, Persson I, Bertilsson L, Johansson I, Rodrigues F, Ingelman-Sundberg M. Frequent distribution of ultrarapid metabolizers of debrisoquine in an Ethiopian population carrying duplicated and multiduplicated functional CYP2D6 alleles. *J Pharmacol Exp Ther* 1996; 278:441–446.
16. Linder MW, Keck PE, Jr. Standards of laboratory practice: antidepressant drug monitoring. National Academy of Clinical Biochemistry. *Clin Chem* 1998; 44:1073–1084.
17. Wilkinson GR. Drug metabolism and variability among patients in drug response. *N Engl J Med* 2005; 352:2211–2221.
18. Nyberg G, Martensson E. Preparation of serum and plasma samples for determination of tricyclic antidepressants: effects of blood collection tubes and storage. *Ther Drug Monit* 1986; 8:478–482.
19. Dasgupta A, Yared MA, Wells A. Time-dependent absorption of therapeutic drugs by the gel of the Greiner Vacuette blood collection tube. *Ther Drug Monit* 2000; 22:427–431.
20. Way BA, Stickle D, Mitchell ME, Koenig JW, Turk J. Isotope dilution gas chromatographic-mass spectrometric measurement of tricyclic antidepressant drugs. Utility of the 4-carbethoxyhexafluorobutyryl derivatives of secondary amines. *J Anal Toxicol* 1998; 22:374–382.
21. Wong SH. Measurement of antidepressants by liquid chromatography: a review of current methodology. *Clin Chem* 1988; 34:848–855.
22. Kollroser M, Schober C. Simultaneous determination of seven tricyclic antidepressant drugs in human plasma by direct-injection HPLC-APCI-MS-MS with an ion trap detector. *Ther Drug Monit* 2002; 24:537–544.
23. Fleischman A, Chiang VW. Carbamazepine overdose recognized by a tricyclic antidepressant assay. *Pediatrics* 2001; 107:176–177.
24. Matos ME, Burns MM, Shannon MW. False-positive tricyclic antidepressant drug screen results leading to the diagnosis of carbamazepine intoxication. *Pediatrics* 2000; 105:E66.
25. Chattergoon DS, Verjee Z, Anderson M, Johnson D, McGuigan MA, Koren G, et al. Carbamazepine interference with an immune assay for tricyclic antidepressants in plasma. *J Toxicol Clin Toxicol* 1998; 36:109–113.
26. Tomaszewski C, Runge J, Gibbs M, Colucciello S, Price M. Evaluation of a rapid bedside toxicology screen in patients suspected of drug toxicity. *J Emerg Med* 2005; 28:389–394.
27. Sloan KL, Haver VM, Saxon AJ. Quetiapine and false-positive urine drug testing for tricyclic antidepressants. *Am J Psychiatry* 2000; 157:148–149.
28. Hendrickson RG, Morocco AP. Quetiapine cross-reactivity among three tricyclic antidepressant immunoassays. *J Toxicol Clin Toxicol* 2003; 41:105–108.
29. Schussler JM, Juenke JM, Schussler I. Quetiapine and falsely elevated nortriptyline level. *Am J Psychiatry* 2003; 160:589.
30. Caravati EM, Juenke JM, Crouch BI, Anderson KT. Quetiapine cross-reactivity with plasma tricyclic antidepressant immunoassays. *Ann Pharmacother* 2005; 39:1446–1469.
31. Ryder KW, Glick MR. The effect of thioridazine on the automatic clinical analyzer serum tricyclic anti-depressant screen. *Am J Clin Pathol* 1986;86:248–249.
32. Maynard GL, Soni P. Thioridazine interferences with imipramine metabolism and measurement. *Ther Drug Monit* 1996; 18:729–731.
33. Benitez J, Dahlqvist R, Gustafsson LL, Magnusson A, Sjoqvist F. Clinical pharmacological evaluation of an assay kit for intoxications with tricyclic antidepressants. *Ther Drug Monit* 1986; 8:102–105.

34. Sorisky A, Watson DC. Positive diphenhydramine interference in the EMIT-st assay for tricyclic antidepressants in serum. *Clin Chem* 1986; 32:715.
35. Wians FH, Jr., Norton JT, Wirebaugh SR. False-positive serum tricyclic antidepressant screen with cyproheptadine. *Clin Chem* 1993; 39:1355–1356.
36. Yuan CM, Spandorfer PR, Miller SL, Henretig FM, Shaw LM. Evaluation of tricyclic antidepressant false positivity in a pediatric case of cyproheptadine (periactin) overdose. *Ther Drug Monit* 2003; 25:299–304.
37. Wong EC, Koenig J, Turk J. Potential interference of cyclobenzaprine and norcyclobenzaprine with HPLC measurement of amitriptyline and nortriptyline: resolution by GC-MS analysis. *J Anal Toxicol* 1995; 19:218–224.
38. Van Hoey NM. Effect of cyclobenzaprine on tricyclic antidepressant assays. *Ann Pharmacother* 2005; 39:1314–1317.
39. Dasgupta A, McNeese C, Wells A. Interference of carbamazepine and carbamazepine 10,11-epoxide in the fluorescence polarization immunoassay for tricyclic antidepressants: estimation of the true tricyclic antidepressant concentration in the presence of carbamazepine using a mathematical model. *Am J Clin Pathol* 2004; 121:418–425.
40. Adamczyk M, Fishpaugh JR, Harrington CA, Hartter DE, Hruska RE, Vanderbilt AS. Immunoassay reagents for psychoactive drugs. Part 3. Removal of phenothiazine interferences in the quantification of tricyclic antidepressants. *Ther Drug Monit* 1993; 15:436–439.
41. Puopolo PR, Flood JG. Detection of interference by cyclobenzaprine in liquid-chromatographic assays of tricyclic antidepressants. *Clin Chem* 1987; 33:819–820.
42. Tserng KY, McPeak RJ, Dejak I, Tserng K. Prevention of tricyclic antidepressant adsorption loss with diethylamine during solvent evaporation. *Ther Drug Monit* 1998; 20:646–651.
43. Buckley NA, Faunce TA. 'Atypical' antidepressants in overdose: clinical considerations with respect to safety. *Drug Saf* 2003; 26:539–551.
44. Maurer HH, Bickeboeller-Friedrich J. Screening procedure for detection of antidepressants of the selective serotonin reuptake inhibitor type and their metabolites in urine as part of a modified systematic toxicological analysis procedure using gas chromatography-mass spectrometry. *J Anal Toxicol* 2000; 24:340–347.
45. Berzas Nevado JJ, Villasenor Llerena MJ, Guiberteau Cabanillas C, Rodriguez Robledo V, Buitrago S. Sensitive capillary GC-MS-SIM determination of selective serotonin reuptake inhibitors: reliability evaluation by validation and robustness study. *J Sep Sci* 2006; 29:103–113.
46. Mandrioli R, Saracino MA, Ferrari S, Berardi D, Kenndler E, Raggi MA. HPLC analysis of the second-generation antidepressant sertraline and its main metabolite N-desmethylsertraline in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; 836:116–119.
47. Loboz KK, Gross AS, Ray J, McLachlan AJ. HPLC assay for bupropion and its major metabolites in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; 823:115–121.
48. Kristoffersen L, Bugge A, Lundanes E, Slordal L. Simultaneous determination of citalopram, fluoxetine, paroxetine and their metabolites in plasma and whole blood by high-performance liquid chromatography with ultraviolet and fluorescence detection. *J Chromatogr B Biomed Sci Appl* 1999; 734:229–246.
49. Joron S, Robert H. Simultaneous determination of antidepressant drugs and metabolites by HPLC. Design and validation of a simple and reliable analytical procedure. *Biomed Chromatogr* 1994; 8:158–164.

# 9

# Immunosuppressive Drugs

## *Pharmacokinetics, Preanalytic Variables, and Analytical Considerations*

*Anthony W. Butch, PhD*

***Contents***



**Summary**

To optimize therapeutic effectiveness and minimize unwanted adverse effects, reliable and precise methods are required for monitoring blood concentrations of immunosuppressive drugs. Therapeutic monitoring of cyclosporine, tacrolimus, and sirolimus is currently considered an integral part of organ transplant programs, and compelling arguments have been made for monitoring mycophenolic acid. Although high-performance liquid chromatography (HPLC) is considered the reference method for monitoring immunosuppressive drugs, most laboratories currently measure these drugs by immunoassay. Immunoassays have gained widespread use because they can be automated, have low start-up costs, and do not require specialized testing personnel. Unfortunately, immunoassays exhibit significant metabolite cross-reactivity that differs among immunoassays and is dependent on the transplanted organ as well as time post-transplant. The advantage of HPLC methods is that they are highly specific and can separate drug metabolites from parent compound. However, HPLC methods can require extensive sample cleanup, have long analytical run times, and require specialized training. Some of these drawbacks can be partially overcome by using HPLC with mass spectrometry (MS) detection systems, although the instrumentation is currently expensive. In view of the high cost of immunoassay reagents, HPLC-MS systems are becoming more cost effective, especially when considering that they can simultaneously measure multiple immunosuppressive drugs in a single whole blood specimen.

**Key Words:** Therapeutic drug monitoring; immunosuppressive drugs; cyclosporine; tacrolimus; sirolimus; mycophenolic acid.

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## 1. INTRODUCTION

It has now been more than 50 years since the first successful kidney transplant was performed between monozygotic twins *(1)*. At that time, the field of immunology was in its infancy, and transplants between non-identical twins ended in organ failure because of acute graft rejection. It was not until the introduction of azathioprine (a nucleotide analogue less toxic than 6-mercaptopurine) in the early 1960s that chemical immunosuppression and prolonged kidney allograft survival became possible *(2)*. Azathioprine by itself was not potent enough to prevent acute graft rejection. However, the combination of azathioprine and corticosteroids was shown to provide effective chemical immunosuppression, with 1-year kidney allograft survival rates ranging from 40 to 50% *(3)*. This combination of chemical immunosuppression continued to be the cornerstone of transplant programs for the next 20 or so years until cyclosporine (CsA) entered the transplantation arena in the late 1970s *(4)*.

In the late 1980s, other immune cell modulators such as tacrolimus and sirolimus were discovered and added to the arsenal of chemical immunosuppressive agents *(5,6)*. Mycophenolic acid (MPA) (as the prodrug mycophenolate mofetil) became available in the mid 1990s based on reports from multicenter clinical trials demonstrating that it could further reduce the incidence of renal graft rejection when used in combination with CsA and steroids *(7–9)*.

The number of solid organ transplants performed in the USA continues to increase each year (Table 1) *(10)*. There has been a 17% increase in kidney, a 29% increase in liver, a 2% reduction in heart, and an overall increase of 17% over the last 5 years, when comparing organ transplants performed in 2005 with 2001 *(10)*. Sadly, the limiting factor in the number of transplanted organs is the availability of donor organs. There were more than 94,000 patients on the U.S. organ transplant waiting list at the end of 2005 *(11)*.

The discovery that CsA had immunosuppressive activity that specifically targeted T lymphocytes was a major breakthrough in organ transplantation because it dramatically reduced acute graft rejection and improved long-term graft and patient survival *(12,13)*. The identification of other immunosuppressive drugs that modulate immune responses by additional molecular pathways enabled treatment options to evolve and has permitted combination therapies to be individualized based on patient requirements. Classes of immunosuppressive drugs along with generic and brand names currently approved

**Table 1**
**Solid Organ Transplants in the USA**

| | *Year* | | | | |
|---|---|---|---|---|---|
| *Organ Transplanted* | *2001* | *2002* | *2003* | *2004* | *2005* |
| Kidney | 14,100 | 14,527 | 14,856 | 15,671 | 16,477 |
| Liver | 4984 | 5061 | 5364 | 5780 | 6441 |
| Heart | 2171 | 2112 | 2026 | 1961 | 2126 |
| All Organs[a] | 23,942 | 24,552 | 25,083 | 26,539 | 28,098 |

[a] Includes pancreas, kidney-pancreas, intestine, lung, and heart-lung transplants.

**Table 2**
**Immunosuppressive Drugs Used in Solid Organ Transplantation**

| *Drug Class* | *Generic Name* | *Brand Names* |
|---|---|---|
| Corticosteroids | Prednisone | Orasone, Deltasone |
| | Methylprednisolone | Solu-Medrol, A-methaPred, Medrol |
| | Dexamethasone | Decadron |
| Anti-metabolites | Azathioprine | Imuran |
| | Cyclophosphamide | Cytoxan, Neosar |
| | Mycophenolate mofetil | CellCept |
| | Mycophenolate sodium | Myfortic |
| Calcineurin inhibitors | Cyclosporine A | Sandimmune, Neoral, many generic forms of Cyclosporines |
| | Tacrolimus (FK-506) | Prograf |
| mTOR inhibitors | Sirolimus (Rapamycin) | Rapamune |
| | Everolimus[a] (RAD0001) | Certican |

mTOR, mammalian target of rapamycin.
[a] Everolimus is currently in phase III clinical trials in the USA and has not been approved by the Food and Drug Administration (FDA) for use as an immunosuppressive agent.

by the United States Food and Drug Administration (FDA) for use in solid organ transplantation are listed in Table 2.

## 2. RATIONALE FOR IMMUNOSUPPRESSIVE DRUG MONITORING

A prerequisite for optimizing and individualizing immunosuppressive therapy is a reliable and precise method for monitoring drug concentrations. However, not all immunosuppressive drugs require routine monitoring of blood concentrations. For instance, corticosteroids are dosed based on empirical guidelines and are not routinely monitored. Although methods have been developed to measure blood concentrations of azathioprine *(14–16)*, this antiproliferative agent is seldom monitored by transplant centers. Blood concentrations of CsA, tacrolimus, sirolimus, and MPA are routinely monitored at transplant centers for the following reasons: (a) there is a clear relationship between drug concentration and clinical response; (b) these drugs have a narrow therapeutic index; (c) these drugs exhibit a high degree of inter- and intrapatient variability; (d) the pharmacological response can be difficult to distinguish from unwanted side effects; (e) there is a risk of poor or non-compliance because the drugs are administered for the lifetime of the graft or patient; and (f) there are significant drug–drug interactions.

The potential for drug interactions is not limited to non-immunosuppressive agents but can also occur among the various classes of immunosuppressive drugs. For instance, CsA inhibits transport of an MPA metabolite from the liver to bile resulting in lower MPA concentrations when the two drugs are used together for immunosuppressive therapy *(17,18)*. The combination of CsA and sirolimus or tacrolimus and sirolimus results in increased blood concentrations of sirolimus *(17,19)*. In 2004, the majority of kidney, liver, and heart transplant patients were receiving tacrolimus and MPA followed

by CsA and MPA for immunosuppression, before hospital discharge *(20)*. Tacrolimus and sirolimus or CsA and sirolimus were less commonly used, and sirolimus and mycophenolate mofetil (MMF) were the least common immunosuppressive regimens. All these drug regimens typically included corticosteroids *(20)*. This illustrates the widespread use of combination immunosuppression and the importance of therapeutic drug monitoring, given the potential for various drug interactions.

This chapter will focus primarily on FDA-approved immunosuppressive drugs that are routinely monitored by clinical laboratories supporting solid organ transplant programs. These include CsA, tacrolimus, sirolimus, and MPA. Everolimus will be briefly discussed because it is currently in phase III clinical trials. Other drugs that are not commonly monitored, such as corticosteroids, azathioprine, and cyclophosphamide, will not be discussed further. Clinical pharmacokinetics, unwanted adverse effects, and various drug interactions will be provided for each of the chemical immunosuppressive agents. A comprehensive review of analytical methods will also be provided, along with detailed information regarding limitations and potential sources of error associated with each of the testing methodologies.

## 3. CALCINEURIN INHIBITORS

The chemical structures of CsA and tacrolimus, calcineurin inhibitors commonly used in organ transplantation, are shown in Fig. 1. The calcineurin inhibitors block the activation and proliferation of $CD4^+$ and $CD8^+$ T lymphocytes by inhibiting IL-2 production *(21,22)*. Under normal circumstances, binding of major histo compatibility complex–peptide complexes to T-cell receptors results in the formation of an activated form of the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin. This leads to de-phosphorylation of the nuclear factor of activated T cells (NF-AT) (among others) and nuclear translocation of NF-AT. Once in the nucleus, NF-AT binds genes encoding pro-inflammatory cytokines such as IL-2, resulting in up-regulated gene transcription *(23)*. CsA and tacrolimus freely cross lymphocyte membranes and form complexes with specific cytoplasmic binding proteins called

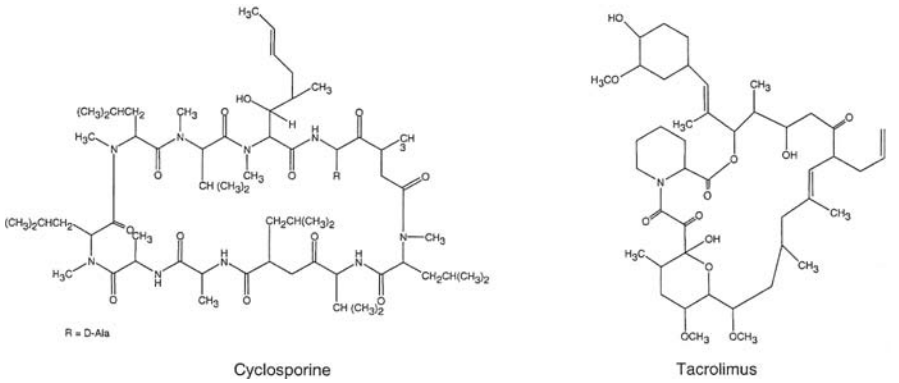


**Fig. 1.** Chemical structures of the calcineurin inhibitors, cyclosporine (CsA) and tacrolimus. This figure was published in Pharmacology & Therapeutics, Volume 112, Masuda S, Inui KI, an up-date review on individualized dosage adjustment of calcineurin inhibitors in organ transplant patients, page 186, Copyright Elsevier 2006.

immunophilins. CsA binds to the immunophilin cyclophilin and tacrolimus binds to the immunophilin FK506-binding protein-12 *(24,25)*. The drug–immunophilin complexes inhibit calcineurin activity, which prevents nuclear translocation of NF-AT. The end result is down-regulated cytokine gene transcription *(26–28)*.

### *3.1. Cyclosporine*

CsA is a small cyclic polypeptide (molecular weight of 1204) that was originally isolated from fungal cultures of *Tolypocladium inflatum Gams* in 1970 *(29)*. It is currently approved in the USA as an immunosuppressive drug to prolong organ and patient survival in kidney, liver, heart and bone marrow transplants. CsA is available for both oral and intravenous administration (Sandimmune). A microemulsion formulation of CsA, called Neoral, exhibiting more reproducible absorption characteristics is also available for oral administration *(30)*. In addition, several generic microemulsion formulations are now available and are often referred to as CsA modified *(31,32)*.

#### 3.1.1. Pharmacokinetics

Oral absorption of Sandimmune is low (5–30%) and highly variable, ranging from 4 to 89% in renal and liver transplant patients *(33,34)*. Absorption of the microemulsion formulation is more consistent, averaging approximately 40% *(35)*. Peak blood concentrations typically occur between 1–3 and 2–6 h following oral administration of Neoral and Sandimmune, respectively *(33,36,37)*. Absorption can be delayed for several hours in a subgroup of patients. Because CsA is lipophilic, it crosses most biologic membranes and has a wide tissue distribution *(38)*. CsA is highly bound to plasma proteins (>90% to lipoproteins), with the majority of CsA localizing in erythrocytes. The distribution of CsA between plasma and erythrocytes is temperature-dependent and varies with changes in hematocrit *(39)*. Because of the potential for artifactural redistribution of CsA during specimen processing because of ambient temperature fluctuations, ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood should be used to measure CsA concentrations *(40–42)*.

CsA is extensively metabolized by cytochrome P450 enzymes (CYP3A isoenzymes) located in the small intestine and liver *(43)*. There is also a cellular transporter of immunosuppressive drugs, called P-glycoprotein, that influences metabolism by regulating CsA bioavailability. P-glycoprotein pumps some of the CsA out of enterocytes back into the lumen of the gut *(44,45)*. This efflux pump probably contributes to the poor absorption rates observed after oral administration of CsA. CYP3A isoenzymes and P-glycoprotein genetic polymorphisms can also influence the oral bioavailability of CsA and are probably involved in the delayed absorption that has been noted in a subset of patients *(44)*. CsA is oxidized or N-demethylated to more than 30 metabolites *(46,47)*. Most of the metabolites do not possess immunosuppressive activity and are not clinically significant *(48)*. However, there is growing evidence to indicate that a few of the inactive metabolites may contribute to CsA toxicity *(48)*. Two of the hydroxylated metabolites, AM1 and AM9, exhibit 10–20% of the immunosuppressive activity of the parent compound *(49,50)* and can account for as much as 33% of the whole blood CsA concentration *(51)*. The major route of CsA elimination is biliary excretion into the feces. As expected, dosage adjustments are necessary in patients with

hepatic dysfunction. Only a small fraction (6%) of CsA and metabolites appear in the urine *(36)*, making dosage adjustments unnecessary in patients with renal insufficiency.

### 3.1.2. Adverse Effects

Serious side effects related to CsA treatment are concentration-dependent and include nephrotoxicity, neurotoxicity, hepatotoxicity, hirsutism, hypertrichosis, gingival hypertrophy, glucose intolerance, hypertension, hyperlipidemia, hypomagnesemia, hyperuricemia, and hypokalemia. In general, over-suppression leads to an increased risk for viral infections and lymphoproliferative disease, especially in children *(52)*.

### 3.1.3. Drug Interactions

Numerous drugs influence the absorption and metabolism of CsA. Any drug that inhibits the cytochrome P-450 system or the P-glycoprotein efflux pump increases blood CsA concentrations because of increased absorption and decreased metabolism. Drugs having the opposite effect (P-450 and/or P-glycoprotein inducers) produce decreased CsA concentrations. Drugs causing increased CsA blood concentrations include calcium channel blockers, several antifungal agents, and the antibiotic erythromycin. Several anticonvulsants and antibiotics, including antituberculosis agents, reduce blood CsA concentrations. In addition, there are many other drugs that synergize with CsA and potentiate nephrotoxicity. There are several excellent reviews that discuss specific drug interactions with CsA *(53,54)*. Not all of the interactions are caused by pharmaceuticals as various foods and herbal remedies can influence CsA concentrations. For instance, grapefruit juice increases CsA blood concentrations by increasing absorption whereas St John's wort decreases CsA concentrations by increasing metabolism *(55)*.

### 3.1.4. Preanalytic Variables

Whole blood anticoagulated with EDTA is the recommended sample type based on numerous consensus documents *(40–42)*. CsA in EDTA whole blood is stable at least 11 days at room temperature or higher temperatures (37°C) *(56)*. For long-term storage, whole blood samples should be placed at −20°C and are stable for at least 3 years *(57)*. As previously mentioned, CsA should only be measured in whole blood samples. Plasma is considered generally not acceptable because partitioning of CsA between plasma and erythrocytes is a temperature- and time-dependent process that can be altered during in vitro specimen processing *(41)*. In addition, plasma CsA concentrations are twofold lower than whole blood concentrations and results in poor analytical precision at low plasma CsA concentrations.

The timing of specimen collection has always been right before administration of the next dose (i.e., trough levels) *(40,41)*. For standardization purposes, the timing should be within 1 h before the next dose *(42)*. However, the introduction of Neoral in 1995, a microemulsion CsA formulation with more predictable absorption kinetics, has resulted in higher peak concentrations and increased drug exposure, based on area under the concentration time curves *(58)*. The highest and most variable CsA concentrations typically occur within the first 4 h after Neoral dosing *(59)*. However, similar trough concentrations are observed for both the conventional and the microemulsion CsA formulations, demonstrating that trough concentrations are not predictive of total

drug exposure *(60)–(62)*. Increased exposure to CsA using Neoral results in decreased rejection rates with slightly higher serum creatinine concentrations compared with conventional CsA therapy *(58,63,64)*. Thus, a better predictor of immunosuppressive efficacy was needed when administering Neoral. Pharmacokinetic and pharmacodynamic studies demonstrated that maximal inhibition of calcineurin and IL-2 production was correlated with the highest CsA concentrations 1–2 h after dosing *(59,65)*, indicating that drug levels shortly after dosing may be a better predictor of total drug exposure and clinical outcome *(66)*. Because multiple time points after dosing are not practical in a clinical setting, different time points were examined and CsA concentrations 2 h after dosing (called C2 monitoring) was shown to correlate best with total drug exposure and result in better clinical outcomes *(67–70)*. These findings have resulted in C2 monitoring of CsA becoming standard practice at many transplant centers. Unfortunately, this creates various nursing/ phlebotomy challenges because blood samples have to be drawn very close to the 2-h time point after dosing, ideally 10 min on either side of the 2-h mark *(71)*. At the author's institution, C2 testing is performed on 16% of all whole blood samples (annual volume ~14,000) received in the laboratory for CsA testing. To avoid confusion and prevent testing delays because of the need for sample dilution of C2 specimens, our laboratory has created a separate test for C2 monitoring and reports all CsA C2 results in μg/mL to avoid mis-interpreting C2 results as tough levels. We still report CsA trough results in ng/mL.

### 3.1.5. Methods of Analysis

Monitoring of CsA is critical for optimizing immunosuppression and organ survival while minimizing unwanted toxic side effects. Improvements in immunosuppressive regimens, along with demands for narrower and tighter control of CsA blood levels, have placed greater demand on clinical laboratories to provide timely and reliable drug concentrations. There are many methods currently available to measure CsA. Factors that need to be considered when selecting a CsA assay include metabolite cross-reactivity, cost of instrumentation and reagents, ease of operation, level of technical expertise required to perform testing, test volume, expected turnaround times, the current method being used when switching methods, and the history/preferences of the transplant physicians. For example, turnaround times can be a critical issue in an outpatient setting when it is desirable to have CsA test results available when patients are being seen by their physicians. Depending on the institution, this may require 2–4 h turnaround times for anywhere from 10 to 50 specimens that have been drawn a few hours before the scheduled clinic visit.

CsA can be measured by radioimmunoassay (RIA), semi-automated and automated non-isotopic immunoassays, and high-performance liquid chromatography (HPLC) with UV (HPLC-UV) or mass spectrometry detection systems (HPLC-MS). There are four companies manufacturing six different CsA assays currently being used in the USA. Assays for CsA and the percentage of laboratories using each method based on the College of American Pathologists Immunosuppressive Drugs Monitoring 1st Survey of 2006 are summarized in Table 3. The Cyclo-Trac SP RIA by Diasorin (Still water, MN, USA) is the least popular and is used by only 1% of all laboratories, most likely because of the manual format and need to handle radioisotopes. Interestingly, the Abbott monoclonal fluorescence polarization immunoassay (FPIA) (Abbott Park, IL, USA) is used by >70% of all laboratories. This is somewhat surprising because the

**Table 3**
**Currently Used Methods to Measure Cyclosporine (CsA)**

| *Method* | *Assay* | *Manufacturer* | *Laboratories Using Assay (%)*[a] |
|---|---|---|---|
| Radioimmunoassay | Cyclo-Trac SP | DiaSorin | 1 |
| Immunoassay | | | |
| Semi-automated | Polyclonal FPIA | Abbott | 2 |
| | Monoclonal FPIA | Abbott | 71 |
| | CEDIA PLUS | Microgenics | 8 |
| | Syva EMIT 2000 | Dade-Behring | 5 |
| Automated | Dimension ACMIA | Dade-Behring | 5 |
| HPLC-UV | | | 2 |
| HPLC-MS | | | 6 |

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay; HPLC-UV, high-performance liquid chromatography with ultraviolet detection; HPLC-MS, high-performance liquid chromatography with mass spectrometry detection.

[a] Percentages are based on the College of American Pathologists Immunosuppressive Drug Monitoring 1st survey of 2006.

Abbott monoclonal FPIA has considerable cross-reactivity with CsA metabolites, and recommendations by numerous consensus panels specify that the analytical method should be specific for parent compound *(40–42)*. HPLC methods to measure CsA are specific for parent compound and, because of this, are considered the "gold standard" for CsA quantitation. Yet, HPLC methods are used by only 8% of all laboratories and are primarily restricted to larger transplant centers. The lack of widespread acceptance of HPLC methods to measure CsA may reflect high initial equipment costs for MS detection systems and the need for specialized training for test performance. HPLC systems with UV detection are considerably less expensive and easier to operate but can suffer from a wide variety of chemical interferences depending on the specific protocol utilized. There are several excellent protocols to measure CsA using HPLC-MS and HPLC-MS/MS systems *(72,73)*. Because sample requirements are the same for analysis of many of the immunosuppressants (CsA, tacrolimus, sirolimus, everolimus), simultaneous measurement of two or more immunosuppressive drugs in a single specimen can be performed using HPLC-MS *(74)*. As therapeutic drug monitoring applications continue to emerge, the use of HPLC-MS will continue to increase and may become commonplace equipment in clinical laboratories in the not too distant future.

All the immunoassays, with the exception of the Dimension antibody conjugated magnetic immunoassay (ACMIA) (Dade Behring, Dearfield, IL, USA), are semi-automated because they require a whole blood pretreatment step. This typically involves preparing a whole blood hemolysate by adding an extraction reagent such as methanol to an aliquot of whole blood. The hemolysate is then centrifuged and the separated supernatant is analyzed by the FPIA or Syva enzyme-multiplied immunoassay (EMIT) (Dade Behring). The cloned enzyme donor immunoassay (CEDIA) PLUS (Microgenics Comp., Fremont, CA, USA) pretreatment step is simpler because a centrifugation step is not required after addition of the extraction reagent. Bayer (Bayer Health care, Tarrytown, NY, USA) has also developed a CsA assay with a simplified pretreatment

**Table 4**
**Instrument Applications for Cyclosporine (CsA) Immunoassays**

| *Immunoassay* | *Instrument Application* | *Manufacturer* |
|---|---|---|
| Monoclonal FPIA | TDx, AxSYM | Abbott Laboratories |
| CEDIA PLUS | MGC240 | Microgenics Corp. |
| | SYNCHRON LX, UniCel Dx | Beckman Coulter |
| | Hitachi 902, 911, 912, 917, Modular P | Roche Diagnostics |
| | AU 400, 640, 2700, 5400 | Olympus America |
| | Aeroset | Abbott Laboratories |
| Syva EMIT 2000 | COBAS Mira[a], INTEGRA 400, 800 | Roche Diagnostics |
| | Dimension RxL Max, Xpand, Xpand Plus, V-Twin, Viva, Viva-E | Dade-Behring |
| Dimension ACMIA | Dimension RxL Max, Xpand, Xpand Plus, V-twin,Viva, Viva-E | Dade-Behring |

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay.
[a] This instrument is no longer manufactured or supported by the company.

step that is pending FDA approval for use on the ADVIA Centaur *(75)*. The Dimension ACMIA does not require a pretreatment step allowing whole blood samples to be placed directly on the instrument. Instruments that currently have applications for the various CsA immunoassays are provided in Table 4.

### 3.1.6. Metabolite Cross-Reactivity

The Abbott polyclonal antibody-based FPIA is non-specific and has extensive cross-reactivity with CsA metabolites. The use of this assay has been declining over the years, and only about 2% of all laboratories currently use this assay (Table 3). CsA results using the Abbott polyclonal FPIA are approximately four times higher than those obtained using HPLC methods *(76)*. Because of the magnitude of metabolite

**Table 5**
**Cyclosporine (CsA) Metabolite Cross-Reactivity of Immunoassays**

| | *Percentage CsA Metabolite Cross-Reactivity*[a] | | | |
|---|---|---|---|---|
| *Immunoassay* | *AM1* | *AM4n* | *AM9* | *AM19* |
| Monoclonal FPIA | 6–12 | $\leq 6$ | 14–27 | $\leq 4$ |
| CEDIA PLUS | 8 | 30 | 18 | 2 |
| Syva EMIT 2000 | $\leq 5$ | 8–13 | $\leq 4$ | 0 |
| Dimension ACMIA | 0 | 4 | 0 | 0 |

FPIA, fluorescence polarization immunoassay; CEDIA, cloned enzyme donor immunoassay; EMIT, enzyme-multiplied immunoassay technique; ACMIA, antibody-conjugated magnetic immunoassay.
[a] Each metabolite was evaluated at 1000 μg/L except AMI, which was tested at 500 μg/L in the CEDIA PLUS assay. Data are derived from references *77–81*.

cross-reactivity and the poor correlation with clinical outcomes and toxicity, the use of this polyclonal assay should be discouraged. Cross-reactivity of the monoclonal immunoassays with CsA metabolites is summarized in Table 5. The Dimension ACMIA has the least overall metabolite cross-reactivity whereas the monoclonal CEDIA PLUS is reported to have the highest overall metabolite cross-reactivity. CsA metabolites, AM1 and AM9, are typically present in the highest concentrations after transplantation *(51)* and cross-reacts the least in the Dimension ACMIA and Syva EMIT, and the most in the monoclonal FPIA (Table 5). The magnitude of metabolite cross-reactivity contributes to the degree of CsA overestimation when comparing immunoassays with HPLC. Mean CsA concentrations have been found to be approximately 12, 13, 17, 22, and 40% higher than HPLC when measured by the Dimension ACMIA, Syva EMIT, CEDIA PLUS, FPIA on the TDx, and FPIA on the AxSYM, respectively *(77–81)*. Thus, it is important to consider metabolite cross-reactivity and the degree of CsA overestimation when selecting the "right" CsA immunoassay to support a solid organ transplant program.

### 3.1.7. Analytical Considerations

Consensus conference recommendations for CsA immunoassays are that the slope of the line should be $1.0 \pm 0.1$, with a *y*-intercept and $S_{y/x} \leq 15\,\mu g/L$, when compared with HPLC *(41)*. None of the current immunoassays satisfy all these requirements *(76–81)*. For instance, the Dimension ACMIA satisfies the slope and intercept requirements but exceeds the $S_{y/x}$ limit, whereas the CEDIA PLUS and Syva EMIT satisfies only one requirement. The FPIA fails to satisfy any of the requirements. Between-day precision recommendations require a coefficient of variation (CV) of $\leq$10% at a CsA concentration of 50 μg/L and a CV of $\leq$5% at 300 μg/L *(41,42)*. Most of the immunoassays satisfy the precision recommendation at 300 μg/L, but it is important that each laboratory determine between-day precision studies at CsA concentrations around 50 μg/L. This is particularly important because recent immunosuppressive drug regimens are designed to reduce CsA trough concentrations to minimize toxicity. Another potential problem is bias because of incorrect assay calibration. Results from the 2003 International Proficiency Testing Scheme have shown that the FPIA using the TDx and CEDIA PLUS overestimates CsA concentrations by 5–10%, whereas the Syva EMIT and Dimension ACMIA slightly underestimate target CsA concentrations by $\leq$5% *(82)*. Lastly, for assays involving a manual extraction step, poor technique can significantly contribute to the overall imprecision of the assay. Careful attention to detail and good technique can minimize variations at this important preanalytical step. This holds true for all whole blood immunosuppressive drug assays requiring a manual extraction step (tacrolimus, sirolimus, and everolimus).

### 3.1.8. C2 Monitoring and Specimen Dilution

Therapeutic ranges for CsA are often organ-specific and can vary widely between transplant centers. They also differ based on various immunosuppressive drug combinations, the time after transplant, and during periods of toxicity and organ rejection. Trough whole blood CsA levels following kidney transplants are typically between 150–250 μg/L shortly after transplant and are tapered down to $<$150 μg/L during maintenance therapy. Recommended levels after liver and heart transplants are

250–350 μg/L shortly after transplant and <150 μg/L during maintenance therapy. These target ranges were determined using HPLC and will vary considerably when measured using immunoassay, depending on the amount of metabolite cross-reactivity.

For C2 monitoring, target concentrations vary between 600 and 1700 μg/L depending on the type of graft and the time after transplantation *(66)*. C2 concentrations often exceed the analytical range of most immunoassays because typical calibration curves are designed to measure trough CsA levels. The FPIA and Syva EMIT have analytical ranges up to 1500 and 500 μg/L, respectively. The CEDIA PLUS and Dimension ACMIA have separate calibration curves for C2 monitoring, with an analytical range from 450 to 2000 and 350 to 2000 μg/L, respectively. However, 28% of laboratories using the CEDIA PLUS reported using only the low-range calibration curve and would have to dilute samples above 450 μg/L *(83)*. Sample dilution can lead to major inaccuracies in test results, and dilution protocols need to be carefully validated before implementation *(83,84)*. This is because CsA metabolites may not dilute in a linear fashion, and there may be differences in the amount of time needed for diluted samples to re-equilibrate, depending on the immunoassay and dilution protocol. Proficiency testing programs have demonstrated that laboratories produce widely varying results when challenged with samples with CsA concentrations outside the analytical range of immunoassays. For instance, at a CsA parent concentration of 2000 μg/L, 125 laboratories participating in the survey reported CsA values ranging from 1082 to 3862 μg/L *(84)*. These findings indicate that laboratories need to develop carefully controlled validated dilution protocols. A validated dilution protocol for the monoclonal FPIA on the TDx has recently been described *(85)*.

Another concern with C2 monitoring is metabolite concentrations and the need for therapeutic ranges that are assay-specific. This clearly is necessary when measuring trough CsA concentrations. A recent study monitoring C2 concentrations in kidney and liver transplant patients found equivalent CsA results when measured using the FPIA, CEDIA PLUS, and Syva EMIT *(86)*. As expected, paired trough samples produced CsA concentrations that differed among the immunoassays. These data indicate that for C2 monitoring, assay-specific therapeutic ranges may not be necessary.

## *3.2. Tacrolimus*

Tacrolimus (also known as FK-506) is a macrolide antibiotic with a molecular weight of 822 (Fig. 1) that was originally isolated from the fungus *Streptomyces tsukubaensis (5)*. In the USA, tacrolimus (brand name Prograf) was approved for use in liver transplantation in 1994 and in kidney transplantation in 1997. It is approximately 100 times more potent than CsA and is associated with a decrease in acute and chronic rejection, and better long-term graft survival *(87)*. In 2004, more than two-thirds of all kidney and liver transplant recipients, and approximately one-half of all heart transplant recipients, were receiving tacrolimus before hospital discharge *(20)*. At the author's institution, approximately 3.5 times more tacrolimus tests are performed compared with CsA.

### 3.2.1. Pharmacokinetics

Tacrolimus is available for both oral and intravenous administration. Similar to CsA, oral absorption of tacrolimus from the gut is poor and highly variable, averaging

25% *(88)*. Peak blood concentrations occur within 1.5–4 h. Tacrolimus is primarily bound to albumin, $\alpha_1$-acid glycoprotein, and lipoproteins in the plasma. However, the majority of tacrolimus is found within erythrocytes *(89)*.

Tacrolimus is metabolized using cytochrome P450 isoenzymes (CYP3A) located in the small intestine and liver. Similar to CsA, the bioavailability of tacrolimus is influenced by CYP3A and the multidrug efflux pump (P-glycoprotein) located in intestinal enterocytes. Biotransformation of tacrolimus occurs by demethylation, hydroxylation, and oxidative reactions *(90)*. At least nine metabolites have been identified based on in vitro studies *(91)*, and all, with the exception of 31-*o*-demethyl tacrolimus (M-II), have very little immunosuppressive activity. M-II has been shown in vitro to have the same immunosuppressive activity as parent compound *(92)*. Metabolites represent 10–20% of whole blood tacrolimus concentrations *(93)*. Tacrolimus is eliminated primarily by biliary excretion into the feces. Patients with hepatic dysfunction require dosage adjustments. Very little tacrolimus is found in urine, and blood concentrations are not altered in renal dysfunction.

### 3.2.2. Adverse Effects

Tacrolimus shares many dose-dependent side effects with CsA *(94)*. These include nephrotoxicity, neurotoxicity, hepatotoxicity, hypertension, and glucose intolerance. Nephrotoxicity with tacrolimus may be less of a problem than with CsA, especially in renal transplantation *(95)*. Diabetogenesis is approximately three times more common with tacrolimus than with CsA *(96)*. Hyperkalemia, hyperuricemia, hyperlipidemia, hirsutism, and gingival hypertrophy are also observed following tacrolimus use, but less commonly than with CsA *(97)*. Alopecia is also associated with tacrolimus use *(94)*.

### 3.2.3. Drug Interactions

Because tacrolimus is metabolized mainly by the cytochrome P450 system, the majority of drug interactions described for CsA also apply to tacrolimus *(88)*. St John's wort also decreases blood tacrolimus concentrations.

### 3.2.4. Preanalytic Variables

For quantitation of tacrolimus, EDTA-anticoagulated whole blood is the specimen of choice for the same reasons provided for CsA. Whole blood samples are stable for 1 week when shipped by mail without coolant *(98,99)*, 1–2 weeks at room temperature *(99,100)*, 2 weeks at refrigerator temperatures *(100)*, and almost 1 year at −70°C *(100)*.

Trough blood tacrolimus concentrations are almost exclusively used for routine monitoring and are believed to be a good indicator of total drug exposure *(101)*. However, recent experience with CsA has challenged this notion, and alternative draw times 1–6 h after dosing have been proposed *(102)*. Whereas some investigators have found a poor correlation between trough tacrolimus concentrations and total drug exposure, others have found good correlation *(103,104)*. Overall, the findings suggest that trough tacrolimus concentrations are predictive of total drug exposure and that measuring tacrolimus at specified times after dosing may not result in dramatic improvements. Until this issue is fully resolved, trough levels will continue to be used for reasons of convenience and reproducibility.

### 3.2.5. Methods of Analysis

Monitoring of tacrolimus is an integral part of any organ transplant program because of variable dose-to-blood concentrations and the narrow therapeutic index. Tacrolimus can be measured using enzyme-linked immunosorbent assay (ELISA), semi-automated and automated immunoassay, and HPLC-MS (Table 6). The ELISA and semi-automated immunoassays require a manual whole blood pre-treatment step. The Dimension ACMIA does not require a pretreatment step allowing whole blood samples to be directly placed on the instrument. Sample extraction can be semi-automated using modern HPLC-MS systems *(105)*.

The ELISA takes about 4 h to complete, requires numerous manual steps, and is used by few clinical laboratories. The Abbott microparticle enzyme immunoassay (MEIA) II on the IMx instrument is currently used by 88% of the laboratories in the USA that participate in the College of American Pathologists immunosuppressive proficiency testing program (Table 6). The MEIA II has a reported detection limit of 2 μg/L and replaced an earlier version (MEIA I) with a detection limit of 5 μg/L. The tacrolimus Syva EMIT has applications for Dade Behring instrumentation, the COBAS Integra 400 *(106)*, the Beckman Synchron LX20 PRO *(107)*, and the Bayer ADVIA 1650 *(108)*. However, the Syva EMIT is currently available only outside the USA. Microgenics has just released a CEDIA for tacrolimus in the USA that has applications for several Hitachi, Olympus, and Beckman instruments. Dade-Behring has just launched (July 2006) an ACMIA to measure tacrolimus using the Dimension family of analyzers and the V-Twin and Viva-E drug-testing analyzers. It uses the same monoclonal antibody used in the Syva EMIT to measure tacrolimus. Lastly, Abbott is developing a chemiluminescent immunoassay for use on their ARCHITECH system *(109)*.

**Table 6**
**Analytical Methods to Measure Tacrolimus**

| *Method* | *Assay* | *Manufacturer* | *Laboratories Using Method (%)*[a] |
|---|---|---|---|
| ELISA | Pro-Trac II | DiaSorin | ≤ 3 |
| Immunoassay | | | |
| Semi-Automated | MEIA II | Abbott | 88 |
| | Syva EMIT | Dade-Behring | –[b] |
| | CEDIA | Microgenics | ≤ 3 |
| Automated | Dimension ACMIA | Dade-Behring | –[c] |
| HPLC-MS | | | 9 |

ELISA, enzyme-linked immunosorbent assay; MEIA, microparticle enzyme immunoassay; EMIT, enzyme-multiplied immunoassay technique; CEDIA, cloned enzyme donor immunoassay; ACMIA, antibody-conjugated magnetic immunoassay; HPLC-MS, high-performance liquid chromatography with mass spectrometry detection.

[a] Percentages are based on the College of American Pathologists Immunosuppressive Drugs Monitoring Survey of 2006.

[b] Currently available only outside the USA.

[c] This assay received Food and Drug Administration (FDA) clearance and was launched in July 2006.

HPLC-MS methods are used by most of the laboratories not using the MEIA II. Tacrolimus cannot be measured by HPLC-UV because the molecule does not possess a chromophore. It is noteworthy that HPLC-MS is the only method that is specific for parent drug and meets the recommendations set forth in Consensus documents *(42)*. There are numerous recently reported assays to quantitate tacrolimus by using HPLC-MS or HPLC-MS/MS with detection limits <0.5 ng/mL *(105,110)*. A major advantage of HPLC-MS over immunoassays is the ability to simultaneously measure other immunosuppressant drugs in the same whole blood sample, such as CsA, sirolimus, and everolimus *(111)*.

### 3.2.6. Metabolite Cross-Reactivity

All the immunoassays have significant cross-reactivities with tacrolimus metabolites. The ELISA, MEIA II, and EMIT cross-react with M-II (31-*o*-demethyl), M-III (15-*o*-demethyl) and M-V (15,13-di-*o*-demethyl) metabolites of tacrolimus *(112)*. The CEDIA has significant cross-reactivity with M-I (13-*o*-demethyl) but does not cross-react with M-II or M-III. Cross-reactivity of the CEDIA with M-V has not been examined *(113)*. The ACMIA is expected to have metabolite cross-reactivity similar to the EMIT because both assays use the same monoclonal antibody. The extent of positive bias because of metabolite cross-reactivity is dependent on the transplant group studied. Metabolite cross-reactivity in patients with good liver function is typically not a problem because metabolite concentrations are relatively low compared with parent drug *(114)*. However, metabolites tend to accumulate during reduced liver function and immediately after liver transplant, resulting in significant assay interference and falsely high blood tacrolimus concentrations *(115)*. Overall, the MEIA II produces tacrolimus results that are 15–20% higher, the EMIT produces results 17% higher, and the CEDIA produces results 19% higher than those obtained by HPLC-MS, in kidney and liver transplant patients *(107,112,113,116,117)*. Calibration error may also contribute to some of the overall positive bias.

### 3.2.7. Analytical Considerations

The recommended therapeutic range for whole blood tacrolimus concentrations after kidney and liver allograft transplants is 5–20 μg/L when measured using HPLC-MS *(118)*. When tacrolimus is used with other immunosuppressive agents such as sirolimus, the desired target concentration for tacrolimus can be considerably <5 μg/L. In view of this, it is important for each laboratory to determine performance characteristics of their tacrolimus assay at concentrations <5 μg/L and make transplant services aware of the lower limit of detection and the imprecision (%CV) at this concentration. The functional sensitivity (between-day CV <20%) of the MEIA II and CEDIA is reported to be around 2 μg/L *(112,116,119,120)*, whereas the detection limit of the EMIT is around 3 μg/L *(107)*. At our institution, we examined functional sensitivity of the MEIA II tacrolimus assay by measuring whole blood pools at various concentrations in duplicate during a 10-day period. As shown in Fig. 2, a 20% CV was observed at a tacrolimus concentration of approximately 2 μg/L. In addition, we found that the MEIA II produced tacrolimus concentrations ranging from 0.8 to 1.7 μg/L when testing samples from patients not receiving tacrolimus ($n = 8$). Homma et al. *(121)* also found false-positive results when measuring tacrolimus in whole blood samples

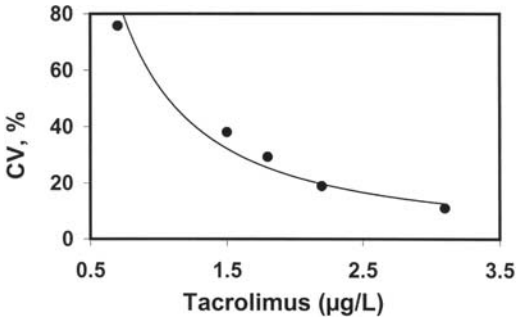


**Fig. 2.** Functional sensitivity of the Abbott tacrolimus microparticle enzyme immunoassay (MEIA) II on the IMx instrument. Whole blood patient pools at varying tacrolimus concentrations were analyzed in duplicate on 10 separate days. The coefficient of variation (CV) is the standard deviation of the mean tacrolimus concentration divided by the mean. The value is multiplied by 100 and is expressed as a percentage (%).

from patients not receiving tacrolimus using the MEIA. Based on our data, we use a cutoff of 2 µg/L for tacrolimus and report values lower than this cutoff as <2 µg/L.

The MEIA II has been shown to produce falsely elevated tacrolimus concentrations when the hematocrit is <25% *(122,123)*. The EMIT for tacrolimus is not affected by changes in hematocrit values *(123)*. Hematocrit bias in the MEIA II could result in therapeutic tacrolimus blood concentrations in under-immunosuppressed patients because of low hematocrit values. This would potentially be most problematic shortly after transplant when hematocrit values are typically at their lowest concentrations. This tacrolimus bias could also make it difficult to appropriately dose patients with widely fluctuating hematocrit values.

The reliability of the MEIA II at low whole blood tacrolimus concentrations has recently been questioned. At tacrolimus concentrations <9 µg/L, the MEIA II exhibited greater between-day imprecision and a weaker correlation with results obtained by HPLC-MS/MS *(124)*. Recovery experiments also demonstrated that the degree of over-estimation of tacrolimus using the MEIA II was more pronounced at lower drug concentrations *(124)*. Poor precision at low tacrolimus concentrations was also noted in the College of American Pathologists longitudinal immunosuppressive drug study. The study found that the major source of imprecision was within-laboratory variation over time, and it was postulated that the variation might be due to changes in assay standardization or reagent lot-to-lot changes *(125)*. Taken together, these performance variables are important to consider when selecting an assay to monitor whole blood tacrolimus concentrations.

## 4. MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS

The chemical structures of the mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus, are shown in Fig. 3. Both are macrocyclic lactones. Sirolimus (also known as rapamycin) is a lipophilic molecule (molecular weight of 914) derived from *Streptomyces hygroscopicus*. This actinomycete fermentation product was identified in the early 1970s and was approved by the FDA in 1999 for use with CsA to reduce the incidence of acute rejection in renal transplantation *(126)*. Everolimus is a

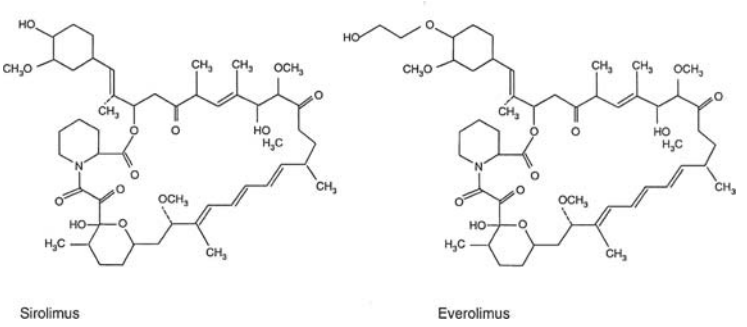


**Fig. 3.** Chemical structures of the mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus. This figure was published in Critical Reviews in Oncology/Hematology, Volume 56, Taylor AL, Watson CJE, Bradley JA, Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy, page 34, Copyright Elsevier 2005.

chemically modified version that is more hydrophilic than sirolimus and has improved pharmacokinetic characteristics and improved bioavailability *(127)*. Everolimus is still in phase III trials and is only available for investigational use in the USA.

Sirolimus and everolimus readily cross the lymphocyte plasma membrane and bind to the intracellular immunophilin, FK506-binding protein-12 *(128)*. In contrast to tacrolimus, sirolimus–immunophilin and everolimus–immunophilin complexes do not inhibit calcineurin activity. Instead, the complexes are highly specific inhibitors of the mTOR, a cell cycle serine/threonine kinase involved in the protein kinase B-signaling pathway. This results in suppressed cytokine-induced T-lymphocyte proliferation, with a block in progression from the G1 to S phase of the cell cycle *(129)*. The mTOR inhibitors work synergistically with the calcineurin inhibitors to produce a profound immunosuppressive effect on T lymphocytes.

## *4.1. Sirolimus*

### 4.1.1. Pharmacokinetics

Sirolimus is available for both oral and intravenous administration. Its long half-life of approximately 60 h allows once-a-day dosing *(130)*. Sirolimus is rapidly absorbed from the gastrointestinal tract, and peak blood concentrations occur 2 h after an oral dose *(131)*. Oral bioavailability is low, ranging from 5 to 15% *(132)* and is considerably reduced (approximately fivefold) when administered within 4 h or concomitantly with CsA *(133)*. There is considerable interpatient variability in total drug exposure that can vary by as much as 50% *(133)*. Sirolimus is primarily found within erythrocytes (95%), with approximately 3 and 1% partitioning into the plasma and lymphocytes/granulocytes, respectively *(134)*. Almost all of the plasma sirolimus is bound to proteins, with lipoproteins being the major binding protein.

Similar to the calcineurin inhibitors, sirolimus is metabolized in the intestine and liver by cytochrome P450 enzymes (CYP3A) *(135)*. The multidrug efflux pump P-glycoprotein in the gastrointestinal tract also controls metabolism by regulating bioavailability. Sirolimus is hydroxylated and demethylated to more than seven metabolites with the hydroxyl forms being the most abundant *(136)*. Metabolites represent

approximately 55% of whole blood sirolimus levels *(136)*. The pharmacological activity of metabolites has not been fully investigated because of difficulties associated with their isolation. However, preliminary studies indicate that the immunosuppressive activity of metabolites is <30% of that observed for the parent compound *(137)*. Sirolimus is eliminated primarily by biliary and fecal pathways, with small quantities appearing in urine *(135)*. As with the calcineurin inhibitors, dosage adjustments are needed in patients with hepatic dysfunction.

### 4.1.2. Adverse Effects

The incidence of adverse effects is dose-related and includes metabolic, hematological, and dermatological effects *(138)*. Metabolic side effects include hypercholesterolemia, hyper- and hypokalemia, hypophosphatemia, hyperlipidema, and increased liver function tests. Anemia can be problematic, with decreases in leukocyte, erythrocytes, and platelet counts being the most common. Skin rashes, acne, and mouth ulcers are also observed in patients being switched to mTOR inhibitors. As with other immunosuppressive drugs, there is an increased risk of infection and an association with lymphoma development. Interstitial pneumonitis is also associated with sirolimus therapy *(139)*.

### 4.1.3. Drug Interactions

CYP3A inhibitors such as antifungal agents (itraconazole, ketoconazole), clarithromycin, erythromycin, and verapamil increase blood levels of sirolimus. CYP3A inducers such as carbamazepine, phenobarbital, phenytoin, and rapamycin may decrease sirolimus blood levels. Grapefruit juice can increase sirolimus by decreasing drug clearance. St John's wort can decrease sirolimus levels. As previously noted, the concomitant use of CsA can result in increased sirolimus concentrations *(140)*. Although tacrolimus and sirolimus compete for sites on the same binding protein, the two drugs do not appear to have significant drug–drug interactions in clinical practice *(104)*.

### 4.1.4. Preanalytic Variables

EDTA-anticoagulated whole blood is the recommended specimen matrix *(132)*. This is because almost all of the sirolimus (~95%) is concentrated in erythrocytes, and plasma levels are too low for most analytical methods *(134)*. Whole blood samples are stable for 10 days at ambient temperature *(141)*, at least 1 week at 30–34°C *(141, 142)*, 30 days at 4°C *(143)*, and at least 2 months at −40°C *(143)*. Whole blood samples can withstand three freeze-thaw cycles without altering measured sirolimus concentrations *(141,142)*.

In contrast to the calcineurin inhibitors, there is good correlation between predose sirolimus concentrations and total drug exposure based on area under the curve measurements *(104,144)*. This also holds true when sirolimus is used in combination with CsA or tacrolimus *(104,144)*. Thus, whole blood 24-h trough specimens are recommended when monitoring sirolimus *(132)*.

### 4.1.5. Methods of Analysis

Therapeutic monitoring of sirolimus is critical because the administered dose is a poor predictor of total drug exposure because of individual patient variables. Because of the long drug half-life, daily monitoring of sirolimus is typically not necessary. Weekly

monitoring of levels may be needed shortly after transplantation followed by monthly monitoring. Target concentrations for sirolimus range between 4 and 12 μg/L when used in combination with a calcineurin inhibitor *(145)*. Similar to tacrolimus, these relatively low whole blood concentrations can be a challenge analytically for some of the currently available methods of analysis. As combination immunosuppressant therapies continue to evolve, target concentrations for sirolimus may become lower, further challenging the analytical performance of some of the currently utilized assays.

Sirolimus can be measured by immunoassay and HPLC with UV or MS detection. According to the College of American Pathologist proficiency testing program (1st survey of 2006), more than 130 laboratories in the USA currently perform sirolimus testing. Approximately 60% of the laboratories measure whole blood sirolimus by the Abbott IMx MEIA that became commercially available in 2004. The original Abbott MEIA kit was only used experimentally to support early clinical studies (investigational use only) and was never available commercially for routine monitoring of sirolimus. The "investigational use only" Abbott immunoassay was discontinued in 2001. A CEDIA for sirolimus (Microgenics) has recently become commercially available for use on several Roche automated analyzers (Hitachi 911, 912, 917, and modular P). The Microgenics sirolimus immunoassay is currently not used by many laboratories in the USA. The majority of laboratories not using the Abbott MEIA (approximately 34%) measure sirolimus by HPLC-MS. The major advantage of HPLC-MS is increased sensitivity and specificity, despite the need for highly skilled personnel. A few laboratories measure sirolimus by HPLC-UV, although this method requires elaborate sample cleanup procedures and long chromatographic run times *(146–148)*. This results in higher labor costs, making HPLC-UV methods unsuitable for laboratories supporting large transplant programs.

### 4.1.6. Metabolite Cross-Reactivity

Both of the currently available immunoassays have significant cross-reactivity with sirolimus metabolites. The MEIA method has 58 and 63% cross-reactivity with 41-*o*-demethyl-sirolimus and 7-*o*-demethyl-sirolimus, respectively *(149)*. The CEDIA has 44% cross-reactivity with 11-hydyroxy-sirolimus and 73% cross-reactivity with 41- and 32-*o*-demethyl-sirolimus *(150)*. This degree of metabolite cross-reactivity results in significant bias between assays. The MEIA produces whole-blood sirolimus concentrations that are 9–49% higher than those obtained by HPLC-UV and HPLC-MS, depending on the study and transplant group studied *(149,151–155)*. One study found that the CEDIA method produces whole blood sirolimus levels with a mean positive bias of 20.4% compared with HPLC-MS *(156)*. However, immunoassay metabolite cross-reactivity may be less of an issue from a clinical standpoint because the distribution of metabolites in whole blood are similar among patients and are relatively stable over long periods of time *(157)*.

### 4.1.7. Analytical Considerations

The therapeutic window for sirolimus appears to be between 5 and 15 μg/L when used in combination with CsA and between 12 and 20 μg/L when used alone *(130)*. Sirolimus levels slightly below the currently used therapeutic range can be a challenge for some of the HPLC-UV methods, with functional sensitivities (based on between-day

CVs of <20%) of 2–3 μg/L *(147,148)*. This is also true for the two currently available immunoassays. The MEIA method has a functional sensitivity that varies among laboratories, with values ranging from 1.3 to 3.0 μg/L *(149,151–155)*. Technical variations at the manual extraction step most likely contribute to the differences in functional sensitivity that were observed among laboratories evaluating the MEIA. One study found that the CEDIA has a functional sensitivity of 3.0 μg/L *(156)*. HPLC-MS methods have excellent sensitivity, with functional sensitivities <1 μg/L *(158,159)*. As previously mentioned, a further advantage of HPLC-MS methods is the ability to measure multiple immunosuppressants in the same whole blood sample. It is important that laboratories experimentally determine their own lower limit of detection based on long-term between day imprecision data (using whole blood samples) and not rely on package insert information or published data.

The sirolimus MEIA is prone to error that is dependent on hematocrit levels. There is an inverse relationship between hematocrit and measured sirolimus levels. At a sirolimus concentration of 5 μg/L, results can be 20% higher for hematocrits of <35% and as much as 20% lower for hematocrits >45% *(149,160)*. When the hematocrit is between 35 and 45%, MEIA bias is <10% at sirolimus concentrations ranging from 5 to 22 μg/L. Incomplete extraction of sirolimus from erythrocyte-binding proteins is the most probable mechanism leading to the hematocrit interference. The CEDIA does not appear to be affected by variations in hematocrit between 20 and 60% *(150)*; however, there are no independently published studies supporting the manufacturer's claim.

### *4.2. Everolimus*

Everolimus (also known as SZD RAD) is a structural analogue of sirolimus with an additional hydroxyethyl group (Fig. 3). Everolimus is currently in phase III clinical trials in the USA and has not received FDA approval for use as an immunosuppressive agent. Because everolimus is still in the experimental stage it will only be briefly discussed.

#### 4.2.1. Pharmacokinetics

Everolimus has improved bioavailability *(161,162)* and a shorter elimination half-life (~24 h) than sirolimus *(163)*. Everolimus also has lower intrapatient drug variability than sirolimus *(144,164)*. Concomitant use of CsA results in increased everolimus blood concentrations due to inhibition of everolimus metabolism *(165)*. Similar to sirolimus, everolimus is metabolized in the intestine and liver by cytochrome P450 enzymes. At least 20 metabolites have been identified *(166)*, with mono-hydroxyl, di-hydroxyl, demethylated, and an open ring form being the major metabolites *(167)*. Metabolites are in relatively low concentrations when monitoring trough blood concentrations *(167)*.

#### 4.2.2. Methods of Analysis

Immunoassays to measure everolimus are not currently available in the USA and most likely will lag behind FDA approval of the drug. Seradyn has developed an FPIA (Innofluor Certican Assay System) to measure whole blood everolimus outside the USA on Abbott TDx instrumentation *(168)*. The FPIA method has a functional sensitivity of 2 μg/L *(168)*, which is just below the therapeutic trough blood concentration lower limit of 3 μg/L *(169)*. When compared with HPLC-MS, the FPIA has a positive

mean bias of 24.4% in renal transplant recipients *(170)*. The positive bias is due to differences in calibrator-assigned values and antibody cross-reactivity with everolimus metabolites *(170)*. Cross-reactivity with metabolites ranges from 5 to 72% *(168)*. HPLC-UV and HPLC-MS methods are also available to measure everolimus *(171,172)*.

## 5. MYCOPHENOLIC ACID

MPA is a fermentation product of *Penicillium* species that was originally shown to have antibacterial, antifungal, and immunosuppressive potential in animal studies *(173)*. To improve the bioavailability of MPA, mycophenolate mofetil (brand name CellCept), the 2-morpholinoethyl ester of MPA was developed for oral and intravenous administration *(174)*. Mycophenolate mofetil received FDA approval for use as an immunosuppressant with corticosteroids and CsA to prevent organ rejection in 1995. The sodium salt of MPA, mycophenolate sodium (brand name Myfortic), has recently become available for oral administration as delayed-release tablets. MPA has primarily replaced azathioprine in organ transplantation. The chemical structure of the active compound MPA and the two parent compounds are shown in Fig. 4.

MPA is a potent non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) enzymatic activity *(175)*. IMPDH is the rate-limiting enzyme in the production of guanosine nucleotides that are required for DNA synthesis and

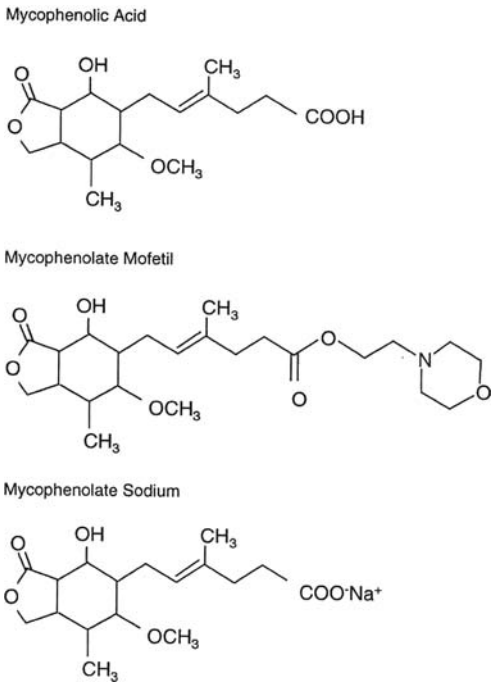


**Fig. 4.** Chemical structures of the active compound mycophenolic acid (MPA), and the two prodrugs, mycophenolate mofetil and mycophenolate sodium. This figure was published in Critical Reviews in Oncology/Hematology, Volume, Taylor AL, Watson CJE, Bradley JA, Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy, page 29, Copyright Elsevier 2005.

cellular proliferation. Guanosine nucleotides are synthesized in most cell types using the IMPDH pathway and a separate salvage pathway. However, the salvage pathway is not found in lymphocytes, and MPA blockage of the IMPDH pathway selectively inhibits lymphocyte proliferation *(176,177)*. There are two isoforms of IMPDH and MPA selectively inhibit the type II isoform, which is predominantly expressed by activated and not resting lymphocytes *(178)*.

### *5.1. Pharmacokinetics*

Mycophenolate mofetil and mycophenolate sodium are rapidly and completely absorbed, and quickly de-esterified in the blood and tissues to MPA, the active form of the drug. The half-life of mycophenolate mofetil during intravenous administration is <2 min *(179)*. Following an oral dose of mycophenolate mofetil, MPA reaches a maximum concentration within 1 h *(180)*. Almost all the drug (>99%) can be found in the plasma compartment *(181)*. For this reason, serum or plasma MPA concentrations are used for routine therapeutic drug monitoring of MPA.

MPA has an elimination half-life of 18 h and is glucuronidated in the liver to the primary inactive metabolite, 7-*o*-glucuronide mycophenolic acid (MPAG) *(182)*. Small quantities of the inactive metabolite 7-*o*-glucoside are also produced in the liver *(180,183)*. Another metabolite produced in small quantities is acyl glucuronide, an active metabolite that may contribute to the adverse gastrointestinal effects of MPA *(184)*. MPAG exhibits significant enterohepatic recirculation with a second MPA plasma peak occurring 4–12 h after drug administration. The kidneys primarily clear MPAG with concentrations rapidly accumulating in patients with severe renal impairment (glomerular filtration rates <25 mL/min) *(185)*. MPA is extensively bound in the circulation to albumin with typical concentrations of free or unbound MPA ranging from 1.25 to 2.5% of the total concentration *(181)*. Free MPA concentrations are increased in hypoalbuminemia, hyperbilirubinemia, and uremia *(186)*. It has been shown that the immunosuppressive effects of MPA are related to free MPA and not the total drug concentration *(181)*. In chronic renal failure, the free concentration of MPA can increase dramatically indicating over immunosuppression when the total MPA concentration is within the therapeutic range *(186,187)*.

### *5.2. Adverse Effects*

Adverse effects from mycophenolate mofetil and mycophenolate sodium are similar. The most common dose-limiting unwanted side effects are diarrhea, nausea, vomiting, and abdominal pain *(188)*. Marrow suppression and anemia can also occur *(94)*. An increased risk of cytomegalovirus, candida, and herpes simplex infections has also been reported *(94,189)*.

### *5.3. Drug Interactions*

Coadministration of CsA results in significantly lower trough concentrations of MPA *(190)*, most likely because of diminished enterohepatic recirculation of MPAG and MPA *(191)*. The antibiotics mycostatin, tobramycin, and cefuroxime also decrease MPA bioavailability by a similar mechanism *(192)*. Tacrolimus may increase the bioavailability of MPA by inhibiting MPAG formation *(193)*; however, additional

studies are needed to confirm this potential drug interaction. Steroids such as dexamethasone lower MPA concentrations by augmenting the activity of the enzyme responsible for MPA metabolism. Several non-steroidal inflammatory drugs such as niflumic acid, diflunisal, flufenamic acid, mefenamic acid, and salicylic acid increase MPA concentrations by inhibiting MPA glucuronidation *(194)*. Antacids (aluminum and magnesium hydroxide) lower total MPA exposure by reducing drug absorption in the gastrointestinal tract. Other drugs such as calcium polycarbophil and iron ion preparations also result in decreased MPA concentrations by the same mechanism *(195)*. Lastly, salicylic acid and furosemide increase the free fraction of MPA by altering albumin binding.

### *5.4. Preanalytic Variables*

Plasma or serum can be used to measure MPA and free MPA blood concentrations *(187)*. However, plasma from EDTA-anticoagulated whole blood is the recommended specimen of choice because the same sample can be used to measure whole blood CsA, tacrolimus, and sirolimus *(196)*. MPA and MPAG are stable in whole blood and plasma samples at room temperature for at least 4 h *(197)*. Plasma samples are stable at 4°C for 4 days and at least 11 months when stored at −20°C *(196)*. Free MPA is stable for at least 6 months when stored at −20°C *(198)*. Thawing and refreezing of plasma samples can be performed up to four times without significant loss of MPA *(199)*. When monitoring MPA during intravenous infusion of mycophenolate mofetil, whole blood samples should be immediately placed in ice and the plasma separated within 30 min *(200,201)*. This is because mycophenolate mofetil is very unstable and rapidly undergoes temperature-dependent degradation to MPA in whole blood samples placed at room temperature *(200)*.

Trough concentrations of MPA are routinely used for drug monitoring and are generally believed to be a relatively good indicator of total drug exposure *(202)*. This is somewhat surprising as numerous studies have shown that area under the curve (0–12 h) measurements are more predictive of total drug exposure and acute graft rejection than trough concentrations *(203–205)*. In addition, MPA trough concentrations can vary considerably depending upon time after transplantation *(205)*. Nevertheless, the superiority of area under the curve measurements is probably overshadowed by practical considerations such as additional testing costs and difficulties associated with the collection of multiply timed samples.

### *5.5. Methods of Analysis*

When MPA was originally approved for use (as mycophenolate mofetil), therapeutic drug monitoring was considered unnecessary. However, recent studies have found wide variations in total drug exposure (as high as 10-fold) following a fixed dose, suggesting that individualized dosing may be of considerable benefit *(206,207)*. A roundtable meeting recently recommended therapeutic drug monitoring based on the interpatient variability and the significant drug interactions associated with combination immunosuppressive therapy *(208)*.

At the present time, fewer than 30 laboratories in the USA measure MPA (1st CAP proficiency survey of 2006). Roughly half the laboratories measure MPA using HPLC,

with the majority of remaining laboratories using HPLC-MS methods. Numerous HPLC methods with UV, fluorimetric, and MS detection systems have been described to measure MPA in plasma samples *(198,199,209–211)*. The HPLC methods primarily differ in sample extraction, analytical column, run-time, and lower limit of detection. Free MPA can be measured using HPLC methods after separation of protein-bound MPA by ultrafiltration *(185,212)*. However, free MPA is typically more difficult to measure and does not appear to be superior to total MPA in predicting clinical outcomes in most transplant patients *(213)*.

Automated assays to measure MPA are currently not available in the USA. Several companies are developing product applications for various automated instruments for either serum and/or plasma samples. For instance, Dade-Behring is developing an enhanced turbidimetric inhibition immunoassay to measure MPA for use on Dimension clinical chemistry analyzers *(214)*. Microgenics is developing a CEDIA to measure MPA on Hitachi, Olympus, and Microgenics (MGC 240) clinical chemistry analyzers *(215)*. Lastly, Roche (Roche Diagnostics, Indianapolis, IN, USA) is developing an enzyme receptor assay to measure total MPA and free MPA using the COBAS INTEGRA system (Roche Diagnostics, Indianapolis, IN, USA) *(216)*. At the time of this writing, none of these assays have been submitted to the FDA for review.

Dade-Behring has an EMIT 2000 MPA immunoassay that is widely used outside the USA. The assay can be performed on Dade-Behring Dimension instruments, the Roche COBAS, and Hitachi automated chemistry analyzers. The antibody used in the EMIT assay has cross-reactivity with acyl glucoronide *(217)* and produces MPA values that are approximately 10–30% higher than those obtained using HPLC *(218–221)*. The bias can be considerably higher in patients with impaired renal function because of accumulation of acyl glucoronide *(218,222)*. The positive bias because of acyl glucoronide cross-reactivity may turn out to be advantageous because metabolite has in vitro anti-IMPDH activity *(206,223)*.

### *5.6. Analytical Considerations*

The generally accepted therapeutic range for trough MPA plasma concentrations is 1.0–3.5 mg/L *(196,224,225)*. This range of values can be easily measured by currently available analytical methods with good precision. Concentrations of free MPA are typically 2% of the total MPA level and can be analytically challenging for some of the HPLC-UV methods *(226)*. In these situations, the functional sensitivity of the free MPA assay needs to be carefully validated.

HPLC is the reference method for measuring MPA that other methods are validated against. This is because HPLC is highly specific for parent compound and is free from coadministered drug interferences *(200,209–211)*. As immunoassays to measure MPA become available in the USA, metabolite cross-reactivity and assay bias will have to be taken into account when interpreting MPA concentrations.

## 6. CONCLUSION

Advances in immunosuppressive therapy are largely responsible for the success and improved outcomes that are now obtained following allogeneic organ transplantation. Today, very few allografts are lost to immune-mediated acute rejection, and there

is remarkable improvement in patient and graft survival. A major goal of immunosuppressive drug therapy is to optimize therapeutic effectiveness while minimizing unwanted adverse effects. Therefore, therapeutic drug monitoring plays a central role because a "one size fits all" approach for immunosuppressive drugs has proved unsuccessful, with optimal drug therapy requiring individualized dosing. Therapeutic monitoring of CsA, tacrolimus, and sirolimus is now considered an integral part of organ transplant programs, and several arguments have been made for monitoring MPA.

Although HPLC is considered the reference method for monitoring immunosuppressive drugs, the majority of laboratories in the USA are currently using immunoassays. Immunoassays are attractive because they can be automated, have low start-up costs, and do not require highly skilled testing personnel. Their major drawback is metabolite cross-reactivity, which results in varying degrees of positive bias that is unique to each immunoassay. Furthermore, cross-reactivity is not always predictable and can vary depending on post-transplant time and type of organ transplanted. The advantage of HPLC is high specificity and the ability to separate metabolites from parent compound. Drawbacks of HPLC include the need for extensive sample cleanup, long analytical run times, and specialized training. This can be partially overcome by using HPLC with MS detection, which requires less sample preparation and has shorter run times than HPLC with UV detection. Unfortunately, HPLC-MS systems are currently very expensive and require highly trained operating personnel. New HPLC-MS systems with automated sample preparation are emerging that are considerably easier to operate. Given the cost of immunoassay reagents, these newer systems are becoming more cost effective, especially when one considers that HPLC-MS can simultaneously measure multiple immunosuppressive drugs in a single whole blood specimen.

**Note:** At the time of this book's printing, the Roche total and free MRA assays had just been cleared for use in the United States.

## REFERENCES

1. Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. *JAMA* 1956;160:277–282.
2. Murray JE, Merrill JP, Harrison JH, Wilson RE, Dammin GJ. Prolonged survival of human-kidney homographs by immunosuppressive drug therapy. *N Engl J Med* 1963;268:1315–1323.
3. Starzl TE, Marchioro TL, Waddell WR. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. *Surg Gynecol Obstet* 1963;117:385–395.
4. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD, Rolles K. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet* 1978;2:1323–1327.
5. Goto T, Kino T, Hatanaka H, Nishiyama M, Okuhara M, Kohsaka M, Aoki H, Imanaka H. Discovery of FK-506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*. *Transplant Proc* 1987;19:4–8.
6. Singh K, Sun S, Vezina C. Rapamycin (AY-22,989), a new antifungal antibiotic. IV. Mechanism of action. *J Antibiot (Tokyo)* 1979;32:630–645.
7. Sollinger HW. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* 1995;60:225–232.

8. A blind, randomized clinical trial of mycophenolate mofetil for the prevention of acute rejection in cadaveric renal transplantation. The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group. *Transplantation* 1996;61:1029–1037.
9. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. European Mycophenolate Mofetil Cooperative Study Group. *Lancet* 1995;345:1321–1325.
10. Organ Procurement and Transplant Network. Transplants by organ and donor type, 1995-2004, and National transplant statistics, national reports. In: *OPTN/SRTR 2005 Annual Report: Summary Table* ? *and Organ Summary Tables* (Accessed August 10, 2006, at http://www.ustransplant.org).
11. Organ Procurement and Transplant Network. National transplant statistics, national reports. In: *OPTN/SRTR 2005 Annual Report: Organ Summary Tables* (Accessed August 10, 2006, at http://www.ustransplant.org).
12. Lindholm A, Albrechtsen D, Tufveson G, Karlberg I, Persson NH, Groth CG. A randomized trial of cyclosporine and prednisolone versus cyclosporine, azathioprine, and prednisolone in primary cadaveric renal transplantation. *Transplantation* 1992;54:624–631.
13. Cyclosporin in cadaveric renal transplantation: one-year follow-up of a multicentre trial. *Lancet* 1983;2:986–989.
14. Bruunshuus I, Schmiegelow K. Analysis of 6-mercaptopurine, 6-thioguanine nucleotides and 6-thiuric acid in biological fluids by high-performance liquid chromatography. *Scand J Clin Invest* 1989;49:779–784.
15. Kreuzenkamp-Jansen CW, De Abreu RA, Bokkerink JPM, Trijbels JMF. Determination of extracellular and intracellular thiopurines and methylthiopurines with HPLC. *J Chromatogr* 1995;672:53–61.
16. Rabel SR, Stobaugh JF, Trueworthy R. Determination of intracellular levels of 6-mercaptopurine metabolites in erythrocytes utilizing capillary electrophoresis with laser-induced fluorescence detection. *Anal Biochem* 1995;224:315–322.
17. Filler G, Lepage N, Delisle B, Mai I. Effect of cyclosporine on mycophenolic acid area under the concentration-curve in pediatric kidney transplant recipients. *Ther Drug Monit* 2001;23:514–519.
18. van Gelder T, Klupp J, Barten MJ, Christians U, Morris RE. Comparison of the effects of tacrolimus and cyclosporine on the pharmacokinetics of mycophenolic acid. *Ther Drug Monit* 2001;23:119–128.
19. Undre NA. Pharmacokinetics of tacrolimus-based combination therapies. *Nephrol Dial Transplant* 2003;18(Suppl 1):i12-i15.
20. Organ Procurement and Transplant Network. Immunosuppression: evolution in practice and trends, 1994-2004. In: *OPTN/SRTR 2005 Annual Report: Table* ? (Accessed June 19, 2006, at http://www.ustransplant.org).
21. Shibasaki F, Hallin U, Uchino H. Calcineurin as a multifunctional regulator. *J Biochem (Tokyo)* 2002;131:1–15.
22. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidylprolyl isomerase activity but is distinct from cyclophilin. *Nature* 1989;341:755–757.
23. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK-506. *Immunol Today* 1992;13:136–142.
24. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. *Nature* 1991;352:803–807.
25. Clipstone NA, Crabtree GR. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 1992;357:695–697.
26. Schreiber SL. Chemistry and biology of immunophilins and their immunosuppressive ligands. *Science* 1991;251:283–287.
27. Gummert JF, Ikonen T, Morris RE. Newer immunosuppressive drugs: a review. *J Am Soc Nephrol* 1999;10:1366–1380.
28. Jorgensen KA, Koefoed-Nielsen PB, Karamperis N. Calcineurin phosphatase activity and immunosuppression. A review on the role of calcineurin phosphatase activity and the immunosuppressive effect of cyclosporin A and tacrolimus. *Scand J Immunol* 2003;57:93–98.
29. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects on cyclosporin A: a new antilymphocytic agent. *Agents Actions* 1976;6:468–475.

30. Vonderscher J, Meinzer A. Rationale for the development of Sandimmune Neoral. *Transplant Proc* 1994;26:2925–2927.
31. Bartucci MR. Issues in cyclosporine drug substitution: implications for patient management. *J Transpl Coord* 1999;9:137–142.
32. Alloway RR. Generic immunosuppressant use in solid organ transplantation. *Transplant Proc* 1999;31(Suppl 3A):2S–5S.
33. Kovarik JM, Mueller EA, van Bree JB, Fluckinger SS, Lange H, Schmidt B, Boesken WH, Lison AE, Kutz K. Cyclosporine pharmacokinetics and variability from a microemulsion formulation-a multicenter investigation in kidney transplant patients. *Transplantation* 1994;58:658–663.
34. Ptachcinski RJ, Venkataramanan R, Burckart GJ. Clinical pharmacokinetics of cyclosporin. *Clin Pharmacokinet* 1986;11:107–132.
35. Hoppu K, Jalanko H, Laine J, Holmberg C. Comparison of conventional oral cyclosporine and cyclosporine microemulsion formulation in children with a liver transplant. *Transplantation* 1996;62:66–71.
36. Faulds D, Goa KL, Benfield P. Cyclosporin. A review of its pharmacodynamic and pharmacotherapeutic properties, and therapeutic use in immunoregulatory disorders. *Drugs* 1993;45:953–1040.
37. Noble S, Markham A. Cyclosporin. A review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (Neoral). *Drugs* 1995;50:924–941.
38. Fahr A. Cyclosporin clinical pharmacokinetics. *Clin Pharmacokinet* 1993;24:472–495.
39. Wenk M, Follath F, Abisch E. Temperature dependency of apparent cyclosporine A concentrations in plasma. *Clin Chem* 1983;29:1865.
40. Kahan BD, Shaw LM, Holt D, Grevel J, Johnston A. Consensus document: Hawk's meeting on therapeutic drug monitoring of cyclosporine. *Clin Chem* 1990;36:1510–1516.
41. Shaw LM, Yatscoff RW, Bowers LD, Freeman DJ, Jeffery JR, Keown PA, McGilveray IJ, Rosano TG, Wong PY. Canadian consensus meeting on cyclosporine monitoring: Report of the consensus panel. *Clin Chem* 1990;36:1841–1846.
42. Ollerich M, Armstrong VW, Kahan B, Shaw L, Holt DW, Yatscoff R, Lindholm A, Halloran P, Gallicano K, Wonigeit K, Schutz E, Schran H, Annesley T. Lake Louise consensus conference on cyclosporin monitoring in organ transplantation: report of the consensus panel. *Ther Drug Monit* 1995;17:642–654.
43. Zhang YC, Benet L. The gut as a barrier to drug absorption. *Clin Pharmacokinet* 2001;40:159–168.
44. Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, Wacher VJ. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clin Pharmacol Ther* 1995;58:492–497.
45. Lown KS, Mayo RR, Leichtman AB, Hsiao HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, Watkins PB. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clin Pharmacol Ther* 1997;62:248–260.
46. Wallemacq PE, Lhoest G, Dumont P. Isolation, purification and structure elucidation of cyclosporine A metabolites in rabbit and man. *Biomed Mass Spectrom* 1989;18:48–56.
47. Christians U, Sewing KF. Cyclosporin metabolism in transplant patients. *Pharmacol Ther* 1993;57:291–345.
48. Yatscoff RW, Rosano TG, Bowers LD. The clinical significance of cyclosporine metabolites. *Clin Biochem* 1991;24:23–35.
49. Radeke HH, Christians U, Sewing KF, Resch K. The synergistic immunosuppressive potential of cyclosporin metabolite combinations. *Int J Immunopharmacol* 1992;14:595–604.
50. Rosano TG, Brooks CA, Dybas MT, Cramer SM, Stevens C, Freed BM. Selection of an optimal assay method for monitoring cyclosporine therapy. *Transplant Proc* 1990;22:1125–1128.
51. Ryffel B, Foxwell BM, Mihatsch MJ, Donatsch P, Maurer G. Biologic significance of cyclosporine metabolites. *Transplant Proc* 1988;20(Suppl 2):575–584.
52. Smets F, Sokal EM. Lymphoproliferation in children after liver transplantation. *J Pediatr Gastroenterol Nutr* 2002;34:499–505.
53. Scott JP, Higenbottam TW. Adverse reactions and interactions of cyclosporin. *Med Toxicol* 1988;3:107–127.

54. Yee GC, McGuire TR. Pharmacokinetic drug interactions with cyclosporin. *Clin Pharmacol* 1990;19:319–332 and 400–415.
55. Durr D, Stieger B, Kullak-Ublick GA, Rentsch KM, Steinert HC, Meier PJ, Fattinger K. St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. *Clin Pharmacol Ther* 2000;68:598–604.
56. Smith MC, Sephel GC. Long-term in vitro stability of cyclosporine in whole-blood samples. *Clin Chem* 1990;36:1991–1992.
57. Schran HF, Rosano TG, Hasse AE, Pell MA. Determination of cyclosporine concentrations with monoclonal antibodies. *Clin Chem* 1987;33:2225–2229.
58. Keown P, Landsberg D, Hollaran P, Shoker A, Rush D, Jeffery J, Russell D, Stiller C, Muirhead N, Cole E, Paul L, Zaltzman J, Loertscher R, Daloze P, Dandavino R, Boucher A, Handa P, Lawen J, Belitsky P, Parfrey P. A randomized, prospective multicenter pharmacoepidemiologic study of cyclosporine microemulsion in stable renal graft recipients. Report of the Canadian Neoral Renal Transplantation Study Group. *Transplantation* 1996;27:1744–1752.
59. Halloran PF, Helms LM, Noujaim J. The temporal profile of calcineurin inhibition by cyclosporine in vivo. *Transplantation* 1999;15:1356–1361.
60. Lindholm A, Kahan BD. Influence of cyclosporine pharmacokinetics, trough concentrations, and AUC monitoring on outcome after kidney transplantation. *Clin Pharmacol Ther* 1993;54: 205–218.
61. Lindholm A. Review: factors influencing the pharmacokinetics of cyclosporine in man. *Ther Drug Monit* 1991;13:465–477.
62. Grevel J, Welsh MS, Kahan B. Cyclosporine monitoring in renal transplantation: area under the curve monitoring is superior to trough-level monitoring. *Ther Drug Monit* 1989;11:246–248.
63. Belitsky P, Levy GA, Johnston A. Neoral absorption profiling: an evolution in effectiveness. *Transplant Proc* 2000;32(Suppl 3A):S45–S52.
64. Keown P, Niese D. Cyclosporine microemulsion increases drug exposure and reduces acute rejection without incremental toxicity in de novo renal transplantation. International Sandimmune Neoral Study Group. *Kidney Int* 1998;54:938–944.
65. Stein CM, Murray JJ, Wood AJ. Inhibition of stimulated interleukin-2 production in whole blood: a practical measure of cyclosporine effect. *Clin Chem* 1999;45:1477–1484.
66. Oellerick M, Armstrong VW. Two-hour cyclosporine concentration determination: An appropriate tool to monitor neoral therapy? *Ther Drug Monit* 2002;24:40–46.
67. Mahalati K, Belitsky P, Sketris I, West K, Panek R. Neoral monitoring by simplified sparse sampling area under the concentration-time curve. *Transplantation* 1999;68:55–62.
68. Grant D, Kneteman N, Tchervenkow J, Roy A, Murphy G, Tan A, Hendricks L, Guilbault N, Levy G. Peak cyclosporine levels (Cmax) correlate with freedom from liver graft rejection: results of a prospective, randomized comparison of neoral and sandimmune for liver transplantation (NOF-8). *Transplantation* 1999;67:1133–1137.
69. Cantarovick M, Barkun JS, Tchervenkov JI, Besner JG, Aspeslet L, Metrakos P. Comparison of Neoral dose monitoring with cyclosporine through levels versus 2-hour postdose levels in stable liver transplant patients. *Transplantation* 1998;66:1621–1627.
70. Cantarovick M, Elstein E, de Varennes B, Barkun JS. Clinical benefit of Neoral dose monitoring with cyclosporine 2-hour post-dose levels compared with trough levels in stable heart transplant patients. *Transplantation* 1999;68:1839–1842.
71. Wallemacq PE. Therapeutic monitoring of immunosuppressant drugs. Where are we? *Clin Chem Lab Med* 2004;42:1204–1211.
72. Whitman DA, Abbott V, Fregien K, Bowers LD. Recent advances in high-performance liquid chromatography/mass spectrometry and high-performance liquid chromatography/tandem mass spectrometry: detection of cyclosporine and metabolites in kidney and liver tissue. *Ther Drug Monit* 1993;15:552–556.
73. Zhou L, Tan D, Theng J, Lim L, Liu YP, Lam KW. Optimized analytical method for cyclosporine A by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2001;754:201–207.

74. Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. *Ther Drug Monit* 2004;26:215–219.
75. Belensky A, Lazzaro P, Shuaib A, Natrajan A, Costello J, Barbarakis M, Connolly P, Hartl T. Bayer ADVIA Centaur® cyclosporine assay: an analytical evaluation. *Clin Chem* 2006;52(Suppl):A60 (Abstract).
76. Tredger JM, Roberts N, Sherwood R, Higgins G, Keating J. Comparison of five cyclosporin immunoassays with HPLC. *Clin Chem Lab Med* 2000;38:1205–1207.
77. Steimer W. Performance and specificity of monoclonal immunoassays for cyclosporine monitoring: How specific is specific? *Clin Chem* 1999;45:371–381.
78. Schutz E, Svinarov D, Shipkova M, Niedmann PD, Armstrong VW, Wieland E, Oellerich M. Cyclosporin whole blood immunoassays (AxSYM, CEDIA, and Emit): a critical overview of performance characteristics and comparison with HPLC. *Clin Chem* 1998;44:2158–2164.
79. Hamwi A, Veitl M, Manner G, Ruzicka K, Schweiger C, Szekeres T. Evaluation of four automated methods for determination of whole blood cyclosporine concentrations. *Am J Clin Pathol* 1999;112:358–365.
80. Terrell AR, Daly TM, Hock KG, Kilgore DC, Wei TQ, Hernandez S, Weibe D, Fields L, Shaw LM, Scott MG. Evaluation of a no-pretreatment cyclosporin A assay on the Dade Behring Dimension RxL clinical analyzer. *Clin Chem* 2002;48:1059–1065.
81. Butch AW, Fukuchi AM. Analytical performance of the CEDIA®cyclosporine PLUS whole blood immunoassay. *J Anal Toxicol* 2004;28:204–210.
82. Immunosuppressive Drugs International Proficiency Testing Scheme URL (Accessed August 16, 2006, at http://www.bioanalytics.co.uk).
83. Morris RG, Holt DW, Armstrong VW, Griesmacher A, Napoli KL, Shaw LM. Analytical aspects of cyclosporine monitoring, on behalf of the IFCC/IATDMCT joint working group. *Ther Drug Monit* 2004;26:227–330.
84. Holt DW, Johnston A, Kahan BD, Morris RG, Oellerich M, Shaw LM. New approaches to cyclosporine monitoring raise further concerns about analytical techniques. *Clin Chem* 2000;46:872–874.
85. Juenke JM, Brown PI, Urry FM, McMillin GA. Specimen dilution for C2 monitoring with the Abbott TDxFLx cyclosporine monoclonal whole blood assay. *Clin Chem* 2004;50:1430–1433.
86. Johnston A, Chusney G, Schutz E, Oellerich M, Lee TD, Holt DW. Monitoring cyclosporin in blood: between-assay differences at trough and 2 hours post-dose (C2). *Ther Drug Monit* 2003;25:167–173.
87. First MR. Tacrolimus based immunosuppression. *J Nephrol* 2004;17:25–31.
88. Venkataramanan R, Swaminathan A, Prasad T, Jain A, Zuckerman S, Warty V, McMichael J, Lever J, Burckart G, Starzl T. Clinical pharmacokinetics of tacrolimus. *Clin Pharmacokinet* 1995;29:404–430.
89. Zahir H, Nand RA, Brown KF, Tattam BN, McLachlan AJ. Validation of methods to study the distribution and protein binding of tacrolimus in human blood. *J Pharmacol Toxicol Methods* 2001;46:27–35.
90. Iwasaki K, Shiraga T, Nagase K, Tozuka Z, Noda K, Sakuma S, Fujitsu T, Shimatani K, Sato A, Fujioka M. Isolation, identification, and biological activities of oxidative metabolites of FK506, a potent immunosuppressive macrolide lactone. *Drug Metab Dispos* 1993;21:971–977.
91. Kelly P, Kahan BD. Review: metabolism of immunosuppressant drugs. *Curr Drug Metab* 2002;3:275–287.
92. Tamura K, Fujimura T, Iwasaki K, Sakuma S, Fujitsu T, Nakamura K, Shimomura K, Kuno T, Tanaka C, Kobayashi M. Interaction of tacrolimus (FK506) and its metabolites with FKBP and calcineurin. *Biochem Biophys Res Commun* 1994;202:437–443.
93. Beysens J, Wigner RMH, Beuman GH, van der Heyden J, Kootstra G, van As H. FK 506: Monitoring in plasma or in whole blood? *Transplant Proc* 1991;23:2745–2747.
94. Taylor AL, Watson CJE, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol* 2005;56:23–46.
95. Artz MA, Boots JM, Ligtenberg G, Roodnat JI, Christiaans MH, Vos PF, Moons P, Borm G, Hilbrands LB. Conversion from cyclosporine to tacrolimus improves quality-of-life indices, renal graft function and cardiovascular risk profile. *Am J Transplant* 2004;4:937–945.

96. Mentzer RM Jr, Jahania MS, Lasley RD. Tacrolimus as a rescue immunosuppressant after heart and lung transplantation. The U.S. Multicenter FK506 Study Group. *Transplantation* 1998;65:109–113.
97. Laskow DA, Neylan JF, Shapiro RS, Pirsch JD, Vergne-Marini PJ, Tomlanovich SJ. The role of tacrolimus in adult kidney transplantation: a review. *Clin Tranplant* 1998;12:489–503.
98. Annesley TM, Hunter BC, Fidler DR, Giacherio DA. Stability of tacrolimus (FK 506) and cyclosporine G in whole blood. *Ther Drug Monit* 1995;17:361–365.
99. Alak AM, Lizak P. Stability of FK506 in blood samples. *Ther Drug Monit* 1996;18:209–211.
100. Freeman DJ, Stawecki M, Howson B. Stability of FK 506 in whole blood samples. *Ther Drug Monit* 1995;17:266–267.
101. Holt DW. Therapeutic drug monitoring of immunosuppressive drugs in kidney transplantation. *Curr Opin Nephrol Hypertens* 2002;11:657–663.
102. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplant. *Clin Pharmacokinet* 2004;43:623–653.
103. Wong KM, Shek CC, Chau KF, Li CS. Abbreviated tacrolimus area-under-the-curve monitoring for renal transplant recipients. *Am J Kidney Dis* 2000;35:660–666.
104. McAlister VC, Mahalati K, Peltekian KM, Fraser A, MacDonald AS. A clinical pharmacokinetic study of tacrolimus and sirolimus combination immunosuppression comparing simultaneous to separated administration. *Ther Drug Monit* 2002;24:346–350.
105. Lensmeyer GL, Poquette MA. Therapeutic monitoring of tacrolimus concentrations in blood: semi-automated extraction and liquid chromatography-electrospray ionization mass spectrometry. *Ther Drug Monit* 2001;23:239–249.
106. Akbas SH, Yavuz A, Tuncer M, Yurdakonar E, Akcit F, Gurkan A, Demirbas A, Gultekin M, Ersoy F, Akaydin M. Evaluation of the new EMIT tacrolimus assay in kidney and liver transplant recipients. *Transplant Proc* 2004;36:86–88.
107. LeGatt DF, Shalapay CE, Cheng SB. The EMIT 2000 tacrolimus assay: an application protocol for the Beckman Synchron LX20 PRO analyzer. *Clin Biochem* 2004;37:1022–1030.
108. Stephen DWS, Rooke P, Clark S, Coutts M, Crowe L, Docherty D. Evaluation of the Dade Behring Syva® EMIT 2000 tacrolimus assay on the Bayer Advia 1650. *Ann Clin Biochem* 2003;40: 697–700.
109. Baugher BW, Drengler S, Rahn T, Ramp J, Liao M, Wong PY. Evaluation of an assay in development for tacrolimus on the Abbott ARCHITECH® analyzer. *Clin Chem* 2006;52(Suppl):A59 (Abstract).
110. Keevil BG, McCann SJ, Cooper DP, Morris MR. Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem* 2002;39:487–492.
111. Deters M, Kirchner G, Rewsch K, Kaever V. Simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine by liquid chromatography-mass spectrometry (LM-MS). *Clin Chem Lab Med* 2002;40:285–292.
112. Iwasaki K, Shiraga T, Matsuda H, Nagase K, Tokuma Y, Hata T, Fujii Y, Sakuma S, Fujitsu T, Fujikawa A, Shimatani K, Sato A, Fujioka M. Further metabolism of FK506 (tacrolimus). Identification and biological activities of the metabolites oxidized at multiple sites of FK506. *Drug Metab Dispos* 1995;23:28–34.
113. CEDIA® Tacrolimus Assay (package insert). Microgenics Corporation, Fremont, CA, 2005.
114. Staatz CE, Taylor PJ, Tett SE. Comparison of an ELISA and an LC/MS/MS method for measuring tacrolimus concentrations and making dosage decisions in transplant recipients. *Ther Drug Monit* 2002;24:607–615.
115. Gonschior AK, Christians U, Winkler M, Linck A, Baumann J, Sewing KF. Tacrolimus (FK506) metabolite patterns in blood from liver and kidney transplant patients. *Clin Chem* 1996;42:1426–1432.
116. Oellerich M, Armstrong VW, Schutz E, Shaw LM. Therapeutic drug monitoring of cyclosporine and tacrolimus. *Clin Biochem* 1998;31:309–316.
117. Cogill JL, Taylor PJ, Westley IS, Morris RG, Lynch SV, Johnson AG. Evaluation of the tacrolimus II microparticle enzyme immunoassay (MEIA II) in liver and renal transplant recipients. *Clin Chem* 1998;44:1942–1946.
118. Busuttil RW, Klintmalm GB, Lake JR, Miller CM, Porayko M. General guidelines for the use of tacrolimus in adult liver transplant patients. *Transplantation* 1996;61:845–847.

119. Schambeck CM, Bedel A, Keller F. Limit of quantification (functional sensitivity) of the new IMx tacrolimus II microparticle enzyme immunoassay. *Clin Chem* 1998;44:2317.
120. Tacrolimus II (package insert). Abbott Laboratories, Diagnostics Division, Abbott Park, IL, 2003.
121. Homma M, Tomita T, Yuzawa K, Takada Y, Kohda Y. False positive blood tacrolimus concentration in microparticle enzyme immunoassay. *Biol Pharm Bull* 2002;25:1119–1120.
122. Kuzuya T, Ogura Y, Motegi Y, Moriyama N, Nabeshima T. Interference of hematocrit in the tacrolimus II microparticle enzyme immunoassay. *Ther Drug Monit* 2002;24:507–511.
123. Akbas SH, Ozdem S, Caglar S, Tuncer M, Gurkan A, Yucetin L, Senol Y, Demirbas A, Gultekin M, Ersoy FF, Akaydin M. Effects of some hematological parameters on whole blood tacrolimus concentration measured by two immunoassay-based analytical methods. *Clin Biochem* 2005;38:552–557.
124. Ghoshal AK, Soldin SJ. IMx tacrolimus II assay: Is it reliable at low blood concentrations? A comparison with tandem MS/MS. *Clin Biochem* 2002;35:389–392.
125. Steele BW, Wang E, Soldin SJ, Klee G, Elin RJ, Witte DL. A longitudinal replicate study of immunosuppressive drugs. A College of American Pathologists Study. *Arch Pathol Lab Med* 2003;127:283–288.
126. Miller JL. Sirolimus approved with renal transplant indication. *Am J Health Syst Pharm* 1999;56:2177–2178.
127. Sedrani R, Cottens S, Kallen J, Schuler W. Chemical modification of rapamycin: the discovery of SZD RAD. *Transplant Proc* 1998;30:2192–2194
128. Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annu Rev Immunol* 1996;14:483–510.
129. Kimball PM, Derman RK, Van Buren CT, Lewis RM, Katz S, Kahan BD. Cyclosporine and rapamycin affect protein kinase C induction of intracellular activation signal, activator of DNA replication. *Transplantation* 1993;55:1128–1132.
130. Mahalati K, Kahan BD. Clinical pharmacokinetics of sirolimus. *Clin Pharmacokinet* 2001;40:573–585.
131. Zimmerman JJ, Kahan BD. Pharmacokinetics of sirolimus in stable renal transplant patients after multiple oral dose administration. *J Clin Pharmacol* 1997;37:405–415.
132. Yatscoff RW, Boeckx R, Holt DW, Kahan BD, LeGatt DF, Sehgal S, Soldin SJ, Napoli K, Stiller C. Consensus guidelines for therapeutic drug monitoring of rapamycin: report of the consensus panel. *Ther Drug Monit* 1995;17:676–680.
133. Cattaneo D, Merlini S, Pellegrino M, Carrara F, Zenoni S, Murgia S, Baldelli S, Gaspari F, Remuzzi G, Perico N. Therapeutic drug monitoring of sirolimus: effect of concomitant immunosuppressive therapy and optimization of drug dosing. *Am J Transplant* 2004;4:1345–1351.
134. Yatscoff R, LeGatt D, Keenan R, Chackowsky P. Blood distribution of rapamycin. *Transplantation* 1993;56:1202–1206.
135. Sattler M, Guengerich FP, Yun CH, Christians U, Sewing KF. Cytochrome P-450 3A enzymes are responsible for biotransformation of FK506 and rapamycin in man and rat. *Drug Metab Dispos* 1992;20:753–761.
136. Gallant-Haidner HL, Trepanier DJ, Freitag DG, Yatscoff RW. Pharmacokinetics and metabolism of sirolimus. *Ther Drug Monit* 2000;22:31–35.
137. Leung LY, Zimmeman J, Lim HK. Metabolic disposition of [14C]-rapamycin (sirolimus) in healthy male subjects after a single oral dose. *Proc Int Soc Stud Xenobiotics* 1997;12:26 (Abstract).
138. Montalbano M, Neff GW, Yamashiki N, Meyer D, Bettiol M, Slapak-Green G, Ruiz P, Manten E, Safdar K, O'Brien C, Tzakis AG. A retrospective review of liver transplant patients treated with sirolimus from a single center: an analysis of sirolimus related complications. *Transplantation* 2004;78:264–268.
139. Haydar AA, Denton M, West A, Rees J, Goldsmith DJ. Sirolimus-induced pneumonitis: three cases and a review of the literature. *Am J Transplant* 2004;4:137–139.
140. Kaplan B, Meier-Kriesche HU, Napoli KL, Kahan BD. The effects of relative timing of sirolimus and cyclosporine microemulsion formulation coadministration on the pharmacokinetics of each agent. *Clin Pharmacol Ther* 1998;63:48–53.
141. Jones K, Saadat-Lajevardi S, Lee T, Horwatt R, Hicks D, Johnston A, Holt DW. An immunoassay for the measurement of sirolimus. *Clin Therapeutics* 2000;22(Suppl):B49–B61.

142. Salm P, Tresillian MJ, Taylor PJ, Pillans PI. Stability of sirolimus (rapamycin) in whole blood. *Ther Drug Monit* 2000;22:423–426.
143. Yatscoff RW, Faraci C, Bolingbroke P. Measurement of rapamycin in whole blood using reverse-phase high-performance liquid chromatography. *Ther Drug Monit* 1992;14:138–141.
144. Kahan BD, Napoli KL, Kelly PA, Podbielski J, Hussein I, Urbauer DL, Katz SH, Van Buren CT. Therapeutic drug monitoring of sirolimus: correlations with efficacy and toxicity. *Clin Transplant* 2000;14:97–109.
145. Holt DW, Denny K, Lee TD, Johnston A. Therapeutic monitoring of sirolimus: its contribution to optimal prescription. *Transplant Proc* 2003;35(Suppl 3):157S–161S.
146. Napoli KL, Kahan BD. Routine clinical monitoring of sirolimus (rapamycin) whole-blood concentrations by HPLC with ultraviolet detection. *Clin Chem* 1996;42:1943–1948.
147. Napoli KL. A practical guide to the analysis of sirolimus using high-performance liquid chromatography with ultraviolet detection. *Clin Ther* 2000;22(Suppl):B14–B24.
148. Maleki S, Graves S, Becker S, Horwatt R, Hicks D, Stroshane RM, Kincaid H. Therapeutic monitoring of sirolimus in human whole-blood samples by high-performance liquid chromatography. *Clin Ther* 2000;22(Suppl):B25–B37.
149. Wilson D, Johnston F, Holt D, Moreton M, Engelmayer J, Gaulier J-M, Luthe H, Marquet P, Moscato D, Oellerich M, Mosso R, Streit F, Brunet M, Brunet M, Fillee C, Schmid R, Wallemacq P, Barnes G. Multi-center evaluation of analytical performance of the microparticle enzyme immunoassay for sirolimus. *Clin Biochem* 2006;39:378–386.
150. CEDIA® Sirolimus Assay (package insert). Microgenics Corporation, Fremont, CA, 2004.
151. Vicente FB, Smith FA, Peng Y, Wang S. Evaluation of an immunoassay of whole blood sirolimus in pediatric transplant patients in comparison with high-performance liquid chromatography/tandem mass spectrometry. *Clin Chem Lab Med* 2006;44:497–499.
152. Holt DW, Laamanen MK, Johnston A. A microparticle enzyme immunoassay to measure sirolimus. *Transplant Proc* 2005;37:182–184.
153. Zochowska D, Bartlomiejczyk I, Kaminska A, Senatorski G, Paczek L. High-performance liquid chromatography versus immunoassay for the measurement of sirolimus: comparison of two methods. *Transplant Proc* 2006;38:78–80.
154. Fillee C, Mourad M, Squifflet JP, Malaise J, Lerut J, Reding R, Borghgraef P, Vanbinst R, Wallemacq PE. Evaluation of a new immunoassay to measure sirolimus blood concentrations compared to a tandem mass-spectrometric chromatographic analysis. *Transplant Proc* 2005;37:2890–2891.
155. Morris RG, Salm P, Taylor PJ, Wicks FA, Theodossi A. Comparison of the reintroduced MEIA® assay with HPLC-MS/MS for the determination of whole-blood sirolimus from transplant recipients. *Ther Drug Monit* 2006;28:164–168.
156. Westley IS, Morris RG, Taylor PJ, Salm P, James MJ. CEDIA® sirolimus assay compared with HPLC-MS/MS and HPLC-UV in transplant recipient specimens. *Ther Drug Monit* 2005;27:309–314.
157. Holt DW, McKeown DA, Lee TD, Hicks D, Cal P, Johnston A. The relative proportions of sirolimus metabolites in blood using HPLC with mass-spectrometric detection. *Transplant Proc* 2004;36:3223–3225.
158. Christians U, Jacobsen W, Serkova N, Benet LZ, Vidal C, Sewing KF, Manns MP, Kirchner GI. Automated, fast and sensitive quantification of drugs in blood by liquid chromatography-mass spectrometry with on-line extraction: immunosuppressants. *J Chromatogr B Biomed Sci Appl* 2000;748:41–53.
159. Holt DW, Lee T, Jones K, Johnston A. Validation of an assay for routine monitoring of sirolimus using HPLC with mass spectrometric detection. *Clin Chem* 2000;46:1179–1183.
160. Salm P, Taylor PJ, Pillans PI. The quantitation of sirolimus by high-performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in renal transplant recipients. *Clin Ther* 2000;22(Suppl):B71–B85.
161. Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet* 2004;43:83–95.
162. Hoyer PF, Ettenger R, Kovarik JM, Webb NJ, Lemire J, Mentser M, Mahan J, Loirat C, Niaudet P, Van Damme-Lombaerts R, Offner G, Wehr S, Moeller V, Mayer H; Everolimus

Pediatric Study Group. Everolimus in pediatric de nova renal transplant patients. *Transplantation* 2003;75:2082–2085.
163. Kahan BD, Kaplan B, Lorber MI, Winkler M, Cambon N, Boger RS. RAD in de novo renal transplantation: comparison of three doses on the incidence and severity of acute rejection. *Transplantation* 2001;71:1400–1406.
164. Kovarik JM, Kahan BD, Kaplan B, Lorber M, Winkler M, Rouilly M, Gerbeau C, Cambon N, Boger R, Rordorf C; Everolimus Phase 2 Study Group. Longitudinal assessment of everolimus in de novo renal transplant recipients over the first post-transplant year: pharmacokinetics, exposure-response relationships, and influence on cyclosporine. *Clin Pharmacol Ther* 2001;69:48–56.
165. Kahan BD, Podbielski J, Napoli KL, Katz SM, Meir-Kriesche HU, Van Buren CT. Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. *Transplantation* 1998;66:1040–1046.
166. Nashan B. Review of the proliferation inhibitor everolimus. *Expert Opin Investig Drug* 2002;11:1842–1850.
167. Kirchner GI, Winkler M, Mueller L, Vidal C, Jacobsen W, Franzke A, Wagner S, Blick S, Manns MP, Sewing KF. Pharmacokinetics of SDZ RAD and cyclosporin including their metabolites in seven kidney graft patients after the first dose of SDZ RAD. *Br J Clin Pharmacol* 2000;50:449–454.
168. Innofluor® Certican® Assay System (package insert). Seradyn Inc, Indianapolis, IN, 2003.
169. Lehmkuhl H, Ross H, Eisen H, Valantine H. Everolimus (Certican) in heart transplantation: optimizing renal function through minimizing cyclosporine exposure. *Transplant Proc* 2005;37:4145–4149.
170. Salm P, Warnholtz C, Boyd J, Arabshahi L, Marbach P, Taylor PJ. Evaluation of a fluorescent polarization immunoassay for whole blood everolimus determination using samples from renal transplant recipients. *Clin Biochem* 2006;39:732–738.
171. Baldelli S, Murgia S, Merlini S, Zenoni S, Perico N, Remuzzi G, Cattaneo D. High-performance liquid chromatography with ultraviolet detection for therapeutic drug monitoring of everolimus. *J Chromatogr B* 2005;816:99–105.
172. Salm P, Taylor PJ, Lynch SV, Pillans PI. Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Anal Technol Biomed Life Sci* 2002;772:283–290.
173. Quinn CM, Bugeja VC, Gallagher JA, Whittaker PA. The effect of mycophenolic acid on the cell cycle of *Candida abicans*. *Mycopathologia* 1990;111:165–168.
174. Lee WA, Gu L, Miksztal AR, Chu N, Leung K, Nelson PH. Bioavailability improvement of mycophenolic acid through amino ester derivatization. *Pharm Res* 1990;7:161–166.
175. Franklin TJ, Cook JM. The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochem J* 1969;113:515–524.
176. Wu JC. Mycophenolate mofetil: molecular mechanisms of action. *Perspect Drug Discov Design* 1994;2:185–204.
177. Eugui EM, Allison A. Immunosuppressive activity of mycophenolate mofetil. *Ann NY Acad Sci* 1993;685:309–329.
178. Allison AC, Eugui EM. Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF). *Clin Transplant* 1996;10:77–84.
179. Bullingham R, Monroe S, Nicholls A, Hale M. Pharmacokinetics and bioavailability of mycophenolate mofetil in healthy subjects after single-dose oral and intravenous administration. *J Clin Pharmacol* 1996;36:315–324.
180. Bullingham RE, Nicholls AJ, Kamm BR. Clinical pharmacokinetics of mycophenolate mofetil. *Clin Pharmacokinet* 1998;34:429–455.
181. Nowak I, Shaw LM. Mycophenolic acid binding to human serum albumin: characterization and relation to pharmacodynamics. *Clin Chem* 1995;41:1011–1017.
182. Korecka M, Nikolic D, van Breemen RB, Shaw LM. Inhibition of inosine monophosphate dehydrogenase by mycophenolic acid glucuronide is attributable to the presence of trace quantities of mycophenolic acid. *Clin Chem* 1999;45:1047–1050.
183. Shipkova M, Armstrong VW, Wieland E, Niedmann PD, Schutz E, Brenner-Weiss G, Voihsel M, Braun F, Oellerich M. Identification of glucoside and carboxyl-linked glucuronide conjugates of

mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Br J Pharmacol* 1999;126:1075–1082.
184. Wieland E, Shipkova M, Schellhaas U, Schutz E, Niedmann PD, Armstrong VW, Oellerich M. Induction of cytokine release by acyl glucuronide of mycophenolic acid: a link to side effects? *Clin Biochem* 2000;33:107–113.
185. Shaw LM, Korecka M, van Breeman R, Nowak I, Brayman KL. Analysis, pharmacokinetics and therapeutic drug monitoring of mycophenolic acid. *Clin Biochem* 1998;31:323–328.
186. Kaplan B, Meier-Kriesche HU, Friedman G, Mulgaonkar S, Gruber S, Korecka M, Brayman KL, Shaw LM. The effect of renal insufficiency on mycophenolic acid protein binding. *J Clin Pharmacol* 1999;39:715–720.
187. Holt DW. Monitoring mycophenolic acid. *Ann Clin Biochem* 2002;39:173–183.
188. Mourad M, Malaise J, Chaib Eddour D, De Meyer M, Konig J, Schepers R, Squifflet JP, Wallemacq P. Pharmacokinetic basis for the efficient and safe use of low-dose mycophenolate mofetil in combination with tacrolimus in kidney transplantation. *Clin Chem* 2001;47:1241–1248.
189. Sollinger HW. Mycophenolates in transplantation. *Clin Transplant* 2004;18:485–492.
190. Vidal E, Cantarell C, Capdevila L, Monforte V, Roman A, Pou L. Mycophenolate mofetil pharmacokinetics in transplant patients receiving cyclosporine or tacrolimus in combination therapy. *Pharmacol Toxicol* 2000;87:182–184.
191. Shipkova M, Armstrong VW, Kuypers D, Perner F, Fabrizi V, Holzer H, Wieland E, Oellerich M; MMF Creeping Creatinine Study Group. *Ther Drug Monit* 2001;23:717–721.
192. Schmidt LE, Rasmussen A, Norrelykke MR, Poulsen HE, Hansen BA. The effect of selective bowel decontamination on the pharmacokinetics of mycophenolate mofetil in liver transplant recipients. *Liver Transplant* 2001;7:739–742.
193. Undre NA, van Hooff J, Christiaans M, Vanrenterghem Y, Donck J, Heeman U, Kohnle M, Zanker B, Land W, Morales JM, Andres A, Schafer A, Stevenson P. Pharmacokinetics of FK 506 and mycophenolic acid after the administration of a FK 506-based regimen in combination with mycophenolate mofetil in kidney transplantation. *Transplant Proc* 1998;30:1299–1302.
194. Vietri M, Pietrabissa A, Mosca F, Pacifici GM. Mycophenolic acid glucuronidation and its inhibition by non-steroidal anti-inflammatory drugs in human liver and kidney. *Eur J Clin Pharmacol* 2000;56:659–664.
195. Kato R, Ooi K, Ikura-Mori M, Tsuchishita Y, Hashimoto H, Yoshimura H, Uenishi K, Kawai M, Tanaka K, Ueno K. Impairment of mycophenolate mofetil absorption by calcium polycarbophil. *J Clin Pharmacol* 2002;42:1275–1280.
196. Shaw LM, Holt DW, Oellerich M, Meiser B, van Gelder T. Current issues in therapeutic drug monitoring of mycophenolic acid: report of a roundtable discussion. *Ther Drug Monit* 2001;23:305–315.
197. Tsina I, Chu F, Hama K, Kaloostian M, Tam YL, Tarnowski T, Wong B. Manual and automated (robotic) high-performance liquid chromatography methods for the determination of mycophenolic acid and its glucuronide conjugate in human plasma. *J Chromatogr B* 1996;675:119–129.
198. Streit F, Shipkova M, Armstrong VW, Oellerich M. Validation of a rapid and sensitive liquid chromatography-tandem mass spectrometry method for free and total mycophenolic acid. *Clin Chem* 2004;50:152–159.
199. Saunders DA. Simple method for the quantitation of mycophenolic acid in human plasma. *J Chromatogr B Biomed Sci Appl* 1997;704:379–382.
200. Tsina I, Kaloostian M, Lee R, Tarnowski T, Wong B. High-performance liquid chromatographic method for the determination of mycophenolate mofetil in human plasma. *J Chromatogr B* 1996;681:347–353.
201. Shipkova M, Armstrong VW, Kiehl MG, Niedmann PD, Schutz E, Oellerich M, Wieland E. Quantification of mycophenolic acid in plasma samples collected during and immediately after intravenous administration of mycophenolate mofetil. *Clin Chem* 2001;47:1485–1488.
202. Mahalati K, Kahan BD. Pharmacological surrogates of allograft outcome. *Ann Transplant* 2005;5:14–23.
203. Filler G, Mai I. Limited sampling strategy for mycophenolic acid area under the curve. *Ther Drug Monit* 2000;22:169–173.

204. Pawinski T, Hale M, Korecka M, Fitzsimmons WE, Shaw LM. Limited sampling strategy for the estimation of mycophenolic acid area under the curve in adult renal transplant patients with concomitant tacrolimus. *Clin Chem* 2002;48:1497–1504.
205. Le Guellec C, Buchler M, Giraudeau B, Le Meur Y, Gakoue JE, Lebranchu Y, Marquet P, Paintaud G. Simultaneous estimation of cyclosporin and mycophenolic acid areas under the curve in stable renal transplant patients using a limited sampling strategy. *Eur J Clin Pharmacol* 2002;57:805–811.
206. van Gleder T, Shaw LM. The rationale for and limitations of therapeutic drug monitoring for mycophenolate mofetil in transplantation. *Transplantation* 2005;80(Suppl 2):S244–S253.
207. Brunet M, Cirera I, Martorell J, Vidal E, Millan O, Jimenez O, Rojo I, Londono MC, Rimola A. Sequential determination of pharmacokinetics and pharmacodynamics of mycophenolic acid in liver transplant patients treated with mycophenolate mofetil. *Transplantation* 2006;81:541–546.
208. van Gelder T, Meur YL, Shaw LM, Oellerich M, DeNofrio D, Holt C, Holt DW, Kaplan B, Kuypers D, Meiser B, Toenshoff B, Mamelok RD. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther Drug Monit* 2006;28:145–154.
209. Teshima D, Kitagawa N, Otsubo K, Makino K, Itoh Y, Oishi R. Simple determination of mycophenolic acid in human serum by column-switching high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;780:21–26.
210. Sparidans RW, Hoetelmans RM, Beijnen JH. Liquid chromatographic assay for simultaneous determination of abacavir and mycophenolic acid in human plasma using dual spectrophotometric detection. *J Chromatogr B Biomed Sci Appl* 2001;750:155–161.
211. Renner UD, Thiede C, Bornhauser M, Ehninger G, Thiede HM. Determination of mycophenolic acid and mycophenolate mofetil by high-performance liquid chromatography using post column derivatization. *Anal Chem* 2001;73:41–46.
212. Jain A, Venkataramanan R, Hamad IS, Zuckerman S, Zhang S, Lever J, Warty VS, Fung JJ. Pharmacokinetics of mycophenolic acid after mycophenolate mofetil administration in liver transplant patients treated with tacrolimus. *J Clin Pharmacol* 2001;41:268–276.
213. Weber LT, Shipkova M, Armstrong VW, Wagner N, Schutz E, Mehls O, Zimmerhackl LB, Oellerich M, Tonshoff B. The pharmacokinetic-pharmacodynamic relationship for total and free mycophenolic acid in pediatric renal transplant recipients: a report of the German Study Group on Mycophenolate Mofetil Therapy. *J Am Soc Nephrol* 2002;13:759–768.
214. Gross SP, Driscoll JA, McClafferty TA, Edwards RL, Chun A. Performance of the mycophenolic acid method on the Dade Behring Dimension® Clinical Chemistry System. *Clin Chem* 2006;52(Suppl):A63 (Abstract).
215. Luo W, Nimmagadda S, Ruzicka R, Tsai A, Loor R. Development of application protocols for CEDIA® mycophenolic acid assay on the Hitachi 911, Olympus AU640, and MGC 240 Clinical Chemistry Analyzers. *Clin Chem* 2006;52(Suppl):A67 (Abstract).
216. Domke I, Engelmayer J, Langmann T, Liebisch G, Streit F, Luthe H, Dorn A, Schmitz G, Oellerich M. Measurement of total and free mycophenolic acid with new enzyme receptor methods on COBAS INTEGRA Systems. *Clin Chem* 2005;51(Suppl):A148 (Abstract).
217. Shipkova M, Schutz E, Armstrong VW, Niedmann PD, Weiland E, Oellerich M. Overestimation of mycophenolic acid by EMIT correlates with MPA metabolite. *Transplant Proc* 1999;31: 1135–1137.
218. Weber LT, Shipkova M, Armstrong VW, Wagner N, Schutz E, Mehls O, Zimmerhackl LB, Oellerich M, Tonshoff B. Comparison of the EMIT immunoassay with HPLC for therapeutic drug monitoring of mycophenolic acid in pediatric renal-transplant recipients on mycophenolate mofetil therapy. *Clin Chem* 2002;48:517–525.
219. Beal JL, Jones CE, Taylor PJ, Tett SE. Evaluation of an immunoassay (EMIT) for mycophenolic acid in plasma from renal transplant recipients compared with a high-performance liquid chromatography assay. *Ther Drug Monit* 1998;20:685–690.
220. Schutz E, Shipkova M, Armstrong VW, Niedmann PD, Weber L, Tonshoff B, Pethig K, Wahlers T, Braun F, Ringe B, Oellerich M. Therapeutic drug monitoring of mycophenolic acid: comparison of HPLC and immunoassay reveals new MPA metabolites. *Transplant Proc* 1998;30:1185–1187.
221. Westley IS, Sallustio BC, Morris RG. Validation of a high-performance liquid chromatography method for the measurement of mycophenolic acid and its glucuronide metabolites in plasma. *Clin Biochem* 2005;38:824–829.

222. Premaud A, Rousseau A, Le Meur Y, Lachatre G, Marquet P. Comparison of liquid chromatography-tandem mass spectrometry with a commercial enzyme-multiplied immunoassay for the determination of plasma MPA in renal transplant recipients and consequences for therapeutic drug monitoring. *Ther Drug Monit* 2004;26:609–619.
223. Schutz E, Shipkova M, Armstrong VW, Wieland E, Oellerich M. Identification of a pharmacologically active metabolite of mycophenolic acid in plasma of transplant recipients treated with mycophenolate mofetil. *Clin Chem* 1999;45:419–422.
224. Oellerich M, Shipkova M, Schutz E, Wieland E, Weber L, Tonshoff B, Armstrong VW. Pharmacokinetic and metabolic investigations of mycophenolic acid in pediatric patients after renal transplantation: implications for therapeutic drug monitoring. German Study Group on Mycophenolate Mofetil Therapy in Pediatric Renal Transplant Recipients. *Ther Drug Monit* 2000;22:20–26.
225. Shaw LM, Korecka M, Aradhye S, Grossman R, Bayer L, Innes C, Cucciara A, Barker C, Naji A, Nicholls A, Brayman K. Mycophenolic acid area under the curve values in African American and Caucasian renal transplant patients are comparable. *J Clin Pharmacol* 2000;40:624–633.
226. Mandla R, Line PD, Midtvedt K, Bergan S. Automated determination of free mycophenolic acid and its glucuronide in plasma from renal allograft recipients. *Ther Drug Monit* 2003;25:407–414.

# 10 Therapeutic Drug Monitoring in Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome

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***Contents***



**Summary**

Human immunodeficiency virus (HIV) causes acquired immunodeficiency syndrome (AIDS), and it is estimated that 42 million people are infected with HIV. Four classes of drugs are used today to treat people with AIDS; nucleoside reverse transcriptase (NRTIs), non-NRTIs (NNRTIs), protease inhibitors (PIs), and entry blockers (EIs). Evidence is accumulating that both PIs and NNRTIs are good candidates for therapeutic drug monitoring (TDM). However, there is little evidence suggesting that TDM of NRTIs would be helpful other than to assess compliance/adherence to the drug regime. There is no commercially available immunoassay for routine monitoring of antiretrovirals in serum. The current methods for TDM of these drugs include high-performance liquid chromatography (HPLC) and tandem mass spectrometry. Tandem mass spectrometry is a superior technique to HPLC for analysis of these antiretrovirals.

**Key Words:** HIV; AIDS; nonnucleoside reverse transcriptase; nucleoside reverse transcriptase; protease inhibitors; entry blockers; HPLC; Tandem-MS.

## 1. INTRODUCTION

Human immunodeficiency virus (HIV) is the virus that causes acquired immunodeficiency syndrome (AIDS). HIV is an RNA virus (i.e., it carries its genetic code in the form of RNA) and falls under the family of retroviruses. Retroviruses use RNA and an enzyme called reverse transcriptase to create DNA and then invade the genome. Worldwide, it is estimated that more than 42 million people are infected with HIV, of which more than 28 million are from Sub-Saharan Africa. In North America, AIDS

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**Table 1**
**Classes of Drugs Used to Treat HIV/AIDS**

| *Nucleoside Analogues (NRTIs)* | *Protease Inhibitors (PIs)* |
|---|---|
| AZT–Zidovudine (1987) | Saquinavir (1995) |
| DdI—Didanosine | Ritonavir |
| DdC—Zalcitabine | Indinavir |
| d4T—Stavudine | Nelfinavir |
| 3TC—Lamivudine (AIDS and hepatitis) | Amprenavir |
| ABC—Abacavir | Lopinavir |
| TNF—Tenofovir | Atazanavir |
| | Tipranavir |
| *Nonnucleoside Analogues (NNRTIs)* | *Entry Inhibitors (EIs)* |
| Nevirapine (1996) | T-20 (2003) |
| Delavirdine | peptide T |
| Efavirenz | |

was first reported in the early 1980s, and today there are more than 940,000 people infected with HIV on the continent *(1,2)*. The two forms of HIV are HIV-1 and HIV-2 *(3)*. Disease caused by the latter tends to be less severe, and it is rarely found outside Africa. HIV kills an important kind of lymphocyte, the CD4 T lymphocyte. These cells are critical for the adequate functioning of the immune system. As the CD4 cells decrease in number, the body becomes vulnerable to opportunistic infections *(4)*. When people with HIV and opportunistic infections have low CD4 counts (200 cells/mL), they are said to have AIDS *(5–9)*. Therapy for HIV/AIDS requires both virologic control and prevention of opportunistic infections. Four classes of drugs are used today

**Table 2**
**Some Pharmacokinetic Parameters for Antiretrovirals**

| *Drug (mg/L)* | *C max (mg/L)* | *C min* | *Half-life (h)* | *Protein Binding Percentage* | *Vd (L/Kg)* |
|---|---|---|---|---|---|
| NRTIs | | | | | |
| Didanosine | 2.4 | 0.1 | 1.5 | <5 | 0.8 |
| Lamivudine | 1.8 | 0.1 | 6 | <5 | ? |
| Stavudine | 2.0 | 0.02 | 1.1 | <5 | 1.0 |
| Zalcitabine | <0.025 | Undetectable | 2.0 | <5 | 0.5 |
| Zidovudine | 1.8 | <0.02 | 1.2 | <5 | 1.4 |
| NNRTIs | | | | | |
| Delavirdine | 16 | 3.0 | 6 | 99 | ? |
| Nevirapine | 20 | 3.0 | 30 | 60 | 1.2 |
| PIs | | | | | |
| Indinavir | 10 | 0.1 | 1.8 | ? | ? |
| Nelfinavir | 4 | 1.0 | 5 | 93 | 4 |
| Ritonavir | 14 | 1.0 | 4 | 98 | 0.4 |
| Saquinavir | 0.5 | 0.015 | 12 | 98 | 10 |

to treat people with AIDS (see Table 1): nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine (also known as AZT), which was the first NRTI to be introduced in 1987, didanosine (also known as ddI), zalcitabine (also known as ddC), stavudine (also known as d4T), and lamivudine (also known as3TC); the non-NRTI inhibitors (NNRTIs), which include nevirapine, introduced in 1996, delavirdine, and efavirenz; the protease inhibitors (PIs), the first of which saquinavir was introduced in 1995, and include ritonavir, indinavir, nelfinavar, amprenavir, lopinavir and atazanavir, and the entry blockers (EIs) such as T-20 introduced in 2003 and peptide T. Table 2 summarizes some pharmacokinetic parameters for HIV drugs.

Treatment of HIV recommended by the Public Health Service Guidelines (2001) calls for the use of multiple drug regimens using 3–9 drugs and both reverse transcriptase inhibitors and PIs; this approach is referred to as highly active antiretroviral therapy. Currently, AIDS drug therapy is followed by measuring both CD4 counts and viral loads.

## 2. ROLE OF THERAPEUTIC DRUG MONITORING IN PATIENTS WITH AIDS

*Rationale*: Several criteria are necessary for therapeutic drug monitoring (TDM). A good relationship should exist between drug concentration and pharmacologic effect. That is, there should be a concentration range at which the drug is subtherapeutic, a range at which it is therapeutic, and a range at which it is toxic. Also, one should not be able to predict a serum concentration for a given drug dose. A reliable method should be available for drug measurement *(10)*, and a procedure should be available to assess the outcome of highly active antiretroviral therapy. Evidence is accumulating that both the PIs and the NNRTIs meet these requirements *(8–16)*. Outcomes are assessed by two different measures, namely, CD4 counts and viral loads. The NRTIs are active as the intracellular triphosphates, and there is little evidence today to suggest that their measurement would be helpful other than to assess compliance/adherence to the drug regimen. Noncompliance is a serious problem and occurs in 33–60% of the patient population *(8–10)*. Common reasons for missing doses include clinical toxicity, forgetfulness, sleeping through the time of prescribed dose, and being away from home.

### *2.1. Reasons for TDM in AIDS*

TDM permits timely dosage adjustments based on individual plasma concentrations to prevent prolonged exposure to toxic or subtherapeutic concentrations of drugs. Numerous studies have shown lack of adequate viral suppression in the absence of TDM *(17–20)*. The PIs and NNRTIs show a concentration–effect relationship *(15,16,21–25)*, although large inter- and intraindividual differences have been observed in many of the HIV drugs with regard to drug disposition, making prediction of a plasma concentration for any given dose exceedingly difficult *(26–29)*. Finally, narrow therapeutic index is seen for the majority of the antiretroviral drugs, with toxicities ranging from nausea and vomiting, to pancreatitis, nephrolithiasis, and neurologic effects *(8–10,12,23)*. The ATHENA study in the Netherlands *(16)* was a randomized, prospective clinical trial and evaluated the role of TDM in the management of HIV-1-infected patients. The outcomes in the TDM cohort were significantly better

than those of the non-TDM group. This finding is all the more impressive, considering <20% of the physicians responded to the dosing recommendations of the intervention group *(13)*. One of the main reasons for TDM of antiretrovirals is the well-known lack of compliance of patients on long-term therapy for chronic illnesses. For HIV/AIDS patients, this lack of adherence to their drug regimens commonly occurs in 33–60% of patients *(1–3)*. When failure of antiretroviral therapy occurs, assessment of compliance (adherence) by TDM, prior to resistance testing is very important. Documentation of ongoing antiretroviral drug presence makes the results of both genotypic and phenotypic HIV resistance tests more meaningful. Drug–drug interactions and interpatient variability in drug handling are other major reasons for TDM in this population.

### *2.2. Methods for Antiretroviral Drug Quantitation*

Methods for antiretroviral drug quantitation include high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS)/MS *(30–34)*. The tandem-MS/MS procedures *(33,34)* developed in our laboratory are simple and allow for the quantitation of 16 HIV drugs simultaneously in less than 5 min. These procedures also afford better sensitivity and specificity than the HPLC methods, which also require lengthy sample preparation steps, relatively large sample volumes, and are far more labor-intensive *(30)*. The tandem-MS/MS method is reliable and has been in routine use at Children's National Medical Center (Washington, DC) since February 2002. Table 3 summarizes a comparison between HPLC and tandem mass spectrometry methods for antiretroviral drug measurement. It is clear that the latter provides for a simultaneous measurement of 16 drugs in a short time frame with minimal sample preparation *(33,34)*, is reliable, more sensitive, and also more specific than HPLC methods *(30,31)*.

### *2.3. Therapeutic Ranges*

The ideal sample is that drawn at steady state just before the next dose (steady-state trough sample). Steady state is reached after an interval of 5 drug half-lives have elapsed, which for most of these drugs is less than 48 h. Exceptions to this rule have to be made for nevirapine (an NNRTI) and saquinavir (a PI), both of which have longer half-lives and should not be monitored until 4 days after initiation of the drug regimen see Table 2. Despite the fact that the majority of HIV/AIDS drugs

**Table 3**
**High-Performance Liquid Chromatography (HPLC) versus Tandem-MS**

| *Parameters* | *HPLC* | *Tandem-MS* |
|---|---|---|
| Sample volume | 0.5–1.0 mL | 0.08 mL |
| Sensitivity | 10–100 μg/L | 1 μg/L |
| Sample preparation | Lengthy | Just precipitation of proteins |
| Chromatography time | 15 min–1 h | <5 min |
| General | Different methods needed for different drugs | Universal method |

have short-half-lives only didonosino is available in a slow release form. Most of the PIs have in vitro 95% inhibitory concentrations of approximately 100 ng/mL. At Children's National Medical Center, the lower limit of the therapeutic range for the PIs is taken as 150 ng/mL. For the upper limit, we recurrently recommend concentrations <6000 ng/mL, except for lopinavir, which we recommend should be maintained at concentrations $<12,000$ ng/mL. These values are gleaned from the literature and are tentative therapeutic ranges *(8,9,12,16,21–29)*. For the NNRTIs, we currently recommend tentative therapeutic ranges between 1200 and 7000 ng/mL. The HIV/AIDS drugs are metabolized by CYP 450, 2B6, 1A2, 2A6, 2C, and 2D6 (efavirenz, nevirapine, nelfinavir, and ritonavir) and by 3A, particularly 3A4 (indinavir, delavirdine, ritonavir, nevirapine, saquinavir, and nelfinavir).

Significant drug–drug interactions occur again, emphasizing the need for TDM. The PIs are also strongly protein-bound to $\alpha_1$-acid glycoprotein, which is an acute-phase reactant (98% except for indinavir 65%), raising the possibility of the need to measure free drug concentrations for this group.

### *2.4. Free Serum and Salivary Drug Measurement for the PIs*

Some two and a half years ago, we developed an Institutional Review Board (IRB)-approved protocol for the assessment of free drug concentrations by measuring both the saliva and the free drug in plasma utilizing the same tandem mass spectrometric methods for plasma or serum measurement as reported earlier. Our specified sample is either plasma or serum, as the methods we published previously work equally well on both. We, however, inject a bigger sample for the free drug measurement (50 vs. 10 μL for plasma/serum). Free drug concentrations can be measured by using either equilibrium dialysis or, more commonly, devices with molecular cutoff filters such as the Amicon Centrifree micropartition system (Millipore Corporation, Bedford, MA) or the Worthington Diagnostics "ultrafree" system (Worthington, Jacksonville, FL). Plasma ultrafiltrate is obtained by centrifuging 1 mL of plasma in Amicon system and

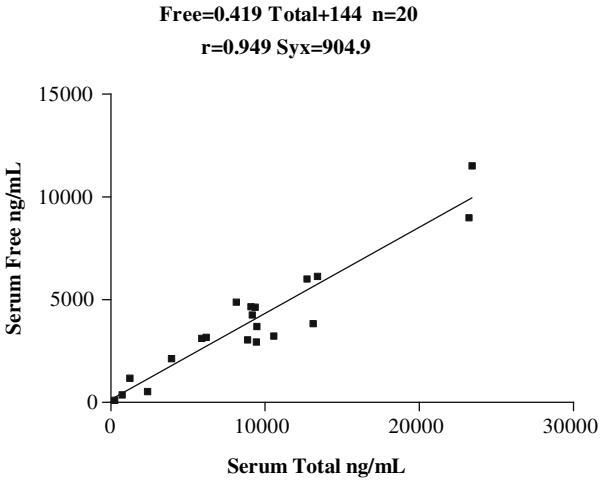


**Fig. 1.** Nevirapine serum total versus serum free (free $= 0.419 \times$ total $+ 144$; $n = 20$; $r = 0.949$; syx $= 904.9$).

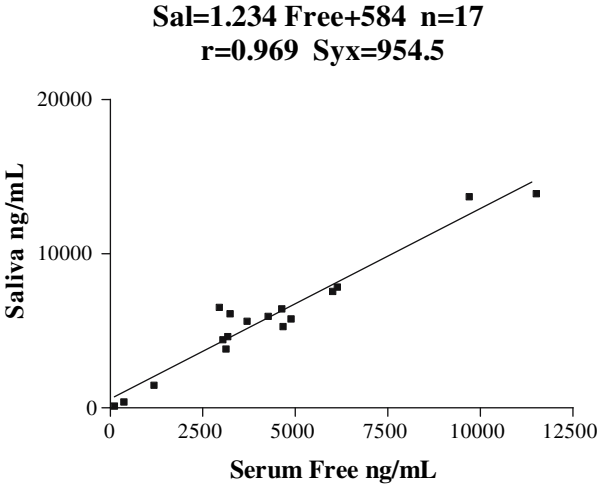


**Fig. 2.** Nevirapine serum free versus saliva (sal = 1.234 × free + 584; $n = 17$; $r = 0.969$; syx = 954.5).

then deproteinizing as described for serum/plasma previously *(33–35)*. Figures 1–3 show the data for nevirapine (an NNRTI). As can be seen, the salivary concentration correlates very well with both serum-total and serum-free concentration. Figures 4 and 5 show the correlations for stavudine (an NRTI). Here the correlation between serum free and serum total is excellent, with a less good correlation between saliva and serum-total drug concentration. Finally, Figs 6 and 7 show the correlations between serum-total and saliva concentrations and between serum-total and serum-free drug concentrations and are adequate at best for abacavir (an NRTI). This is exciting as it opens up the possibility of salivary monitoring in place of plasma monitoring to

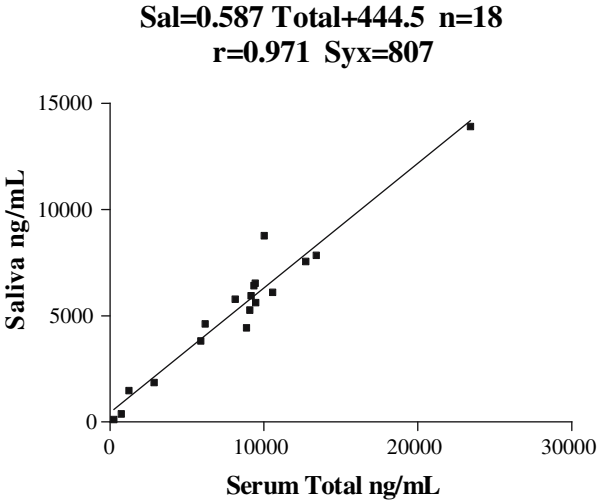


**Fig. 3.** Nevirapine serum total versus saliva (sal = 0.587 × total + 444.5; $n = 18$; $r = 0.971$; syx = 807).

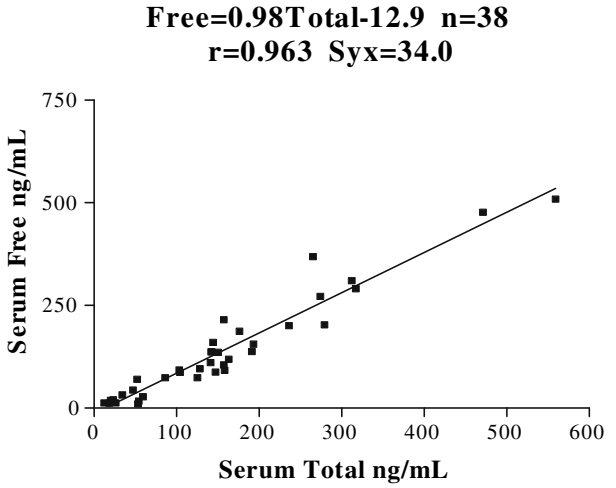


**Fig. 4.** Stavudine serum total versus serum free (free $= 0.98 \times$ total $- 12.9$; $n = 38$; $r = 0.963$; syx $= 34.0$).

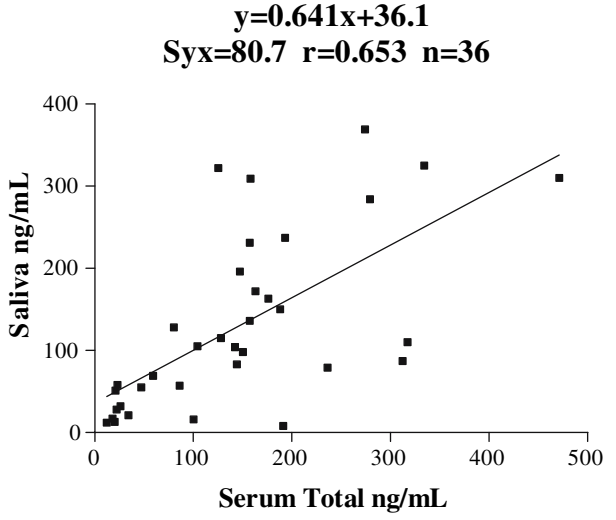


**Fig. 5.** Stavudine serum total versus saliva ($y = 0.641x + 36.1$; syx $= 80.7$; $r = 0.653$; $n = 36$).

assess patient compliance with the drug regimen and also may allow for optimization of patient outcomes employing salivary TDM *(35,36)*.

### *2.5. Proficiency Testing and Drug Standards*

External proficiency testing for the AIDS drugs is available from the International Quality Control Program For TDM in HIV Infection (University Medical Center Nijmegan, Department of Clinical Pharmacy, Nijmegan, The Netherlands). Currently, more than 80 laboratories worldwide are enrolled in this program. Drug standards can

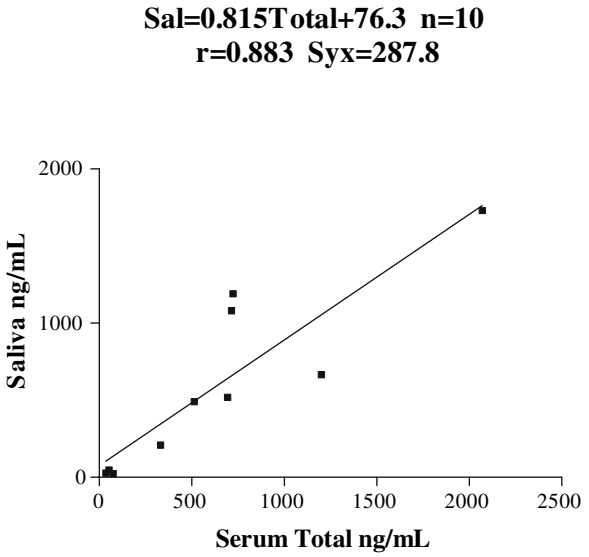


**Fig. 6.** Abacavir serum total versus saliva (sal = 0.815 × total + 76.3; $n = 10$; $r = 0.883$; syx = 287.8).

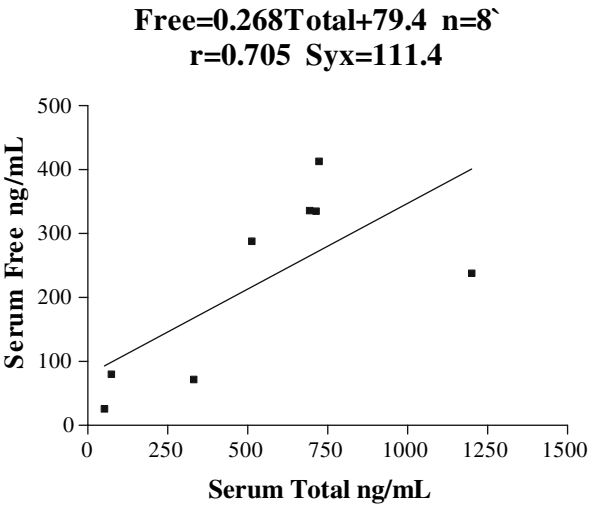


**Fig. 7.** Abacavir serum total versus serum free (free = 0.268 × total + 79.4; $n = 8$; $r = 0.705$; syx = 111.4).

be obtained from the National Institutes of Health AIDS Reagent Reference Program (McKesson HBOC BioServices, Rockville, MD).

## 3. CONCLUSION

TDM of PIs and NNRTIs has clinical significance, and these antiretrovirals are emerging as new drugs that will be monitored in more and more medical centers in the future. Eventually, TDM of these drugs will be employed routinely in the management

of patients with HIV/AIDS along with currently monitored parameters such as CD4 counts and viral load assessments.

## REFERENCES


1. The economics of HIV in Africa [editorial]. *Lancet* 2002: 360;1.
2. UNAIDS. AIDS epidemic update: December 2001. Available at http://www.unaids.org/epidemicpupdate/reportpdec01/index.html (accessed June 2002).
3. Centers for Disease Control and Prevention, Divisions of HIV/AIDS Prevention. Human immunodeficiency virus type 2. Available at http://www.cdc.gov/hiv/pubs/facts/hiv2.htm (accessed June 2002).
4. Ledergerber B, Egger M, Erard V, et al. AIDS-related opportunistic illnesses occurring after initiation of potent antiretroviral therapy: the Swiss HIV Cohort Study. *JAMA* 2000;283: 2653–2654.
5. Office of Communications and Public Liaison, National Institute of Allergy and Infectious Diseases. Fact Sheet. Bethesda, MD: National Institutes of Health; May 2001.
6. The National Institute of Allergy and Infectious Diseases (March 1999). Available at http://www.niaid.nih.gov/factsheets/hiv_inf.htm (accessed June 2002).
7. Centers for Disease Control and Prevention. HIV/AIDS surveillance report.1999;11: 1–44.
8. Dasgupta A, Okhuysen PC. Pharmacokinetic and other drug interactions in patients with AIDS. *Ther Drug Monit* 2001;23: 591–605.
9. van Heeswick RPG. Critical issues in therapeutic drug monitoring of antiretroviral drugs. *Ther Drug Monit* 2002;24: 323–331.
10. Soldin OP, Soldin SJ. Therapeutic drug monitoring in pediatrics. *Ther Drug Monit* 2002;24: 1–8.
11. Gerber JG. Using pharmacokinetics to optimize antiretroviral drug-drug interactions in the treatment of human immunodeficiency virus infection. *Clin Infect Dis* 2000;30(Suppl 2): S123–S129.
12. Back DJ, Khoo SH, Gibbons SE, et al. Therapeutic drug monitoring of antiretrovirals in human immunodeficiency virus infection. *Ther Drug Monit* 2000;22: 122–126.
13. Merry C. Therapeutic drug monitoring of antiretroviral drugs. *Northwest Univ Rep HIV/AIDS* 2000;4: 1–8.
14. van Heeswick RPG, Veldkamp AI, Mulder JW, et al. The steady-state pharmacokinetics of nevirapine during once daily and twice daily dosing in HIV-1-infected individuals. *AIDS* 2000;14: F77–F82.
15. Durant J, Clevenbergh P, Garraffo R, et al. Importance of protease inhibitor plasma levels in HIV infected patients treated with genotypic-guided therapy: pharmacological data from the Viradept study. *AIDS* 2000;14: 1333–1339.
16. Burger D, Hugen P, Droste J, et al. Therapeutic drug monitoring (TDM) of nelfinavir (NFV) and indinavir (IDV) in treatment-naïve patients improves therapeutic outcome after 1 year: results from ATHENA. In: *Abstracts of the First IAS Conference on HIV Pathogenesis and Treatment*; 2001; Buenos Aires, Argentina. Abstract 30.
17. Lebergeber B, Egger M, Opravil M, et al. Clinical progression and virological failure on highly active antiretroviral therapy in HIV-1 patients: a prospective cohort study. *Lancet* 1999;353: 863–868.
18. Erb P, Battegay M, Zimmerli W, et al. Effect of antiretroviral therapy on viral load, CD4 cell count, and progression to acquired immunodeficiency syndrome in a community human immunodeficiency virus-infected cohort: Swiss Cohort Study. *Arch Intern Med* 2000;160: 1134–1140.
19. Junghans C, Low N, Chan P, et al. Uniform risk of clinical progression despite differences in utilization of highly active antiretroviral therapy: Swiss HIV Cohort Study. *AIDS* 1999;13: 2547–2554.
20. Lucas GM, Chaisson RE, Moore RD. Highly active antiretroviral therapy in a large urban clinic: risk of virologic failure and adverse drug reactions. *Ann Intern Med* 1999;131: 81–87.
21. Veldkamp AI, Weverling GJ, Lange JM, et al. High exposure to nevirapine in plasma is associated with an improved virological response in HIV-1-infected individuals. *AIDS* 2001;15: 1089–1095.
22. Hoetelmans RMW, Reijers MHE, Weverling GJ, et al. The effect of plasma drug concentrations on HIV-1 clearance rate during quadruple drug therapy. *AIDS* 1998;12: F111–F115.
23. Gatti G, Di Biagio A, Casazza R, et al. The relationship between ritonavir plasma levels and side effects: implications for therapeutic drug monitoring. *AIDS* 1999;13: 2083–2089.

24. Sadler BM, Gillotin C, Lou C, et al. Pharmacokinetic and pharmacodynamic study of the immunodeficiency virus protease inhibitor amprenavir after multiple oral dosing. *Antimicrob Agents Chemother* 2001;45: 30–37.
25. Marzolini C, Telenti A, Decosterd LA, et al. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1–infected patients. *AIDS* 2001;15: 71–75.
26. Fletcher CV, Anderson PL, Kakuda TN, et al. Concentration controlled compared with conventional antiretroviral therapy for HIV infection. *AIDS* 2002;16: 551–560.
27. Anderson PL, Fletcher CV. Clinical pharmacologic considerations for HIV-1 protease inhibitors. *Curr Infect Dis Rep* 2001;3: 381–387.
28. Fletcher CV, Balfour HH Jr. Variability in zidovudine serum concentrations. *Pharmacotherapy* 1996;16: 1154–1158.
29. Barry MG, Merry C, Lloyd J, et al. Variability in trough plasma saquinavir concentrations in HIV case for therapeutic drug monitoring. *Br J Clin Pharmacol* 1998;45: 501–502.
30. Moyer TP, Temesgen Z, Enger R, et al. Drug monitoring of antiretroviral therapy for HIV-1 infection: method validation and results of a pilot study. *Clin Chem* 1999;45: 1465–1476.
31. Titier K, Lagrange F, Pehourcq F, et al. High-performance liquid chromatographic method for the simultaneous determination of the six HIV-protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in human plasma. *Ther Drug Monit* 2002;24: 417–424.
32. Villani P, Feroggio M, Gianelli L, et al. Antiretrovirals: simultaneous determination of five protease inhibitors and three nonnucleoside transcriptase inhibitors in human plasma by a rapid high-performance liquid chromatography-mass spectrometry assay. *Ther Drug Monit* 2002;24: 380–388.
33. Volosov A, Alexander C, Ting L, Soldin SJ. Simple rapid method for quantification of antiretrovirals by liquid chromatography–tandem mass-spectrometry. *Clin Biochem* 2002;35: 99–103.
34. Ghoshal AK, Soldin SJ. Improved method for concurrent quantification of antiretrovirals by liquid chromatography-tandem mass spectrometry. *Ther Drug Monit* 2003;25: 541–543.
35. Soldin SJ, Rakhmanina NY, Spiegel HML, Sever JL. Therapeutic drug monitoring for patients with HIV infection: Children's National Medical Center, Washington, DC Experience. *Ther Drug Monit* 2004;26: 107–109.
36. Rakhmanina NY, Cappardli EV, Van den Pocker JN, Williams K, Sever JL, Spiegel HML and Soldin SJ. Nevirapine Concentration in Nonstimulated Saliva: An Alternative to Plasma Sampling in Children with Human. Immunodeficiency Virus Infection. *Ther Drug Monit* 2007: 29; 110–117.

# 11 Pharmacogenomics and Personalized Medicine

## *Issues and Methodology*

*Steven H. Y. Wong, PhD, DABCC (TC), FACB*

***CONTENTS***



**Summary**

With the completion of the human genome project, pharmacogenomics is regarded as part of genomic medicine and personalized medicine. While pharmacogenomics is concerned with the whole genome effect on drug metabolism and efficacy, pharmacogenetics is readily defined as the study of the genetic effect, for example, single-nucleotide polymorphism, on an individual's ability to metabolize a drug or compound. Pharmacogenomic biomarkers, in combination with other biomarkers, enable personalized medicine by identifying the right patient, with the right diagnosis/treatment, matching with the right drug, the right dose and at the right time, thus achieving clinical efficacy with no or minimized toxicity. This chapter would attempt to present pharmacogenomics, not only as an emerging, inter-dependent discipline, but as a complementing field in enhancing drug therapy. It includes the introduction, the pharmacogenomics space and the enabling drivers, the principles of pharmacogenetics/pharmacogenomics, with update on the candidate pharmacogenomics testing. Recent examples would include warfarin and irinotecan, showing that pharmacogenomic biomarkers would serve as adjuncts to other functional biomarkers for enhanced drug therapy.

**Key Words:** Biomarkers; cytochrome P450; methodology; personalized medicine; pharmacogenomics.

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## 1. INTRODUCTION—PHARMACOGENOMICS AND PERSONALIZED MEDICINE

With the completion of the human genome project, one of the most tangible benefits is the emerging practice of pharmacogenomics as part of genomic medicine *(1–11)* and personalized medicine *(12)*. While pharmacogenomics and pharmacogenetics are currently used interchangeably, pharmacogenetics is readily defined as the study of the genetic effect, for example, single-nucleotide polymorphism (SNP), on an individual's ability to metabolize a drug or compound, whereas pharmacogenomics is concerned with the whole genome effect on drug metabolism and efficacy. Pharmacogenomic biomarker, in combination with well-accepted biomarkers such as therapeutic drug monitoring and other functional testing, emerging proteomic biomarkers and possibly molecular imaging, enable personalized medicine to identify the right patient, with the right diagnosis/treatment, matching with the right drug, the right dose and at the right time, thus achieving clinical efficacy with no or minimal toxicity.

The emerging clinical applications of pharmacogenetics/pharmacogenomics may be directly verified by several US Food Drug Administration (FDA)-approved genotyping methodologies/platforms, the frequent inclusion of this topic in scientific and clinical meetings, and the upcoming availability of a 2007 pharmacogenomics survey program offered by the College of American Pathologists. All these positive developments, however, should be interpreted with some probing questions of the available evidence-based studies to support clinical pharmacogenomic applications. What is emerging is the reality that pharmacogenomics serves as an "adjunct" to other testings and practice. The term "convergence" is often mentioned in its use in combination with functional testing such as therapeutic drug management. Thus, this chapter would attempt to present pharmacogenomics, not only as an emerging, inter-dependent discipline, but as a complementing field in enhancing drug therapy. Following the introduction, a section is devoted to the pharmacogenomics space and the enabling drivers. Then, the principles of pharmacogenetics/pharmacogenomics are introduced, with update on the candidate pharmacogenomics testings. References are provided for the readers interested in the details of the clinical findings of the applications of pharmacogenomics and the technical details of pharmacogenomics protocol. Recent examples would include warfarin and irinotecan.

## 2. PHARMACOGENOMICS "SPACE"

In assessing the status of pharmacogenomics for drug discovery/development and clinical adaptation, a recent market analysis was completed by O'Dell and Doyle in 2004 *(13)*. The market analysis was conducted by surveying 53 out of 200 person-contact databases. These individuals were representatives in pharmaceutical and diagnostic companies, regulatory and clinical colleagues, and others. The market was small, $800 million in 2002 as compared to the pharmaceutical market of $433 billion in 2003. Some of the key findings included the encouraging and engaging roles of the FDA and the need for physician education. It concluded that the pharmacogenomics approached a "tipping" point in 2003/2004. With the recent developments of several new FDA-approved pharmacogenomics testing/devices, pharmacogenomics "tipped" forward in the clinical pharmacogenomics/personalized medicine space, in part due to

the proactive roles of the FDA in collaboration with other professional organizations for the past several years.

Another recent study funded by the European Commission examined the status of pharmacogenetics and the challenges for its applications *(14)*. It reviewed the science and industry base in the United States, Europe, and Japan. Pharmacogenomics is regarded as interdisciplinary in 60 research institutions. The countries with more than 10 institutions are United States, 73; Germany, 35; UK, 27; Japan, 25; The Netherlands, 21; Sweden, 14; Italy and Switzerland, 13; and France, 12. The US institutions are usually better funded by National Institute of Health. European institutions received funding from the Government but not from the European Union. The major areas of research study are drug metabolism, disease mechanisms, and disease predisposition. These institutions collaborated more with other research groups than with companies, with the possible outcome of limiting the clinical applications. The commercial sector was comprised of about 47 companies, mostly small to medium sizes with a high turn-over rate of about 40%. However, the influx of new companies seemed to have maintained the total number. The business model may be divided into 12 options under the areas: drug discovery, drug safety in development, drug efficacy in development, marketed drug safety, and stratification of diseases and infectious agents. Furthermore, the top five areas are listed below.

1. Central nervous system.
2. Drug metabolism/toxicity.
3. Cardiovascular.
4. Cancer.
5. Infection.

Both FDA and the European Agency for the Evaluation of Medicinal Products (EMEA) are proactive but follow different paths. Whereas FDA provides guidance documents, EMEA conducts meetings with sponsors. The established clinical pharmacogenomics tests for the European countries would include HER2 testing for breast cancer and thiopurine methyltransferase (TPMT) for acute lymphoblastic leukemia. It concluded that many interdependent variables would contribute to clinical applications of pharmacogenomics.

By applying the genomic medicine for drug discovery and development, pharmacogenomics has been regarded as a "new science." FDA has proactively outreached to the scientific colleagues in pharmaceutical companies, partially for the purpose of developing a rational approval process. A series of workshops were held in the Washington, D.C. area (Pharmacogenomics: first workshop, May, 2002, second workshop, November, 2003, and third workshop, April, 2005; Pharmacogenomic drug-diagnostic co-development workshop, July 2004; and Application and Validation of Genomic Markers, October 2005), followed by publications of guidance documents (first draft, November 2003 and final draft, March, 2005), and white/concept papers (Critical Path Initiative, March 2004 and Drug-diagnostic co-development, April, 2005) *(15–26)*.

The topics included co-development of drug and diagnostics, sometimes regarded as "theranostics," and the voluntary genomics data submission (VGDS) process. More recently, VGDS was modified to VXDS—voluntary "X" data submission, with "X" representing diagnostic proteomic and other "omics" biomarkers in the future. In September

2006, the Center for Clinical Device and Radiological Health (FDA-CDRH) issued a draft of a guidance document "In Vitro Diagnostic Multivariate Index Assays" which addresses test system and data process, with implications to genetic testing. For practitioners of clinical pharmacogenomics, it would be important to follow the outcome of the final draft of this and other documents. Two recent chapters by the FDA-CDRH *(27)* and FDA-Center for Drug Evaluation and Research *(28)* provided guidance of the various regulatory issues related to use of pharmacogenomic biomarkers. In summary, the FDA workshops, the guidance documents, and publications of concept and white papers serve as enabling tools toward the practice of clinical pharmacogenomics.

As a result of the decreasing number of submissions as shown in Fig. 1, the critical path has been advocated by the FDA to facilitate the co-development of drugs along with genomic and proteomic diagnostic biomarkers *(29)*. As an example and extension of that concept/practice, the Critical Path Institute (C-Path), founded by the University of Arizona as part of the Arizona Biosciences Roadmap in July 2005, is an independent, neutral, community-funded, non-profit/tax exempt organization. Other key members are SRI International and FDA. Funding sources included public sector, foundations, FDA, and Agency for Healthcare Research on Quality. It also has a partnership with universities such as George Washington University and professional organizations such as the Drug Information Association, the American College of Clinical Pharmacology, and others. It has a consortium of 13 pharmaceutical companies: Merck, Johnson & Johnson, Pfizer, Novartis, GlaxoSmithKline, Schering, Roche, AstraZeneca, Boehringer-Ingelheikm, Amge, Sanofi-Aventis, Bristol-Myers Squibb, and Abbott.

As shown by the model of collaboration in Fig. 2, the C-Path Institute would share methods, data, and strategies in order to ensure the safety of newly marketed drugs. Some of projects enlisted consortium of diagnostic and pharmaceutical companies to develop better treatment of lung cancer and to develop an approach for strokes treatment and to lower the incidence of death due to embolism via warfarin dosing therapy possibly by using pharmacogenomic biomarkers. According to the article by Feigal et al. *(29)*, C-Path is currently undertaking 76 projects in six main categories.

1. Better evaluation tools.
2. Streamlining clinical trials.
3. Harnessing bioinformatics.
4. Modernizing manufacturing.

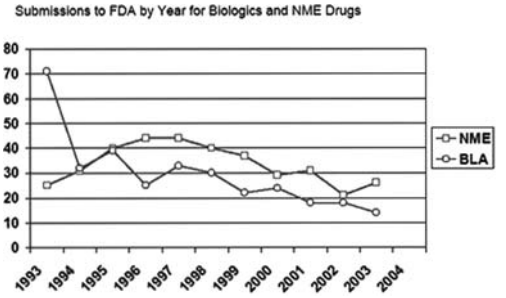



**Fig. 1.** Submissions to Food Drug Administration by year for biologics and New Molecular Entities (NME) drugs *(29)*. Reprinted with permission.

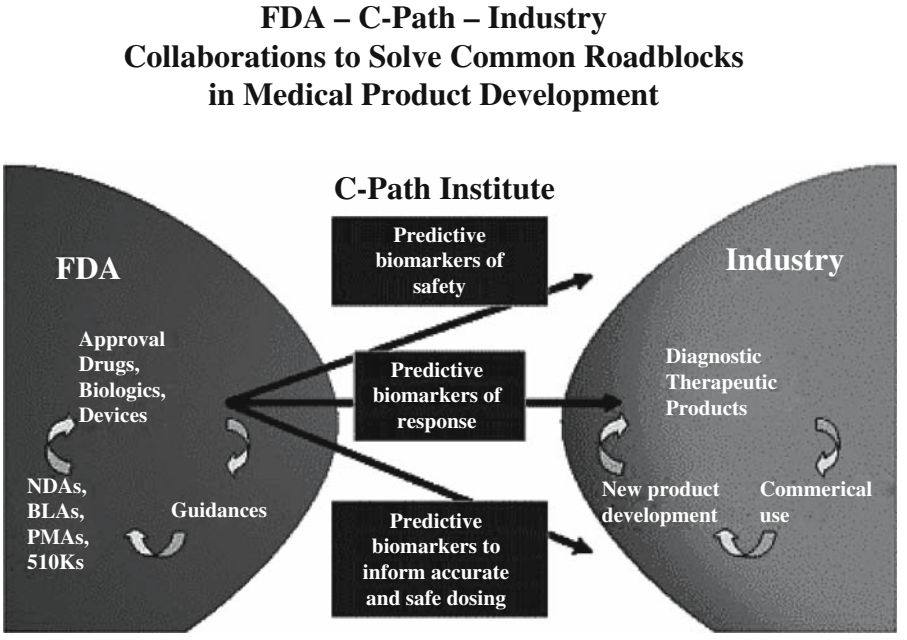



**Fig. 2.** FDA-C-Path-Industry *(29)*. Reprinted with permission.

5. Developing products to address urgent public health needs.
6. Specific at-risk populations—pediatrics.

Another major factor in adapting clinical pharmacogenomics is the education outreach to both patient and clinician. To that end, the International Society of Pharmacogenomics recently published a position paper recommending the education effort for the deans of schools of medicine, pharmacy, and allied healthcare *(30)*. The following 10 recommendations are proposed.

1. Encourage the deans to include the teaching of pharmacogenomics.
2. Global outreach to policy makers and government leaders to educate physicians, pharmacists, and nurses.
3. Basic medical teaching to include 4–8 h of lecture.
4. Graduate school.
5. Continuing medical education.
6. Pharmacogenomics for oncology.
7. Pharmacogenomics update.
8. Pharmacogenomics dedicated issues in journals.
9. Educational tools using web-based learning.
10. Better general education to outreach to patient and general public.

If these recommendations are adopted by the deans, it would pave way to prepare graduating physicians for clinical pharmacogenomics. For example, a Personalized Medicine e-Symposium held on June 21, 2006 addressed the various issues [http://www.e-symposium.com/pm/archive.php *(12)*].

Another major educational effort in enhancing the practice is the Laboratory Medicine Practice Guidelines for Clinical Pharmacogenetics, prepared by the National

Academy of Clinical Biochemistry (NACB) the academy of the American Association for Clinical Chemistry (AACC) *(31)*. The document was drafted with input from NACB, AACC members as well as selected colleagues from other professional societies and regulatory agencies both in the United States and in the Europe.

## 3. PRINCIPLES OF PHARMACOGENETICS/PHARMACOGENOMICS

The basic principles of pharmacogenetics and pharmacogenomics have been reviewed in depth by recent articles and chapters *(4–11)*. According to the central dogma of molecular biology, the genetic code of DNA is passed through transcription, onto mRNA. The information in mRNA is passed through translation, in protein synthesis. These proteins may be drug metabolizing enzymes, transporters, and receptors. As a result, DNA genetic variations would determine the enzyme activity, or transporters and receptor sensitivity. For drug metabolizing enzyme, the lack of and the presence of genetic variation would result in normal to deficient or higher enzyme activities. Genetic variations might include SNPs, deletion, duplications, and other variations. The polygenic determinants of drug response are illustrated in Fig. 3

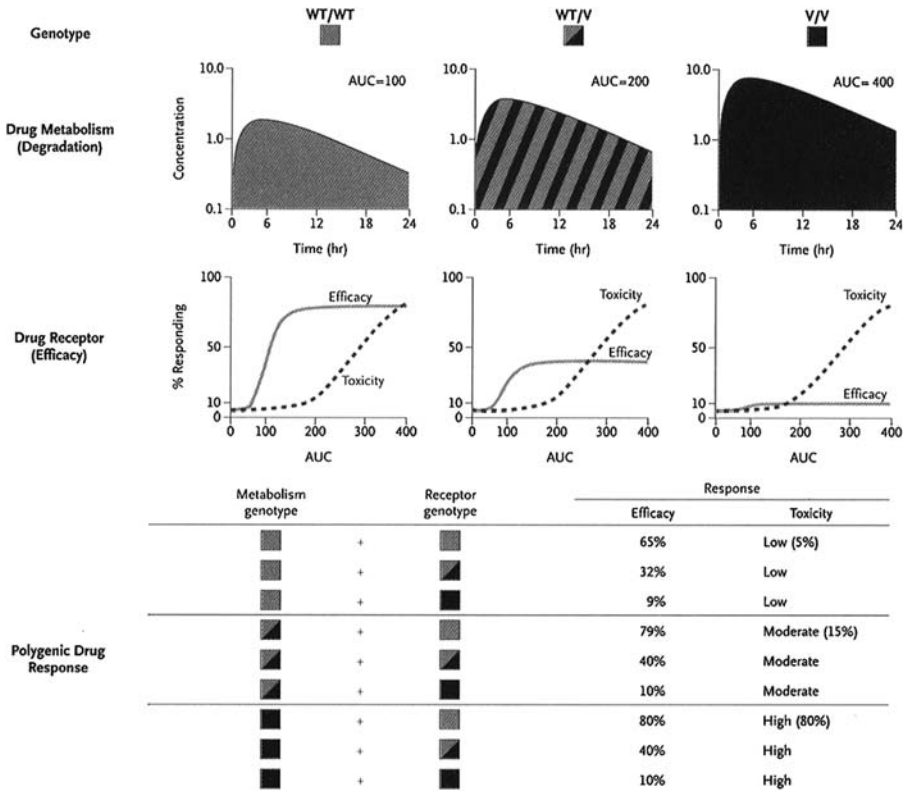



**Fig. 3.** Polygenic determinants of drug response *(10)*. (With Permission from ref. 10, Copyright 2003 Massachusetts Medical Society).

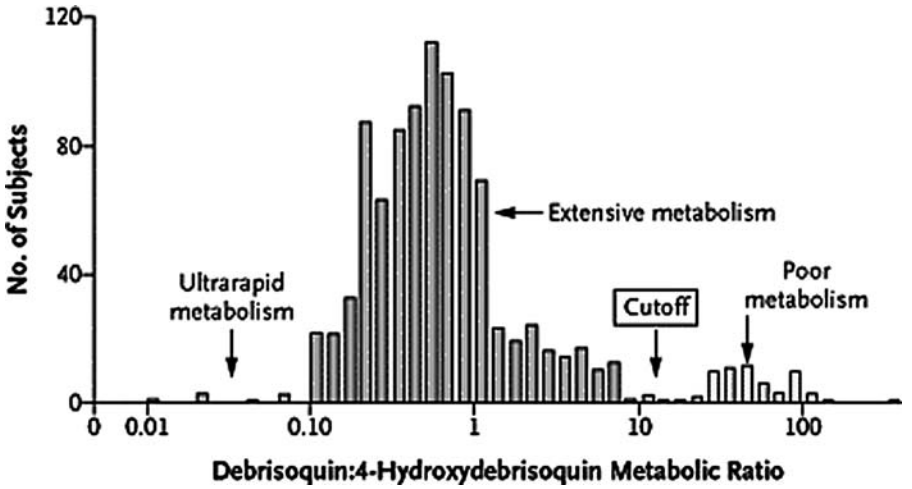
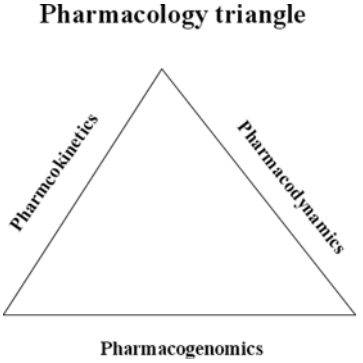



**Fig. 4.** Pharmacogenetics of CYP 2D6 *(32)*. Reprinted with permission.

[according to Evans and McLeod *(10)*]. By comparing an individual with two wild-type alleles, an extensive metabolizer, on the left to an individual with two variant alleles, and a poor metabolizer, on the right, the genetic variations would result in lower enzymes activity and elevated area under curve (AUC) with corresponding increased toxicity and decreased receptor sensitivity and efficacy. The heterozygous individual in the middle with one variant allele, an intermediate metabolizer, with resultant AUC, toxicity and efficacy intermediate between those of the extensive and poor metabolizers. With a possible combination of nine metabolism and receptor genotypes, the therapeutic index would range from 13 to 0.125. Furthermore, individuals with multiple copies of the genes correspond to ultra-rapid metabolizers, and Fig. 4 shows the debrisoquin metabolic ratios of these phenotypes *(32)*.

This relationship might be readily further conceptualized by a pharmacology triangle, proposed by Linder and Valdes, Jr. Pharmacogenomics provides the fundamental basis, the independent variable for the two interrelated, dependent variables pharmacokinetics (drug metabolism) and pharmacodynamics (drug action) *(33)*.

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Weinshilboum reviewed the pharmacogenetics of phase I drug metabolism enzymes including cytochrome P450 (CYP) 2D6, 2C9, 2C19, dihydropyrimidine dehydrogenase, and butyrylcholinesterase and phase II enzymes *N*-acetyltransferase 2, uridine diphosphate-glucuronosyltransferase 1A1, thiopurine *S*-methyltransferase, and catechol *O*-methyltransferase *(9)*. Evans and McLeod reviewed the effect of polymorphism of drug target gene on drug effect—angiotensin-converting enzyme, arachidonate 5-lipoxygenase, β2-adrenergic receptor, bradykinin B2 receptor, dopamine receptors, estrogen receptor-α, glycoprotein, and serotonin transporter—and the effect of polymorphism in disease, or treatment-modifying genes—adducin, apolipoprotein E, HLA, cholesterol ester transfer protein, ion channels, methylguanine methyl transferase, parkin, prothrombin and factor V, and stromelysin-1*(10)*. Wong and Jannetto *(34)* supplemented the information in a recent publication.

## 4. PHARMACOGENOMICS TESTS AND METHODOLOGIES

The majority of the current testing might be classified as pharmacogenetics (PGx). In attempting to guide the possible planning of clinical pharmacogenomics testing, AACC conducted a survey of the top 10 tests in 2005, showing the following.

1. *CYP 2D6*
2. *TPMT*
3. *CYP 2C9*
4. *CYP 2C19*
5. *NAT*
6. *CYP 3A5*
7. *UGT1A1*
8. *MDR1*
9. *CYP 2B6*
10. *MTHFR*

With recent pharmacogenomics findings for warfarin therapy, it would be appropriate to add the *VKORC1* gene to this list *(35)*. Within the list, CYP and other phase II enzymes such as UGT1A1 accounted for the majority of drug/substrate metabolism for drugs approved in the United States, about 75% involving CYP 3A4 and CYP 2D6 enzymes. According to the draft of LMPG by NACB, listed in the website http://www.nacb.org/lmpg/LMPG_Pharmacogenetics.pdf, the proposed alleles to be initially included for clinical pharmacogenetics were *CYP 2D6*1* to *CYP 2D6*12, CYP 2D6*17* and *CYP 2D6*2A*, *2C9*1* to *2C9*6*, and *2C19*1* to *2C19*8* and *2C19*17*. The final recommendations are pending. During the third FDA-DIA workshop, O'Kane presented an assessment of possible routine pharmacogenomics testing for medical care *(36)*. He summarized some of the barriers including the current findings of more than 160 alterations for *CYP 2D6* genes. Assay problems would include allelic drop-out, intra-allelic recombination, the need for specific assays not affected by pseudogenes *CYP 2D7* and *CYP 2D8* and to address gene conversion of *CYP 2D6* from *CYP 2D7*. Cautions were recommended that *CYP 2D6* genotyping might not be that routine.

Some of the AACC top 10 pharmacogenomic tests are readily performed either by home-brew assay or by some commercially available, FDA-approved test/platform. Payne *(37)* recently reviewed how to choose a method; Jannetto et al. *(38)*, Weber *(39)*,

**Table 1**
**Methodologies for Pharmacogenetics Testing *(37)***

| *Method* | *Company* | *FDA Cleared or Approved* |
|---|---|---|
| Sequencing[a] | Abbott (Abbott Park, IL) | Yes |
| Real-time PCR | Applied Biosystems (Foster City, CA) | – |
| PCR Arrays | Autogenomics (Carlsbad, CA) | – |
| Sequencing[a] | Bayer Healthcare (Tarrytown, NY) | Yes |
| Pyrosequencing | Biotage AB (Uppsala, Sweden) | – |
| Real-time PCR | Celera Diagnostics (Alemeda, CA) | – |
| Real-time, allele-specific PCR | DxS Genotyping (Manchester, UK) | – |
| PCR | Gentris (Morrisville, NC) | – |
| User-developed PCR arrays | Nanogen (San Diego, CA) | – |
| Nanoparticles | Nanosphere (Northbrook, IL) | – |
| PCR arrays | Roche Diagnostics (Indianapolis, IN) | Yes |
| Invader assay | Third Wave Technologies (Madison, WI) | Yes |
| PCR bead-based detection | Tm Biosciences Corp (Toronto, Ontario) | – |
| FISH | Vysis (Des Plaines, IL)[b] | Yes |

FDA, Food Drug Administration; FISH, fluorescent in-situ hybridization; PCR, polymerase chain reaction.
[a] Sequencing for HIV drug resistance.
[b] Vysis is now Abbott Molecular Diagnostics.

and other recently published chapters *(6)* also summarized the currently available technologies. The approaches included non-amplification, for example, fluorescent in-situ hybridization, target and signal amplification methods including end-point polymerase chain reaction (PCR) detection, allele-specific primers, length analysis using restiction fragment length polymorphism (RFLP) and Oligomer Ligase Assays (OLA), real-time PCR, signal amplifications, and new methods including solid-phase microarray and fluorescent-based bead assay (liquid microarray). Other technologies reviewed included Pyrosequencing and, in the future, nanotechnology. The manufacturers and the status of FDA approval are listed in Table 1.

From personal communications, some labs performing genotyping has adapted the PCR liquid bead-based detection. The choice of the platform and assays seem to reflect, similar to the selection of clinical chemistry analyzers and tests, on the ease of "home-brew" assay development and the cost of the instrument and reagents.

## 5. CLINICAL APPLICATIONS

In the recently published book *(6)*, the clinical applications of pharmacogenomics are classified according drug group, specialties, and diseases including opioids, pain management, nicotine addiction, HIV treatment, immunosuppressants, psychiatry, and clinical and forensic toxicology. Another previous publication by Jicinio and Wong *(4)* offered extensive basic and clinical information for pharmacogenomics. Readers are

directed to these references for detail. Other important and emerging areas include cancer, cardiovascular disorders, and hematology. It would be important to recognize the role of therapeutic drug monitoring (TDM) as a global phenotypic index including contributing pharmacokinetic, pharmacodynamic, drug–drug interaction, and other environmental factors. Thus, pharmacogenomic biomarkers might be readily characterized as an adjunct to enable the practice of personalized medicine. In order to update on these applications, a summary of recent examples would include pharmacogenomics for warfarin therapy and the treatment of colorectal cancer by irinotecan.

Warfarin, an antithrombotic agent, has narrow index and large inter-individual variation. Recent publications proposed a new dosing regimen based on pharmacogenetics of genes of *CYP 2C9* and vitamin K epoxide reductase complex protein 1 (*VKORC1*) *(35,41,42)*. Warfarin is racemic, with the active enantiomer, S-warfarin metabolized by CYP 2C9. Variant *CYP 2C9*2* and *CYP 2C9*3* correspond to decreased enzyme activities. For Caucasians, the prevalence of extensive, intermediate, poor, and ultra-rapid metabolizers are 58, 38, 4, and 4–18%, respectively. *CYP 2C9* genotype accounts for 6–10% of warfarin dosing variability *(40,41)*. VKORC1 mediates the reduction of vitamin K, and its genetic variations account for 25% of warfarin dose variability. Mean dose for *VKCORC1 A/A*, *A/B*, and *B/B* genotypes are 2.7, 4.8, and 6.1 mg/day *(35)*. The additional contribution from CYP 2C9 and non-genetic factor account for up to 60% of warfarin variability *(42)*. A dosage adjustment model is proposed along with International normalized ratio (INR) measurement with dosage reduction to 33% for *CYP 2C9 *3/*3* genotype.

In using pharmacogenomics for cancer, the latest example is the FDA-approved test for uridine diphosphate-glucuronosyltransferase 1A1 (Third Wave Technologies, Madison, WI) for stratifying patients undergoing colorectal cancer treatment with irinotecan. UGT1A1 medicates the conjugation of irinotecan active metabolite, SN-38 to a glucuronide metabolite *(43–46)*. Individual homozygous for *UGT1A1*28* allele would have reduced enzyme activity, therefore requiring lower dose.

## 6. CONCLUSION

The emerging practice of personalized medicine, dependent on pharmacogenomic and other biomarkers, is expected to take longer time. With the upcoming availability of proficiency survey program and quality assurance/control from commercial sources, the clinical adaptations will soon be readily achieved by clinical laboratories. NACB guidelines would certainly pave the way. Challenges remain for adequate reimbursement, clinical interpretation, and ethical guidelines. Finally, it would be important to regard pharmacogenomics as an adjunct to biomarkers such as TDM and others in enhancing drug therapy.

## REFERENCES

1. International Human Genome Sequencing consortium. Initial sequencing and analysis of the human genome. Nature 2001;409:860–921.
2. Venter JC, Adams MD, Myers EW, et al. The sequence of human genome. Science 2001;291: 1304–1351.

3. Guttmacher AE, Collins FS, Drazen JM, eds. Genomic medicine - articles form the New England Journal of Medicine. Johns Hopkins University Press, Baltimore, MD, and New England Journal of Medicine, Boston, MA, 2004, pp. 1–179.
4. Jicinio J, Wong M-L. Pharmacogenomics. Wiley-VCH, Weinheim, Germany, 2002, pp. 1–559.
5. Linder MW, Prough RA, Valdes R, Jr. Pharmacogenetics: a laboratory tool for optimizing therapeutic efficiency (review). Clin Chem 1997;43(2):254–266.
6. Wong SHY, Linder MW, Valdes R, Jr, ed. Pharmacogenomics and Proteomics – Enabling the Practice of Personalized Medicine. AACC Press, Washington, D.C., 2006, pp. 1–386.
7. White R, Wong SHY. Pharmacogenomics and its clinical applications. MLO Med Lab Obs 2005;37:20–27.
8. Weber WW. Pharmacogenetics. Oxford University Press, Oxford, UK, 1997, pp. 1–344.
9. Weinshilboum R. Inheritance and drug response. N Engl J Med, 2003;348:529–537.
10. Evans WE, McLeod HL. Pharmacogenomics – drug disposition, drug targets and side effects. N Engl J Med, 2003;348:538–549.
11. Schmitz G, Aslanidis C, Lackner KJ. Pharmacogenomics: implications for laboratory medicine. Clin Chem Acta 2001;308:43–53.
12. Personalized Medicine e-Symposium, June 21, 2006 (http://www.e-symposium.com/pm/archive.php)
13. O'Dell L, Doyle J. Opportunities in Pharmacogenomics – Market Research Analysis. May 2004.
14. Hopkins MM, Ibarreta D, Gaisser S, et al. Putting pharmacogenetics into practice. Nat Biotechnol 2006;24:403–410.
15. Salerno RA, Lesko LJ. Pharmacogenomics in drug development and regulatory decision-making: the Genomic data submission (GDS) proposal. Pharmacogenomics 2004;5:25–30.
16. Personalized Medicine: What is it? How Will it Affect Health Care? Felix W. Frueh, presented April 26, 2005 to the FDA Science Forum, Concepts and Tools in Pharmacogenomics, Pharmacogenomics from the Ground Up: Submissions and Labels in Regulatory Pharmacogenomics (http://www.fda.gov/cder/genomics/).
17. Lesko LJ, Woodcock J. Translation of pharmacogenomics and pharmacogenetics: a regulatory perspective. *Nat Rev Drug Discovery* 2004;3:763–769.
18. Salerno RA. Developing the regulatory pathway for pharmacogenomics. Regulatory Affairs Focus 2004;8:12–15.
19. Lesko LJ, Salerno RA, Spear BB, et al. Pharmacogenetics and pharmacogenomics in drug development and regulatory decision making: report of the first FDA-PWG-PhRMA-DruSafe workshop. J Clin Pharmacol 2003;34:342–358.
20. Salerno RA, Lesko LJ. Pharmacogenomic data: FDA Voluntary and Required Submission Guidance. Pharmacogenomics 2004;5:503–505.
21. Leighton JK, DeGeorge J, Jacobson-Kram D, MacGregor J, Mendrick D, Worobec A. Pharmacogenomic data submissions to FDA: non-clinical case studies. Pharmacogenomics 2004;5:507–511.
22. Gualberto R, Collins J, Dorner AJ, Wang SJ, Guerciolini R, Huang SM. Pharmacogenomic data submissions to FDA: clinical pharmacology case studies. Pharmacogenomics 2004;5:513–517.
23. Trepicchio WL, Williams GA, Essayan D, Hall ST, Harty LC, Shaw PM, Spear BB, Wang SJ, Watson ML. Pharmacogenomic data submissions to FDA: clinical case studies. Pharmacogenomics 2004;5:519–524.
24. Salerno RA, Lesko LJ. Three years of promise, proposals and progress on optimizing the benefit/risk of medicines: a commentary on the 3rd FDA-DIA-PWG-PhRMA-BIO pharmacogenomics workshop. Pharmacogenomics J 2006;6:1–4.
25. Wang SJ, Cohen N, Katz DA, et al. Retrospective validation of genomic biomarkers – What are the questions, challenges and strategies for developing useful relationships to clinical outcomes – workshop summary. Pharmacogenomics J. 2006;6:82–8.
26. Trepicchio WL, Essayan D, Hall ST, et al. Designing prospective clinical pharmacogenomic (PG) trials: meeting report on drug development strategies to enhance therapeutic decision making. Pharmacogenomics J. 2006;6:89–94.
27. Tezak Z, Hackett J. Biomarker-based diagnostic devices in therapeutic applications (Marketed therapeutics). In Wong SHY, Linder M, Valdes R, Jr, ed. Pharmacogenomics and Proteomics: Enabling the Practice of Personalized Medicine. AACC Press, Washington, D.C. 2006, pp. 37–40.

28. Goodsaid F, Huang S-M, Frueh F, Temple R, Lesko JJ. Regulatory guidance and application of genomic biomarkers in drug development. In Wong SHY, Linder M, Valdes R, Jr, ed. Pharmacogenomics and Proteomics: Enabling the Practice of Personalized Medicine. AACC Press, Washington, D.C. 2006, pp. 41–52.
29. Feigal EG, Cossman J, Woosley RL. Clearing road blocks on critical path. Drug Discovery and Development 2006;9:28–34.
30. Gurwitz D, Lunshof JE, Dedoussis D, Flordellis CS, Fuhr U, Kirchheiner J, Licinio J, Llerena L, Manolopoulos VG, Sheffield LJ, Siest G, Torricelli FT, Vasiliou V, Wong S. Pharmacogenomics education: International Society of Pharmacogenomics Recommendations for Medical, Pharmaceutical, and Health Schools Deans of Education. Pharmacogenomics J 2005;5:221–225.
31. Valdes R Jr, Payne D, Linder MW, et al. Guidelines and recommendations for Laboratory analysis and application of pharmacogenetics to clinical practice (National Academy of Clinical Biochemistry). http://www.nacb.org/lmpg/LMPG_Pharmacogenetics.pdf.
32. Bertillsson L, Lou YW, Du YL, et al. Pronounced differences between Chinese and Swedish populations n the polymorphic hydroxylations of debrisoquin and S-mephenytoin. Clin Pharmacol Ther 1992;51:388–397.
33. Linder MW, Valdes R, Jr. Fundamentals and applications of pharmacogenetics for the clinical laboratory. Ann Clin Lab Sci 1999;29:140–149.
34. Wong SHY, Jannetto PJ. Pharmacogenomics. In Wu A, ed. Tietz's Applied Laboratory Medicine, 4th edition. Saunders Elsevier, St. Louis, 2006, pp. 1713–1742.
35. Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, Blough DK, Thummel KE, Veenstra DL, Rettie AE. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. N Engl J Med 2005;352:2285–2293.
36. O'Kane DJ. Use of PG in routine medical care. 3rd FDA-DIA Worksop, Bethesda, MD, April 13, 2005.
37. Payne D. Pharmacogenetic testing: how to choose a method to analyze genetic changes. Clin Lab News 2006;7:14–16.
38. Jannetto PJ, Laleli-Sahin E, Schur BC, Wong SH. Enabling pharmacogenomics: methodologies for genotyping. In Wong SHY, Linder MW, Valdes R, Jr., ed. Pharmacogenomics and Proteomics - Enabling the Practice of Personalized Medicine. AACC Press, Washington, D.C., 2006, pp. 1–386.
39. Weber WW. Techniques for analyzing pharmacogenetic variation. In Wong SHY, Linder MW, Valdes R, Jr, ed. Pharmacogenomics and Proteomics – Enabling the Practice of Personalized Medicine. AACC Press, Washington, D.C., 2006, pp. 1–386.
40. Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanaprachanh SL, Farin FM, Rettie AE. Association between CYP 2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. JAMA 2002;287(13):1690–1698.
41. Sconce EA, Kahn TI, Wynne HA, et al. The impact of CYP 2D9 and VKORC1 genetic polymorphism and patient characteristics on warfarin dose requirements: proposal for a new dose regimen. Blood 2005;106:2329–2333.
42. Marsh S, McLeod HL. Pharmacogenomics: from bedside to clinical practice. Hum Mol Genet 2006;15:R89–R93.
43. Rivory LP, Bowles MR, Robert J, et al. Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. Biochem Pharmacol 1996;52:1103–1111.
44. Iyer L, Hall D, Das S, et al. Phenotype-genotype correlation of in vitro SN-38 (active metabolite of irinotecan) and bilirubin glucuronidation in human liver tissue with UGT1A1) promoter polymorphism. Clin Pharmacol Ther 1999;65:576–582.
45. Araki E, Ishikawa M, Iigo M, et al. Relationship between development of diarrhea and the concentration of SN-38, an active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11. Jpn J Cancer Res 1993;84: 697–702.
46. Iyer L, Das S, Janisch L, et al. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. Pharmacogenetics J 2002;2:43–47.

http://www.genome.gov/glossary.cfm
http://www.geneclinics.org
http://www.cdc.gov/genomics/hugenet/reviews.htm
http://www.cancer.gov/cancer_information/pdq
http://www.ncbi.nlm.nih.gov/omin/
http://www4.od.nih.gov/oba/sacgt.htm
http://www.nhlbi.nih.gov/resources/docs/cht-book.htm
http://www.nhlbi.nih.gov/about/factpdf.htm
http://www.cardiogenomics.med.harvard.edu
http://www.nhgri.nih.gov/Policy_and _public_affairs/Legislation/insure.htm
http://medicine.iupui.edu/flockhart/table.htm
http://www.imm.ki.se/CYPalleles/
http://www.aidsinfonyc.org/tag/science/pgp.html

# 12 Interference of Heterophilic and Other Antibodies in Measurement of Therapeutic Drugs by Immunoassays

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***Contents***



**Summary**

Heterophilic antibodies are human antibodies in a specimen which interact with assay antibodies to give false-positive or false-negative results. The heterophilic antibody may arise in a patient in response to exposure to certain animals or animal products, or to infection by bacterial or viral agents, or non-specifically. Among the anti-animal antibodies, the most common occurrence is of human anti-mouse antibody because of the wide use of murine monoclonal antibody products in therapy or imaging. Interferences from heterophilic antibody and anti-animal antibody in immunoassays are often grouped together as heterophilic antibody interference. Interference from auto-antibodies therapeutic anti-bodies, and rheumatoid factors (RF) is included in such interference. Such interferences are common with immunometric sandwich assays, but encountered only infrequently with competition (most common assay used in therapeutic drug monitoring/drugs of abuse testing) assays. Sample dilution, removal of interfering antibodies, or treatment of specimen with a heterophilic blocking agent prior to assay, has been recommended to remove heterophilic antibody interference. Thus, heterophilic antibodies are absent in the protein-free ultrafiltrate, and monitoring free drug concentration also eliminates this interference.

**Key Words:** Autoantibody; digoxin; HAMA; heterophilic antibody; false positive; therapeutic antibody.

## 1. INTRODUCTION

Most therapeutic drug monitoring (TDM) and drugs of abuse (DAU) testing are now performed by immunoassays on automated systems. Most methods do not require pretreatment of specimens and are run on continuous, random access systems. In

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immunoassays, the analyte is detected by its interaction with a specific antibody (or a pair of specific antibodies). This reaction is further utilized in various formats and labels. The common formats of immunoassays have been described in Chapter 3.

In addition to the common sources of interference, such as exogenous factors, immunoassays additionally may also suffer from interference of endogenous human antibodies to the analyte or one (or more) components of the assay reagents. Such antibody interference can be categorized in four groups.

a. Autoantibody (human endogenous antibody to the analyte).
b. Heterophilic antibody (human endogenous, non-specific antibody that interact with assay antibodies).
c. Anti-animal antibody (human endogenous, specific antibody that interacts with assay antibodies).
d. Therapeutic antibody (antibody or its fragments used therapeutically).

Because it is difficult to distinguish between the effects of the group b and c, many investigators term them in general as heterophilic antibodies. Although many of the immunoglobulin (Ig) clones in normal human serum may display anti-animal antibody properties, only those antibodies with sufficient titer and affinity toward the reagent antibody used in an assay may cause interference.

Interference from such antibodies has been observed in all kinds of immunoassays, competition and non-competition (sandwich) types. In general, competition immunoassays, using a single primary antibody, are less affected by such interference than the sandwich immunoassays (which use separate capture and label antibodies). Furthermore, types of label used (enzyme, fluorescent, or chemiluminescent tag) have lesser effect with respect to such interference. The commonly used assay formats can be summarized as below.

1. Competition (limited binding site or primary antibody):
    a. Homogeneous (no separation; bound label has different signal than unbound ones):
        a. Immunoturbidimetric: regular or latex (latex-antibody or latex-antigen).
        b. Fluorescence: fluoroimmunoassay or fluorescence polarization immunoassay (FPIA).
        c. Label can be conjugated to the antigen or the antibody.
    b. Heterogeneous (bound label is separated from unbound ones):
        a. Capture reagent may incorporate the primary antibody with the label conjugated to the antigen or vice versa.
        b. A secondary antibody may be used to isolate the immune complex or to amplify signal.
2. Immunometric ("Sandwich") assays are mostly heterogeneous and more susceptible to interference from heterophilic antibody.
    a. Both primary antibodies (capture and label) from same species or different species.
    b. There is no report of any interference from heterophilic antibody in assay using avian (chicken) primary antibodies. However, few commercial assays use chicken primary antibody.

c. Use of Fab or Fab' fragments or chimeric antibody may reduce interference from heterophilic antibody.

Because heterophilic antibodies are found mainly in serum, plasma, or whole blood, but not in urine, tests involving urine as a specimen are not subjected to interference from heterophilic antibodies. Therefore, comparison of values of an analyte in serum and urine (provided the analyte is present in both biological matrixes) provides a clue of the presence of heterophilic antibody in serum. For example, many case studies with false-positive human chorionic gonadotropin (hCG) in serum/plasma have been described in the literature where such interferences were not observed when urine specimens were analyzed *(1)*.

## 2. MECHANISM OF INTERFERENCE

In the competition type immunoassays, interference from antibody can be explained from the following mechanisms.

1. If the assay uses labeled analyte (or its analog), the interfering antibody may interact with the label causing reduced signal and false-positive results. The same phenomenon may occur even if the assay antibody is labeled and the competing analyte (or its analogs) on the "capture reagent" binds to the interfering antibody thus reducing signal.
2. However, if only the analyte (when the assay uses an analog of the analyte in the reagent) interacts with the interfering endogenous antibody, then analyte concentration in the assay reaction decreases, causing false-negative results
3. Heterophilic antibody may interact with the assay antibody, reducing its effective concentration (via steric hindrance). Such interaction decreases assay signal generating a false-positive result.

The competition immunoassays, which are commonly used in analyzing small (TDM/DAU) molecules, are in general less affected by heterophilic antibody. In contrast, two-site immunometric assays (sandwich type assays) which are used for analytes with relatively higher molecular weights than drug molecules (cardiac troponin I, hCG, and so on) suffer more from interference of heterophilic antibodies. In these type of assays, the heterophilic antibody commonly bridges between the two assay antibodies ("capture" and "label") used, creating false sandwich complexes and false positive results.

## 3. INTERFERENCE FROM AUTOANTIBODY AND THERAPEUTIC ANTIBODY

Autoantibodies are endogenous antibodies of the patient that bind to either the analyte itself or one of the reagents used in the assay. Such antibodies are more common in patients with auto-immune disease.

### *3.1. Autoantibody to the Analyte*

The autoantibodies may bind to the analyte-label conjugate in the competition type immunoassay, reducing signal and producing a false-positive result. Conversely, it

may bind to the analyte in a sandwich assay or competition assay which uses a label containing an analog of the analyte, generating false-negative results. Interference of circulating troponin autoantibody causing false-negative results in a cardiac troponin I assay has been reported *(2)*.

### *3.2. Autoantibody to a Component in the Reagent*

In one report, endogenous anti-avidin antibody interfered in a theophylline assay which used the avidin–biotin system *(3)*. In this competition type immunoassay, the autoantibody interacted with avidin in the reagent, interfering in complex formation, thus lowering signal and causing false-positive result. The observed theophylline concentration using the avidin-based assay was 27.2 μg/mL compared to the theophylline value of 8.4 μg/mL observed using a non-avidin assay.

### *3.3. Therapeutic Antibodies*

These are antibodies used in therapy. One example is Digibind™ (Glaxo/Burroughs Wellcome), the Fab fragments from ovine anti-digoxin antibodies which is a specific antidote for life threatening digoxin overdose. Digibind interferes with most digoxin immunoassays that do not require sample pretreatment. This type of interference in digoxin immunoassay is discussed in Chapter 6. However, interference from Digibind in serum digoxin measurement can be eliminated by ultrafiltration and measuring digoxin in protein-free ultrafiltrate *(4)*.

## 4. INTERFERENCE FROM HETEROPHILIC ANTIBODY

Heterophilic antibodies are poorly defined polyreactive human antibodies recognizing IgG from different species. These antibodies are non-specific without having a clearly identifiable immunogen. These antibodies bind mostly to the Fc region of assay antibodies causing interference. Moreover, incidences of heterophilic antibody binding to other parts of the assay antibody (e.g., idiotope or the "hinge" region) have been also reported. Such interference is normally more significant if the assay antibody pair are from the same (animal) source. Heterophilic antibodies are found more in sick and hospitalized patients with a reported prevalence from 0.2 to 15%. However, during the last decade, most commercial assays also included blocking reagents against the heterophilic antibodies in their assay reagent formulations, thus reducing this interference from heterophilic antibodies. A 2005 report lists 0–4% heterophilic antibody interference (measured by assay responses with and without an interference-blocking reagent) in eight automated tumor marker immunoassays *(5)*. Because of the heterogeneous nature of heterophilic antibodies, their concentrations may differ significantly among individuals. Therefore, despite improved assay design, interferences from heterophilic antibodies are still reported in the literature.

As most of the TDM/DAU assays are competitive immunoassay using only a single analyte-specific antibody in the reagent, reported cases of heterophilic antibody interference in such types of immunoassays are infrequent. Liendo, Ghali, and Graves *(6)* reported a case where a high serum digoxin level of 4.2 ng/mL was observed in a patient 24 h after his last digoxin dose, but there was no clinical symptom of digitalis toxicity. The patient was receiving spironolactone that may interfere with serum

digoxin measurement using immunoassays. Therefore, spironolactone was substituted by furosemide, but even 24 days after discontinuation of spironolactone and 29 days after discontinuation of digoxin the serum digoxin level was still 3.3 ng/mL and his underlying tachycardia began to reemerge. The initial digoxin assay was performed using a Roche assay (FARA 2 digoxin assay). Dilution of a specimen showed marked deviation from linearity. When the authors analyzed these digoxin specimens using other digoxin immunoassays, discordant results were also observed. For example, a digoxin value of 3.6 ng/mL observed by the Roche assay was 0.2 ng/mL using a FPIA and a TDx analyzer. Authors ruled out spironolactone and endogenous digoxin-like immunoreactive substances as the sources of interference and concluded that the marked elevation of digoxin concentration was due to the interference from heterophilic antibody *(6)*. This was further validated by observing a significant drop in measured digoxin concentration in the protein-free ultrafiltrate (0.0 ng/mL) by the same Roche assay compared to a digoxin concentration of 3.1 ng/mL in the original serum. The authors prepared protein-free ultrafiltrate using filtration of serum through a 30,000-Da exclusion membrane that removed heterophilic antibody due to its large molecular weight. Incubation of sera from this patient with Protein A, a polypeptide isolated from *Staphylococcus aureus* that tightly binds the Fc region of human IgG molecules, also reduced the magnitude of this interference using the same Roche assay, further validating the presence of heterophilic antibody in the specimen. Authors concluded that this false-positive digoxin level was due to binding of heterophilic antibody to murine monoclonal antibody used in the digoxin assay and warned that because newer digoxin assays now use murine monoclonal antibodies, the possibility of the presence of anti-mouse antibody in the sera of patients should be considered for interpreting an unexpected high value of digoxin obtained by an immunoassay *(6)*.

RFs are IgM type antibodies which interact with assay antibodies at the Fc area. RF is present in serum of over 70% patients with rheumatoid arthritis. RF is also found in patients with other auto-immune diseases. RF concentration increases in infection or inflammation. The interference of RF in immunoassays follows the same mechanism as interference from other types of antibodies. Thus, in two-antibody immunometric assays, RF bridges the capture and label antibodies without involving the antigen and generates false-positive signal. In single-antibody competition type immunoassays, RF binds to assay antibody, preventing its reaction to the label reagent through steric hindrance, thus reducing signal and generating false-positive results. RF concentration in the specimen may be measured by many of the commercial RF assays available. RF can be removed from the sample by pretreatment of specimen with anti-RF antibody. In one report, interference of RF in a troponin I assay was eliminated by such pre-treatment of specimen *(7)*.

### *4.1. Interference from Human Anti-Animal Antibody*

Human anti-animal antibodies (HAAAs) are different from heterophilic antibody by their specificity to antibody from certain species and by their stronger avidity. HAAAs arise mostly when the patient is exposed to a defined animal antigen. In the majority of the cases, the exposure is from diagnostic (i.e., tumor-targeted imaging agent) or therapeutic applications of tumor-specific monoclonal antibodies. Examples of animal-derived pharmaceuticals which may contain anti-animal antibody are given in Table 1.

**Table 1**
**Animal-Derived Pharmaceuticals**

| *Drug* | *Source animal* | *Reference* |
|---|---|---|
| Antibody-targeted imaging agents | Mouse, rat | *(8)*, *(9)* |
| Antibody-targeted drugs | Mouse, rat | *(10)*, *(10)* |
| Anti-thymocyte globulin | Horse, rabbit | *(11)*, *(12)* |
| Calcitonin | Salmon | *(13)* |
| Digibind (anti-digoxin Fab) | Sheep | *(14)* |
| Factor VIII | Pig | *(15)* |
| Insulin | Pig | *(16)*, *(17)* |
| Vaccines | Rabbit, chicken | *(18)*, *(19)* |
| Patent medicines | Rabbit | *(20)* |

Anti-animal antibodies may also arise from contact with the animals (e.g., animal husbandry or keeping of animals as pets) *(21)* and the transfer of dietary antigens across the gut wall in conditions such as celiac disease *(22)*.

Serum concentrations of HAAA may range from microgram to gram per liter. The HAAA may be transient, lasting a few days to months and sometimes even years. Sometimes it is difficult to validate whether a particular interference in an assay is from HAAA especially if the presence of antibody in the patient is transient *(23)*. The prevalence of HAAA, especially human anti-mouse antibody (HAMA), may vary from $<1\%$ up to 80% among hospitalized patients or outpatients depending on the population studied. Several commercial assay kits for HAMA estimation are available but a negative result may also be observed even if HAMA is present due to its heterogeneous nature *(24)*

HAMA interferes in immunoassays that use murine antibodies to the analyte. As more and more monoclonal (most common source is mouse) antibodies are used in commercial and other immunoassays (because of the specificity of the antibody and the reliability of the antibody supply), impact of HAMA interference in terms of incorrect result and resulting inaccuracy in diagnosis and therapy has become more serious. As expected, the HAMA can be of varied prevalence, specificity, titer, and binding capacity. The most common HAMA concentration is $<10\,\mu g/mL$, however HAMA concentration as high as $1000\,\mu g/mL$ has been reported. As HAMA arises from exposure of patients to mouse antibodies, cancer patients who may have used such antibodies as part of imaging or therapeutic agents have higher prevalence of HAMA occurrences (40–70%). HAMA can be IgG (most common), IgM, IgA, or IgE and can be directed to any part of the monoclonal antibody used in the assay (Fc, Fab, idiotope, and so on).

### *4.2. Antibodies to Other Species*

After mouse, rabbit and goat are the most common animals used to generate assay antibodies for utilization in assays. Therefore, like HAMA, immunoassay interference caused by human anti-rabbit (HARA) and anti-goat antibodies have been described. While HARA interference was shown in transthyretin, haptoglobin, and C-reactive

protein assays, there is no report in the literature describing interference of these types of antibodies in immunoassays for TDM.

An interesting case study showed that the IgM antibodies to *Escherichia coli* in a septimea patient's serum showed heterophilic antibody like interference in many sandwich assays *(25)*.

## 5. HOW TO DETECT AND REMOVE ANTIBODY INTERFERENCE

If a test result is unexpected and heterophilic antibody is suspected as the source of interference, several strategies can be adopted for investigation.

1. Dilution linearity study with the specimen is the simplest way to document interference when observed values after dilution deviate significantly from the target values. Figure 1 illustrates the effect of successive dilutions of a HAMA containing sample (spiked with 32 μg/mL of theophylline but observed value was 59 μg/mL) compared to a serum-based calibrator for the assay (60 μg/mL) for a theophylline immunoassay that uses mouse anti-theophylline antibody. The HAMA in the specimen interfered with the assay and initial value observed was 59 μg/mL. After successive dilutions (with the assay diluent), the interfering antibody was diluted enough and with high dilution the interference was minimal (Datta, unpublished data). However, dilutions do not always correct the analyte value in the sample because of increased imprecision in the low end of the assay and the "matrix effect" between the calibrator matrix and the matrix of the original specimen.
2. Careful examination of the patient history (exposure to immunogenic animals or animal products; history of hyperactive immune system) may provide an important clue.
3. The treatment of the sample to block the interference or remove the interfering antibody and then repeating the assay.

There are various types of commercial or home-brew blockers for heterophilic antibody *(26)*. The blocker can be a non-immune animal serum, polyclonal antibody,

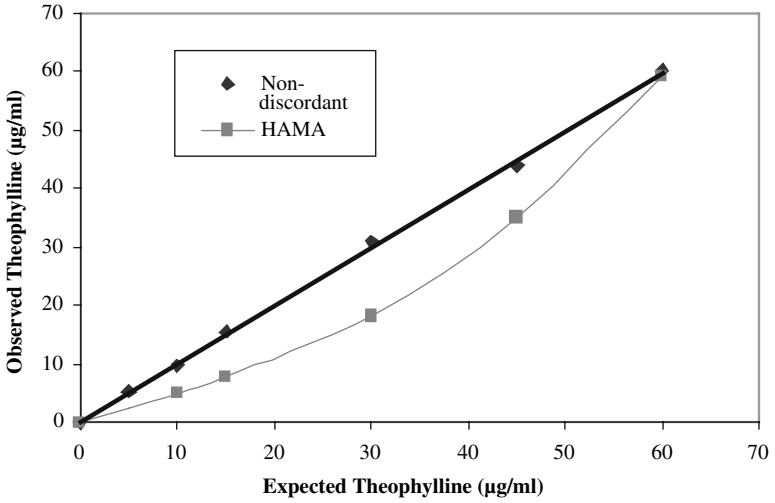


**Fig. 1.** Human anti-mouse antibody (HAMA) interference detected by sample serial dilution.

polymerized IgG, nonimmune mouse monoclonals, and a mixture of monoclonal antibodies or fragments of IgG [Fc, Fab, F(ab')$_2$] preferably from the same species used to raise the reagent antibodies. Commercially available blocking agents are immunoglobulin inhibiting reagent (IIR; Bioreclamation), heterophililic blocking reagent (HBR; Scantibodies), heteroblock (Omega Biologicals), MAB 33 (monoclonal mouse IgG1) and poly MAB 33 (polymeric monoclonal IgG1/Fab; Boehringer Mannheim). IIR is a proprietary formulation of high-affinity anti-animal antibody, and HBR is monoclonal mouse anti-human IgM. A suspected discordant sample, for example, sample giving false-positive hCG, may be separately incubated with the blocker and then re-assayed *(26)*. Most commercial assay reagents now include such blockers. However, because of the heterogeneous nature of the interfering antibodies, no blocker can guarantee that there should be no interference from heterophilic antibody in all specimens analyzed by that particular assay.

Other approach to remove antibody interference is selective removal of the antibodies from a specimen. This can be achieved by selective adsorption of human IgG by a solid phase containing protein A or protein G *(6)*. However, this method does not work if majority of the interfering antibodies are of IgM type. Alternately, the antibody fraction in the sample may be precipitated out with polyethylene glycol (preferable PEG 6000) reagent *(27)*.

Because most drugs routinely monitored in clinical laboratories have small molecular weight, a simple approach to eliminate interference of heterophilic antibody is to analyze drug concentration in protein-free ultrafiltrate (free drug monitoring). The centrifugal ultrafiltration is a fast and relatively easy method to prepare protein-free ultrafiltrate of serum using ultra-centrifugal cartridges with 10,000- or 30,000-D molecular weight cutoff filter membrane (e.g., Centrifree Micropartition System). Assay kits are commercially available for determination of free concentrations of phenytoin, carbamazepine, and valproic acid. For a drug with poor protein binding, for example, digoxin, assay kit for determination of total digoxin can be used for the determination of free digoxin concentration.

## 6. CONCLUSION

Interference from heterophilic antibody to an immunoassay resulting in a false-positive or false-negative result is more frequently observed with immunometric or sandwich assays than in competition assays. Because competition assays are commonly used in assays for drugs in TDM, interference of heterophilic antibody is only infrequently encountered in TDM. Sample pretreatment with blocking agents may eliminate this interference. Alternatively monitoring free drug concentration in protein-free ultrafiltrate eliminates this interference.

## REFERENCES

1. Braunstein G. False positive serum human chorionic gonadotropin results: causes, characteristics, and recognition. Am J Obstet Gynecol 2002;187:217–224.
2. Eriksson S, Halenius H, Pulkki K, et al. Negative interference in cardiac Troponin I immunoassays by circulating troponin autoantibodies. Clin Chem 2005;51:839–847.
3. Banfi G, Pontillo M, Sidoli A, et al. Interference from antiavidin antibodies in thyroid testing in a woman with multi endocrine neoplasia syndrome type 2B. J Clin Ligand Assay 1995;18:248–251.

4. McMillin GA, Qwen W, Lambert TL, De B, et al. Comparable effects of DIGIBIND and DigiFab in thirteen digoxin immunoassays. Clin Chem 2002;48:1580–1584.
5. Preissner CM, Dodge LA, O'Kane DJ, et al. Prevalence of heterophilic antibody interference in eight automated tumor marker immunoassays. Clin Chem 2005;51:208–210.
6. Liendo C, Ghali JK, Graves SW. A new interference in some digoxin assays: anti-murine heterophilic antibodies. Clin Pharmacol Ther 1996;60:593–598.
7. Dasgupta A, Banerjee SK, Datta P. False positive Troponin I in the MEIA due to the presence of rheumatoid factors in serum. Am J Clin Pathol 1999;112:753–756.
8. Miller RA, Maloney DG, McKillop J, Levy R. In vivo effects of murine hybridoma monoclonal antibody in a patient with T-cell leukemia. Blood 1981;58:78–86.
9. Grossman H. Clinical applications of monoclonal antibody technology. Urol Clin North Am 1986;13:465–474.
10. Kuus-Reichel K, Grauer LS, Karavodin LM, et al. Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal antibodies? Clin Diagn Lab Immunol 1994;1: 365–372.
11. Gilbert C. Clinical uses of anti-thymocyte globulin (ATGAM). Part I. N C Med J 1984;45:737–739.
12. Belitsky P, MacDonald AS, Lawen J, et al. Use of rabbit anti-thymocyte globulin for induction of immunosuppression in high-risk kidney transplant recipients. Transplant Proc 1997;29:16S–17S.
13. Plosker GL, McTavish D. Intranasal salcatonin (salmon calcitonin). A review of its pharmacological properties and role in the management of postmenopausal osteoporosis. Drugs Aging 1996;8:378–400.
14. Azrin MA. The use of antibodies in clinical cardiology. Am Heart J 1992;124:753–768.
15. Hay CR, Lozier JN, Lee CA, et al. Safety profile of porcine factor VIII and its use as hospital and home-therapy for patients with hemophilia-A and inhibitors: the results of an international survey. Thromb Haemost 1996;75:25–29.
16. Schernthaner G. Immunogenicity and allergenic potential of animal and human insulins. Diabetes Care 1993;16(Suppl 3):155–165.
17. Padova G, Briguglia G, Tita P, et al. Hypergonadotropinemia not associated to ovarian failure and induced by factors interfering in radioimmunoassay. Fertil Steril 1991;55:637–639.
18. Palache AM, Brands R, van Scharrenburg GJ. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells of fertilized chicken eggs. J Infect Dis 1997;176(Suppl 1):S20–S23.
19. Schaison G, Thomopoulos P, Moulias R, Feinstein MC. False hyperthyrotropinemia induced by heterophilic antibodies against rabbit serum. J Clin Endocrinol Metab 1981;53:200–202.
20. Smid WM, va der Meer J. Five-year follow-up of human anti-mouse antibody in multitransfused HIV negative hemophiliacs treated with a monoclonal purified plasma derived factor VIII concentrate [Letter]. Thromb Haemost 1995;74:1203.
21. Berglund L, Holmberg NG. Heterophilic antibodies against rabbit serum causing falsely elevated gonadotropin levels. Acta Obstet Gynecol Scand 1989;68:377–378.
22. Kazmierczak SC, Catrou PG, Briley KP. Transient nature of interference effects from heterophilic antibodies: examples of interference with cardiac marker measurements. Clin Chem Lab Med 2000;38:33–39.
23. Falchuck KR, Iselbacher KJ. Circulating antibodies to bovine albumin in ulcerative colitis and Crohn's disease: characterization of the antibody response. Gastroenterology 1976;70:5–8.
24. Kricka LJ. Human anti-animal antibody interferences in immunological assays. Clin Chem 1999;45:942–956.
25. Vaidya HC, Beatty BJ. Eliminate interference from heterophilic antibodies in a two-site immunoassay for CKMB by using $F(ab')_2$ conjugate and polyclonal mouse IgG. Clin Chem 1992;38:1737.
26. Butler SA, Cole LA. Use of heterophilic antibody blocking agent (hbt) in reducing false-positive hCG results. Clin Chem 2001;47:1332–1333.
27. Schnorr GK, Hachmann H, Harthus HP, et al. Interferences of human anti-mouse antibodies in mouse monoclonal antibody based immunoassays [Abstract]. Clin Chem 1989;35:1188.

# 13 Drug–Herb and Drug–Food Interactions

## *Impact on Therapeutic Drug Monitoring*

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***Contents***



**Summary**

Interactions between herbal supplements and drugs and between food and drugs are of significant clinical importance. St. John's wort, a popular herbal supplement, induces cytochrome P 450 mixed function oxidase as well as modulated P-glycoprotein in intestine and reduces plasma concentrations of cyclosporine, tacrolimus, amitriptyline, digoxin, fexofenadine, indinavir, methadone, midazolam, nevirapine, phenoprocoumon, squinavir, simvastatin, theophylline and warfarin. These reductions may cause treatment failures. Interaction of warfarin with herbal supplements may increase or decrease the anticoagulant effect of warfarin. Important interactions of various drugs with ginseng, ginkgo biloba, kava and garlic have also been reported. Consumption of a single glass of grapefruit juice caused a twofold to threefold increase in the plasma concentration of felodipine, and pharmocokinetics of approximately 40 other drugs are also affected by intake of grapefruit juice. In contrast, bioavailability of fexofinadine was significantly reduced by grapefruit juice. Orange, cranberry, pomelo and pomegranate juice also may alter

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bioavailability of certain drugs. Interactions between piperine, a constituent of black pepper and certain drugs, have also been reported.

**Key Words:** Bioavailability; drug; food; herbal supplements; interactions; pharmacokinetics.

## 1. INTRODUCTION

Complementary and alternative medicines are becoming increasing popular in the United States, Europe and other parts of the world. These medicines include herbal remedies, Chinese herbal products, ayurvedic medicines (herbal remedies originated in India) as well as homeopathic remedies and are freely available to the general population from health food stores and herbal drug stores without any prescription. In developing countries, as much as 80% of the indigenous populations depend on local traditional systems of medicines. Within the European market, herbal medicines represent an important pharmaceutical market with annual sales of 7 billion US dollars. In the United States, the sale of herbal medicine increased from 200 million dollars in 1988 to over 3.3 billion dollars in 1997 *(1)*. The majority of the population using herbal medicines in the United States have a college degree and falls in the age group

**Table 1**
**Toxicity of Common Herbal Supplements**

| *Herb* | *Toxicity* | *Intended use?* (use may cause death) |
|---|---|---|
| Ephedra | Cardiovascular | Herbal weight loss |
| Chan Su | Cardiovascular | Tonic for heart |
| Oleander tea | Cardiovascular | May cause severe toxicity and even death. |
| Kava-Kava | Hepatotoxicity | Sleeping aid, anti-anxiety |
| Comfrey | Hepatotoxicity | Repairing of bone and muscle<br>Prevention of kidney stone |
| Germander | Hepatotoxicity | Weight loss/General tonic |
| Chaparral | Hepatotoxicity<br>Nephrotoxicity<br>Carcinogenic | General cleansing tonic, blood thinner, arthritis remedies and weight loss product |
| Borage | Hepatotoxicity<br>Hepatocarcinogenic | Source of essential fatty acids<br>Rheumatoid arthritis, hypertension |
| Calamus | Carcinogenic | Psychoactive, not promoted in US |
| Senna | Carcinogenic<br>Hepatotoxic | Laxative |
| Cat's Claw | Renal toxicity | Promoted as anticancer, anti-HIV, treatment of diabetes, chronic fatigue syndrome |
| Sassafras | Carcinogenic | FDA prohibits the use of sassafras as food additives. |
| Licorice | Psudoaldosteronism (Sodium and water retention, hypertension, heart failure) | Treatment of peptic ulcer, flavoring agent |

FDA, Food and Drug Administration.

of 25–49 years. In one study, 65% people thought that herbal medicines are safe *(1)*. In another recent study, Honda and Jacobson *(2)* reported that individual psychological characteristics such as personality, coping mechanisms and perceived social support may influence the use of herbal medicines. Race and ethnic origin also play a role in demographic of usage of complementary and alternative medicine. In another recent study involving 13,436 subjects, the authors found that prevalence of using herbal or natural supplements was lowest among African Americans (9.5%), intermediate in Hispanics (12%) and highest among Whites (19%). More women use complementary and alternative medicines compared to men, and the use was also higher for subjects between 45 and 64 years of age regardless of race and ethnicity. The use of these products also increased with increasing years of education *(3)*. Gulla et al. *(4)* performed a survey of 369 patient-escort pairs and found that ginseng was most commonly used (20%) followed by echinacea (19%), ginkgo biloba (15%) and St John's wort (14%).

The general concept often portrayed in marketing and media that anything natural is safe is not true. Herbal remedies can be toxic and inappropriate use or overuse may even cause fatality. Deaths have been reported from use of dietary supplements containing ephedra alkaloids and kava-kava *(5,6)*. In another report, the authors provided their opinion regarding relatively safe and unsafe herbal products. Many herbs that have been classified as unsafe include comfrey, life root, borage, calamus, chaparral, licorice and ma huang. Relatively safe herbs are feverfew, garlic, ginkgo, Asian ginseng, saw palmetto, St John's wort and valerian *(7)*. Toxicities of common herbal remedies are listed in Table 1.

## 2. REGULATORY ISSUES AFFECTING HERBAL MEDICINES

The US Food and Drug Administration (FDA) regulates drugs and requires that they be both safe and effective. Most herbal products are classified as dietary supplements or foods and are marketed pursuant to the Dietary Supplement Health and Education Act of 1994. FDA requires these to be safe for consumers but does not require demonstration of efficacy as long as they are not marketed for prevention or treatment of disease *(8,9)*.

Herbal products are regulated differently in other countries. In the United Kingdom, any product not granted a license as a medical product by the Control Agency is treated as a food and cannot carry any health claim or medical advice on the label. Similarly, herbal products are sold as dietary supplements in the Netherlands. In Germany, herbal monographs called the German Commission E monographs are prepared by an interdisciplinary committee using historic information, chemical, pharmacological, clinical and toxicological studies, case reports, epidemiological data and unpublished data from manufacturers. If an herb has an approved monograph, it can be marketed. Australia created a Complementary Medicine Evaluation Committee in 1997 to address regulatory issues regarding herbal remedies, and Canada has created a Natural Health Products Directorate after restructuring Therapeutic Products and Foods Branch in 2000 *(8,9)*.

## 3. DRUG–HERB INTERACTIONS

Mechanism of drug–herb interactions can be classified under several categories:

1. Herbal product may increase the clearance of a Western drug leading to unexpected lower concentration of a therapeutic drug. St. John's wort, a herbal anti-depressant, increased clearance of many Western drugs.
2. An herbal remedy may have a synergistic effect of increasing pharmacological activity of a Western drug or may decrease therapeutic efficacy of a drug.
3. An herbal remedy may displace a Western drug from serum protein increasing free drug concentration, the pharmacologically active component of a drug. Common drug-herb interaction are summarized in Table 2.

## 4. ST. JOHN'S WORT

Herbal remedies are often taken in conjunction with therapeutic drugs. Patients diagnosed with cancer or human immunodeficiency virus (HIV) often take various herbal antidepressants such as St. John's wort and energy pills. Cho et al. *(10)* recently reported that in their study population of patients visiting outpatient HIV clinic, 74% use herbal supplements or visit herbalists, and St. John's wort was used among these patients. Most commercially available St John's wort preparations in the United States are dried alcoholic extract of hypercian. Other preparations are liquid extract of the plant. St John's wort is licensed in Europe for treatment of depression and anxiety and is sold over-the-counter in the United Kingdom *(11)*. Many chemicals have been isolated from St John's wort including hypericin, pseudohypericin, quercetin, isoquercitrin, rutin, amentoflavone, hyperforin, other flavonoids and xanthones. However, hypericin, hyperforin and 1,3,5,7-tetrahydroxyxanthone are unique to St. John's wort. Hypericin, a naturally occurring substance found in St. John's wort, has antidepressant effect. In addition, hypericin may also have antitumor, antineoplastic and antiviral effect (hepatitis C virus and human immunodeficiency virus) *(12)*. However, more investigations are needed to confirm these claims.

Although the adverse effects of St. John's wort appear to be less than prescription antidepressants, side effects and toxicity have been reported. St. John's wort may induce photosensitivity. Therefore, fair skinned persons should be cautious about exposure to bright sun light. Photosensitivity may also be present as neuropathy, possibly due to demyelination of cutaneous axons by photo-activated hypericins. After taking St. John's wort for 4 weeks, a 35-year-old woman complained about stinging pain on sun-exposed areas. The neuropathy improved 2 months after she discontinued the product *(13)*. There are few case reports describing episodes of hypomania (irritability, agitation, anger, insomnia and difficulty in concentrating) after using St. John's wort. O'Breasail and Argouarch *(14)* reported two cases of hypomania occurring 6 weeks and after 3 months usage of St. John's wort. Other adverse effects reported with St. John's wort include gastrointestinal irritations, headache, allergic reactions, tiredness and restlessness. Demiroglu et al. *(15)* recently described a case where hematological toxicity and bone marrow necrosis due to use of St. John's wort (100 mg/day) for 3 weeks caused death in a patient.

**Table 2**
**Common Drug–Herb Interactions**

| *Herbal product* | *Interacting drug* | *Comments* |
|---|---|---|
| St. John's wort | Paroxetine | Lethargy, incoherent, nausea |
| | Digoxin | Decreased AUC, peak and trough concentration of digoxin, may reduce effectiveness of digoxin |
| | Cyclosporine/ tacrolimus | Lower cyclosporine/cyclosporine levels due to increased clearance may cause transplant rejection |
| | Theophylline | Lower concentration thus decreases the efficacy of theophylline |
| | Alprazolam/ midazolam | Reduced plasma concentration |
| | Indinavir, lopinavir | Lower concentration may cause treatment failure in ritonavir, atazanavir patients with HIV |
| | Statins | Reduced plasma concentration of simvastatin but no effect on pravastatin |
| | Irinotecan, imatinib | Reduced efficacy |
| | R-and S-verapamil | Increased clearance |
| | Methadone | Lower concentrations/ withdrawal symptoms |
| | Oral contraceptives | Lower concentration/Failed birth control |
| | Carbamazepine | No effect on plasma concentration and clearance |
| Ginkgo Biloba | Aspirin | Bleeding because ginkgo can inhibit PAF |
| | Warfarin | Hemorrhage |
| | Thiazide | Hypertension |
| | Phenytoin | Lower serum level caused death in a patient |
| | Valproic acid | Lower serum concentration of valproic acid |
| Ginkgo/Onion | Cyclosporine | Lower blood concentration of cyclosporine |
| Ginseng | Alcohol | Lower blood alcohol in the presence of ginseng |
| Ginseng | Warfarin | Ginseng may decrease effectiveness of warfarin |
| Ginger | Warfarin | Increases effectiveness of warfarin, bleeding |
| Dong quai | Warfarin | Dong quai contains coumarin/increases INR |
| Garlic | Warfarin | Increases effectiveness of warfarin, bleeding |
| Garlic | Saquinavir | Reduced plasma concentration due to reduced absorption of saquinavir |
| Kava | Alprazolam | Additive effects with CNS depressants, alcohol |
| Comfrey | Phenobarbital | Increases metabolism of Comfrey producing a lethal metabolite from pyrrolizidine/severe hepatotoxicity |
| Evening Primrose oil | Phenobarbital | May lower seizure threshold, need dose increase |
| Valerian | Sedatives | Increased effect of sedatives |

AUC, area under the curve; CNS, central nervous system; HIV, human immunodefeciency virus; INR, international normalization ratio; PAF, Platelet activating factor.

### 4.1. Drug—St John's Wort Interactions: Mechanism

Primary mechanism of drug–herb interaction involves induction or inhibition of hepatic or intestinal metabolism of drugs by cytochrome P 450 (CYP). Another mechanism of drug–herb interaction involves induction or inhibition of intestinal drug efflux pumps including P-glycoprotein and multiple resistance proteins. Any inhibitory effect of herbs on efflux proteins and CYP may result in increased plasma and tissue concentrations of a drug leading to toxicity. On the other hand, any induction of efflux proteins and CYP may lead to significantly reduced concentrations of a drug in plasma and tissue, thus causing treatment failure *(16)*. CYP3A4 is the most abundant isoenzyme of cytochrome P 450 and is responsible for metabolism of more than 73 drugs and numerous endogenous compounds *(17)*. The active components of St. John's wort induce CYP3A4 and CYP2B6 *(18,19)*. In particular, hyperforin is thought to be responsible for isoenzyme induction through activation of a nuclear steroid/pregnane and xenobiotic receptor *(20)*. St. John's wort also induces P-glycoprotein drug transporter and may reduce efficacy of drugs where hepatic metabolism may not be the major pathway of clearance. The component hypericin may be the active ingredient that modulates P-glycoprotein *(21)*. Based on the reported studies, it can be concluded that St. John's wort induces hepatic and intestinal CYP3A4 and modulates intestinal P-glycoprotein pumps. Therefore, it is likely that St. John's wort will interact with drugs that are metabolized via CYP3A4 and P-glycoprotein pumps. However, not all drugs interact with St. John's wort *(22)*.

### 4.2. Drug—St John's Wort Interactions: Lower Concentrations of Therapeutic Drugs

Self-medication with St. John's wort may cause treatment failures due to significant reductions in plasma drug concentrations because of increase in clearance of drugs. Unrecognized use of St. John's wort is frequent among patients and may have important influences on the effectiveness and safety of drug therapy during hospital stay *(23)*. Published reports indicate that St. John's wort significantly reduces steady-state plasma concentrations of cyclosporine, tacrolimus, amitriptyline, digoxin, fexofenadine, indinavir, methadone, midazolam, nevirapine, phenoprocoumon, squinavir, simvastatin, theophylline and warfarin *(24)*. This long list of drugs includes immunosuppressant drugs (cyclosporine and tacrolimus), HIV protease inhibitors and HIV non-nucleoside reverse transcriptase inhibitors metabolized via CYP3A4. Increased clearance of oral contraceptives has also been reported.

### 4.3. Theophylline and St. John's Wort

A 42-year-old female taking an unusually high dose of theophylline (1600 mg per day) indicated that prior to her recent hospitalization she was stabilized on a twice daily theophylline dose of 300 mg. This dosage produces a theophylline serum concentration of 9.2 μg/mL. The patient informed during interview that she was taking St John's wort (0.3% hypericin) 300 mg per day for the last 2 months. She had been decided to discontinue St. John's wort, and 7 days later her theophylline level was increased to 19.6 μg/mL and her dosage was consequently adjusted downwards *(25)*. In vitro study with intestinal LS 180 cells indicted that St. John's wort increased the expression of

CYP1A2 in a concentration-dependent manner, and this induction maybe responsible for reduced plasma theophylline concentrations upon co-administration of St. John's wort *(26)*. However, Morimoto et al. *(27)* found no significant interaction between St. John's wort and theophylline where healthy volunteers consumed St. John's wort for (300 mg three times a day) 15 days, and on day 14, the subjects received a single oral dose of 400 mg theophylline.

### *4.4. Immunosuppressants and St. John's Wort*

Barone et al. reported two cases where renal transplant recipients started self-medication with St. John's wort. Both patients experienced sub-therapeutic concentrations of cyclosporine, and one patient developed acute graft rejection due to low cyclosporine concentration. Upon termination of use of St. John's wort, both patients' cyclosporine concentrations returned to therapeutic levels *(28)*. Interaction between St. John's wort and cyclosporine is well documented in the literature *(29)*. Bauer et al. concluded that St John's wort caused rapid and significant reduction in plasma cyclosporine concentrations. Additionally, substantial alteration in cyclosporine metabolite kinetics was also observed *(30)*. Alschner and Klotz reported a case study where a 57-year-old kidney transplant recipient with a long-term regular intake of cyclosporine (125–150 mg/day) and prednisolone (5 mg/day) and routinely monitored cyclosporine trough level (100–130 ng/mL) over the past 2 years showed a sudden drop in cyclosporine blood level to 70 ng/mL despite the daily cyclosporine dose increased to 250 mg per day. The patient admitted of taking a herbal tea mixture for depression, which contained St. John's wort. Five days after discontinuing the herbal tea his cyclosporine level was increased from 70 to 170 ng/mL (250 mg of cyclosporine per day). The dose was reduced to 175 mg per day and his trough cyclosporine level again returned to around 130 ng/mL *(31)*. Mai et al. reported that hyperforin content of St. John's wort determines the magnitude of interaction between St. John's wort and cyclosporine. Patients who received low hyperforin-containing St. John's wort showed minimal changes in pharmacokinetic parameters and needed no dose adjustment. In contrast, the patients who received high amounts of hyperforin-containing St. John's wort needed dose increases within 3 days in order to maintain trough therapeutic concentration of cyclosporine *(32)*.

Significant reduction in area under the curve (AUC) for tacrolimus was also observed in 10 stable renal transplant patients receiving St. John's wort. The maximum concentration of tacrolimus was also reduced from a mean value of 29.0–22.4 ng/mL following co-administration of St. John's wort *(33)*. Bolley et al. reported a case where a 65-year-old patient who received a renal transplant in November 1998 had a trough whole blood level tracrolimus concentration between 6 and 10 ng/mL. The patient started self-medication with St. John's wort in July 2000 (600 mg per day) because of depression and in August 2000 showed an unexpected low tracolimus concentration of 1.6 ng/mL. Interestingly, the serum creatinine was also decreased to 0.8 mg/dL from an initial value of between 1.6 and 1.7 mg/dL. When the patient stopped taking St. John's wort, tracrolimus level returned to the previous range of 6–10 ng/mL. After 1 month, the creatinine value was also gradually increased to 1.3 ng/mL. Because the patient showed no rejection episode, the new tacrolimus target level was set to 4–6 ng/mL *(34)*. Mai et al. studied interaction of St. John's wort with tracrolimus

and mycophenolic acid using 10 stable renal transplant patients. Co-administration of St. John's wort significantly reduced the AUC as well as both peak and trough blood concentrations of tracrolimus. In order to achieve sufficient immunosuppression, tacrolimus doses were increased in all patients (median 4.5–8.0 mg/day). The tacrolimus trough levels after corrected for dose decreased from a median value of 10.8 ng/mL (pre St John's wort) to 3.8 ng/mL 2 weeks after initiation of use of St. John's wort. Two weeks after discontinuation of St. John's wort treatment, trough concentrations were increased again to 7.6 ng/mL, and patients were adjusted again to their previous doses approximately 4 weeks after the end of the study. Interestingly, pharmacokinetic parameters of mycophenolic acid, another immunosuppressant, were not affected by co-administration of St. John's wort *(35)*.

### *4.5. Digoxin and St. John's Wort*

Interaction between St. John's wort and digoxin is of clinical significance. Johne et al. reported that 10-day usage of St. John's wort resulted in a 33% decrease of peak and 26% decrease in trough serum digoxin concentrations. The mean peak digoxin concentration was 1.9 ng/mL in the placebo group and 1.4 ng/mL in the group taking St. John's wort. The AUC between 0 and 24 h was 25% lower in the group consuming St. John's wort compared to the placebo group *(36)*. Digoxin is a substrate for P-glycoprotein, which is induced by St. John's wort. Durr *(37)* et al. also confirmed the lower digoxin concentrations in healthy volunteers who concurrently took St. John's wort. St John's wort's dose and preparation also affect phramacokinetics of digoxin. Mueller et al. reported that low daily dose of hyperforin containing St. John's wort does not affect pharmacokinetics of digoxin. In contrast, co-medication with high-dose hyperforin-rich extract resulted in a 24.8% decrease in AUC from time 0 to 24 h. A reduction of 37% was also observed in digoxin maximal plasma concentrations *(38)*.

### *4.6. Antiretrovirals and St. John's Wort*

A patient positive for HIV and taking antiviral agents should not consume St. John's wort, Echinacea, garlic, ginkgo and milk thistle because of interactions between these herbal remedies and antiretrovirals *(39)*. St John's wort was shown to reduce the AUC of the HIV-1 protease inhibitor indinavir by a mean of 57% and decreased the extrapolated trough by 81%. The subjects received 300 mg of St. John's wort three times a day for 14 days. The mean peak concentration ($C_{max}$) decreased from 12.3 to 8.9 ng/mL in healthy volunteers taking both indinavir and St. John's wort. More significant effect was observed in C8 concentrations where the mean value was reduced from 0.494 to 0.048 ng/mL in the group taking both St. John's wort and indinavir. Reduction in indinavir concentrations of these magnitudes are clinically significant and could lead to treatment failure *(40)*. Busti et al. *(41)* reported that atazanavir therapy can also be affected due to simultaneous use of St. John's wort. Co-administration of lopinavir/ritonavir with St. John's wort is also not recommended because of substantial reduction in lopinavir plasma concentrations *(42)*.

### *4.7. Tricyclic Antidepressants and St. John's Wort*

There is a high probability that a person suffering from depression and being treated with a tricyclic antidepressant is also taking St. John's wort. Concomitant intake of

St. John's wort (hypericum extract LI160) for at least 2 weeks in 12 depressed patients decreased the AUC between 0 and 12 h) of amitriptyline by 22% and nortriptyline by 41%. The AUC of all hydroxylated metabolites except 10-E-hydroxynortriptyline was also reduced. The mean peak concentration of amitriptyline was reduced from 69.8 to 54.1 ng/mL in patients also receiving St. John's wort, and significant reductions were also observed in peak nortriptyline concentrations among subjects taking St. John's wort *(43)*. The demethylation of amitriptyline to nortriptyline is catalyzed by CYP3A4 and CYP2C19, while further metabolism of nortriptyline through hydroxylation at position 10 is mediated by CYP3A4 and CYP2D6.

### *4.8. Benzodiazepines, Fexofenadine and St. John's Wort*

Benzodiazepines alprazolam and midazolam are metabolized by CYP3A4. Although short-term ingestion of St. John's wort (900 mg/day for 1–3 days) does not alter the pharmacokinetics of alprazolam and midazolam in healthy volunteers, long-term ingestion (900 mg/day for 2 weeks) significantly increased oral clearance of midazolam and decreased oral bioavailability by 39.3% *(44)*. Fexofenadine is a non-sedating antihistamine. A single dose of St. John's wort (900 mg) significantly increased the maximum plasma concentration of fexofenadine by 45% and significantly decreased the oral clearance by 20% without any significant change in half-life or renal clearance. However, long-term use of St. John's wort (2 weeks) caused a significant decrease of 35% in maximum plasma concentration and a significant increase (47%) in oral clearance. This is probably due to inhibition of intestinal P-glycoprotein when a single dose of St. John's wort was given, but a long-term use reversed the changes in fexofenadine disposition *(45)*.

### *4.9. Methadone and St. John's Wort*

Reduced plasma level of methadone was also observed in the presence of St. John's wort. Long-term treatment with St. John's wort (900 mg/day) for a median period of 31 days (range 14–47 days) decreased the trough concentrations of methadone by an average of 47% in four patients. Two patients experienced withdrawal symptoms due to reduced plasma levels of methadone *(46)*.

### *4.10. Anticancer Agents and St. John's Wort*

Clearance of imatinib mesylate, an anticancer drug, is also increased due to administration of St. John's wort resulting in reduced clinical efficacy of the drug. Imatinib is used in the treatment of Philadelphia chromosome positive chronic myeloid leukemia and gastrointestinal stromal tumors. In one study involving 10 healthy volunteers, 2-week treatment with St. John's wort significantly reduced maximum plasma concentration by 29%, AUC by 32. The half-life of the drug was reduced by 21% *(47)*. St. John's wort also showed significant interaction with another anticancer drug irinotecan. In one study involving five patients, ingestion of St. John's wort (900 mg/day) for 18 days resulted in an average 42% reduction in concentration of SN-39, the active metabolite of irinotecan. This reduction also caused decreased myelosuppression *(48)*.

### 4.11. Oral Contraceptives and St. John's Wort

Oral contraceptives are divided into two types: progestogen only and combined estrogen and progestogen. Most oral contraceptives are substrates for CYP3A4 *(49)*. 17-Ethynylestradiol is a major component of oral contraceptive pill and is also used in hormonal replacement therapy in postmenopausal women. It is metabolized through hydroxylation in position 2 by CYP3A4 *(50)*. St. John's wort has significant interaction with oral contraceptives *(51)*. Muprhy et al. studied interaction between St. John's wort and oral contraceptives by investigating phramacokinetics of norethindrone and ethinyl estradiol using 16 healthy women. Treatment with St. John's wort (300 mg three times a day for 28 days) resulted in a 13–15% reduction in dose exposure from oral contraceptives. Breakthrough bleeding increased in treatment cycle as did evidence of follicle growth and probable ovulation. Authors concluded that St. John's wort increased metabolism of norethindrone and ethinyl estradiol and thus interfered with contraceptive effectiveness *(52)*.

### 4.12. Other Drug—St. John's Wort Interactions

Sugimoto et al. reported interactions of St. John's wort with cholesterol-lowering drugs simvastatin and pravastatin. In a double blind crossover study using 16 healthy male volunteers, the authors demonstrated that use of St. John's wort (900 mg/day) for 14 days decreased peak serum concentration of simvastatin hydroxyl acid, the active metabolite of simvastatin, from an average of 2.3 ng/mL in the placebo group to 1.1 ng/mL in the group taking St. John's wort. The AUC was also reduced in the group of volunteers taking St. John's wort compared to the placebo group. Simvastatin is extensively metabolized by CYP3A4 in the intestinal wall and liver, and St. John's wort induces this enzyme. On the other hand, St. John's wort did not influence plasma pravastatin concentration *(53)*. St. John's wort also induces both CYP3A4-catalyzed sulfoxidation and 2C19-dependent hydroxylation of omeprazole. In a study involving 12 healthy adult men, a group of volunteers received St. John's wort (900 mg/day) for 14 days. Then, both control groups and volunteers taking St. John's wort consumed a single dose of omeprazole (20 mg) orally. Significant decreases in peak plasma concentrations of omeprazole were observed in volunteers taking St. John's wort indicating significant interactions between St. John's wort and omeprazole *(54)*. Tannergren et al. reported that repeated administration of St. John's wort significantly decreases bioavailability of R and S-verapamil. This effect is caused by induction of first pass metabolism by CYP3A4 most likely in the gut *(55)*.

Interestingly, St. John's wort does not interact with carbamazepine. Burstein et al. *(56)* reported that intake of St. John's wort (900 mg/day) for two weeks did not alter pharmacokinetics of the antiepileptic drug carbamazepine. Carbamazepine is metabolized by CYP3A4, but the lack of interaction may be due to the inducing property of carbamazepine itself on cytochrome P 450 enzymes, and therefore, further induction by St. John's wort may not occur.

St. John's wort is as effective as paroxetine for treating mild to moderate depression *(57)*. A patient taking paroxetine (Paxil, 40 mg) for 8 months stopped taking paroxetine and started taking St. John's wort (600 mg per day). She experienced no adverse effect from switching medication. However, one night when she felt tired she took 20 mg of paroxetine and felt lethargic and ended up in a hospital. The authors

conclude that St. John's wort is a monoamine oxidase inhibitor and interacted with paroxetine, a selective serotonin reuptake inhibitor *(58)*.

### *4.13. Drug—St. John's Wort Interactions: Impact on Therapeutic Drug Monitoring*

Interactions of St. John's wort with various drugs depend on concentrations of active components. Herbal remedies are not prepared following rigorous pharmaceutical standards. Wide variations in the active component of St. John's wort in various commercial preparations have been reported. Draves and Walker *(59)* reported that in commercial tablets of St. John's wort, the percentage of active components varied from 31.3 to 80.2% of the claim of active ingredients based on labeling of the bottle. Studies have demonstrated that cytochrome P 450 enzyme induction by St. John's wort depends on the hyperforin content, and products that do not contain substantial amount of hyperforin (<1%) may not show clinically significant interactions with drugs *(22)*. Arold et al. *(60)* demonstrated that low hyperforin containing St. John's wort had no significant interaction with alprazolam, caffeine, tolbutamide and digoxin. Moreover, hyperforin is photosensitive and unstable in aqueous solution while degradation is dependent on the pH of the solution *(61)*.

Because interaction between St. John's wort and drugs may depend on the concentrations of active components of St. John's wort, measurement of active components of St. John's wort in human serum may have clinical implications. Bauer et al. described a high performance liquid chromatographic method (isocratic reverse phased-HPLC) for determination of hypericin, pseudohypericin using fluorimetric detection and hyperforin by UV detection. The limit of detection was 0.25 ng/mL of hypericin and pseudohypericin and 10 ng/mL for hyperforin in human plasma *(62)*. Pirker et al. also used liquid-liquid extraction using n-hexane and ethyl acetate (70:30 by vol) and reverse phase HPLC with UV fluorescence and mass spectrometric (electrospray ionization) detection for quantification of active components of St. John's wort in human plasma. The linearity for hypericin determination was 8.2–28.7 ng/mL for hypericin and 21.6–242.6 ng/mL for hyperforin *(63)*. Several liquid chromatographic methods have also been reported for determination of active components of St. John's wort in various commercial preparations *(64,65)*.

## 5. INTERACTION OF WARFARIN WITH HERBAL SUPPLEMENTS

Warfarin acts by antagonizing the cofactor function of vitamin K. Variability in the anticoagulant response to warfarin is an ongoing clinical dilemma. Although clinical efficacy of warfarin varies with intake of vitamin K, genetic polymorphisms that modulate expression of CYP2C9, the isoform mediating clearance of S-warfarin, may have significant effect on warfarin therapy. Moreover, several herbal remedies also interact with warfarin. St. John's wort may have the potential to diminish warfarin's anticoagulation effect by increasing clearance through inducing CYP2C9 *(66)*. Another report indicates that St. John's wort increases clearance of both R- and S-warfarin but ginseng has no effect *(67)*.

Anticoagulant effect of warfarin increases if combined with coumarin-containing herbal remedies such as bilba, fenugreek and dong quai or with antiplatelet herbs such

as danshen, garlic and ginkgo biloba. Conversely, vitamin K-containing supplement such as green tea may antagonize the anticoagulant effect of warfarin. The international normalization ratio (INR) was increased in a patient treated with warfarin for atrial fibrillation when he started taking coumarin-containing herbal products boldo and fenugreek. After discontinuation of herbal supplements, his INR returned to normal after 1 week *(68,69)*. Increased anticoagulation due to interaction between warfarin and danshen has been reported. *(70,71)*. Two cases of increased INR were mentioned in patients taking garlic previously stabilized on warfarin *(72)*. A likely mechanism is an additive effect because garlic has antiplatelet activity.

## 6. INTERACTION OF GARLIC (*ALLIUM SATIVUM*) WITH DRUGS

Garlic is widely used as a herbal supplement promoted to lower cholesterol and blood pressure. Garlic is rich in the sulfur-containing compounds allicin and alliin. Piscitelli et al. studied the effect of garlic on pharmacokinetics of saquinavir, a protease inhibitor, with 10 health volunteers. In the presence of garlic, the mean saquinavir AUC during 8-h dosing interval decreased by 51%, and trough serum concentration 8 h after dosing reduced by 54%. After a 10-day washout period, the AUC and trough serum concentrations returned to 60–70% of the baseline values. The altered pharmacokinetics of saquinavir were considered to be related to decreased bioavailability of saquinavir *(73)*. This may be due to inhibition of P-glycoprotein pump in the gut mucosa by garlic. Although interaction between warfarin and garlic has been reported, Macan et al. *(74)* recently reported that aged garlic extract may not have any interaction with warfarin.

## 7. INTERACTIONS OF GINSENG WITH DRUGS

Ginseng is a widely used herbal product in China, other Asian countries and also in the United States. For thousands of years, the common people in China have used ginseng as a tonic. The Chinese ginseng that grows in Manchuria is *Panax ginseng*. However, the ginseng that grows in North America is *Panax quinquefolius*. The common preparation of ginseng is ginseng root. Ginseng is promoted as a tonic and also as a reliever of stress. Ginseng may also be effective in the treatment of mild hyperglycemia. In Germany, it is indicated to combat lack of energy. Ginseng contains saponins known as ginsenosides.

Lee et al. studied interaction between *P. ginseng* and alcohol in 14 healthy male volunteers utilizing each subject as their own control. At 40 min after the last drink, the blood alcohol in the test group receiving ginseng extract (3 gm/65 kg bodyweight) along with alcohol (72 g/65 kg bodyweight) was about 35% lower than the control value *(75)*. A study using mice indicated that decreased plasma concentrations of alcohol in the presence of ginseng may be due to a delay in gastric emptying *(76)*. Interaction between ginseng and phenelzine, a monoamine oxidase inhibitor, has been reported. The interaction may be related to the psychoactive effect of ginseng *(77)*.

## 8. INTERACTIONS OF GINKGO WITH DRUGS

Ginkgo biloba is prepared from dried leaves of the ginkgo tree by organic extraction (acetone/water). After the solvent is removed, the extract is dried and standardized. Most commercial dosage forms contain 40 mg of this extract. Ginkgo biloba is sold

in the United States as a dietary supplement in order to improve blood flow in brain and peripheral circulation. It is used mainly to sharpen mental focus and to improve diabetes related circulatory disorder. The German Commission E approved the use of ginkgo for memory deficit, disturbances in concentration, depression, dizziness, vertigo and headache. Ginkgo leaf contains kaempterol-3-rhamnoglucoside, ginkgetin, isoginketin and bilobetin. Several glycosides have also been isolated (ginkgolide A and B). Other substances isolated were shikimic acid, D-glucarica acid and anacardic acid. Several chemicals found in ginkgo extracts, especially ginkgolide B, are potent antagonist of platelet activity factor and also have antioxidant effect.

Ginkgo biloba exerts inductive effect on CYP2C19 activity. Yin et al. investigated interaction of ginkgo biloba with omeprazole in 18 healthy subjects. All subjects received a single dose of omeprazole (40 mg) at baseline and after 12 days treatment with ginkgo biloba (140 mg, twice daily). Plasma concentrations of omeprazole and omeprazole sulfone were significantly decreased and concentration of 5-hydroxyomeprazole was significantly increased following treatment with ginkgo biloba. The authors concluded that ginkgo biloba can induce omeprazole hydroxylation in a CYP2C19 genotype-dependent manner and concurrently reduce renal clearance of 5-hydroxyomeprazole *(78)*. On the other hand, ginkgo biloba has no significant interaction with digoxin *(79)*.

Yang et al. studied bioavailability of cyclosporine in the presence of ginkgo and onion in rats. Cyclosporine was administered both orally and intravenously with or without ginkgo or onion in crossover design. Oral administration of ginkgo and onion significantly decreased the maximum serum concentration ($C_{max}$) by 62 and 60% and also reduced the AUC by 51 and 68%, respectively. The average maximum serum concentration of cyclosporine in the control group was 169.4 ng/mL and in the group receiving ginkgo was 65.2 ng/mL. In contrast, no effect was seen on pharmacokinetics of cyclosporine in the presence of ginkgo and onion when cyclosporine was given intravenously *(80)*.

A recent case report indicates that fatal seizures in a 55-year-old male may be due to interaction of ginkgo biloba with antiepileptic drugs. The patient suffered a fatal breakthrough seizure with no evidence of his non-compliance with anticonvulsant medications. The post-mortem femoral blood concentrations of both phenytoin (2.5 μg/mL) and valproic acid (<26 μg/mL) were sub-therapeutic. Interestingly, his phenytoin serum concentrations were within therapeutic in the last 6 months (range 9.6–21.2 μg/mL) and the last phenytoin value prior to his death was 13.9 μg/mL. The patient was taking a variety of herbal supplements but ginkgo was a main component. Phenytoin is primarily metabolized by CYP2C9 and secondarily by CYP2C19 whereas valproic acid metabolism is also modulated by CYP29 and CYP2C19. Ginkgo biloba induces CYP2C19 activity and thus may be responsible for sub-therapeutic levels of anticonvulsant medications in this patient *(81)*. Granger reported cases of two patients who were stable with valproic acid but developed seizure within 2 weeks of using ginkgo products. After discontinuation of ginkgo, both patients were again seizure free without any increases in dose of valproic acid *(82)*.

Using rat model, Kubota et al. demonstrated that ginkgo biloba extract when orally administered to rats for 2 weeks reduced the hypotensive effect of nicardipine. This

may be due to increased hepatic metabolism of nicardipine *(83)*. Sugiyama et al. reported that intake of ginkgo attenuated hypoglycemic action of tolbutamide in aged rats *(84)*.

## 9. INTERACTION OF KAVA WITH DRUGS

Kava is a herbal sedative with purported calming effect. Kava is prepared from a South Pacific plant (*Piper mesthysticum*). There are 72 different kava plants, which differ in appearance as well as in their chemical composition. Kava drink is prepared by mixing fresh or dried root with cold water or coconut milk. Kava is available from a variety of manufacturers. The neurological effects of kava are attributed to a group of substituted dihydropyrones called kava lactones.

Heavy consumption of kava has been associated with increased concentrations of γ-glutamyltransferase suggesting potential hepatotoxicity. Escher et al. described a case in which severe hepatitis was associated with kava use. A 50-year-old man took three to four kava capsules daily for 2 months (maximum recommended dose three capsules). Liver function tests showed 60-fold to 70-fold increases in AST and ALT. Tests for viral hepatitis were all negative as were tests for cytomegalovirus and HIV. The patient eventually received a liver transplant *(85)*. Humberston et al. *(86)* also reported a case of acute hepatitis induced by kava-kava. Other cases of hepatotoxicity due to the use of kava have been documented *(87)*. In January 2003, kava extracts were banned in the entire European Union, Canada and also in the United States (the FDA strongly cautioned against using kava). There are at least 11 cases of serious hepatic failure and four deaths directly linked to kava extract consumption, and there are also 23 reports indirectly linking kava with hepatotoxicity *(88)*.

Recently, it has been demonstrated that several kava lactones are potent inhibitor of several enzymes of cytochrome P 450 system (CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4 and CYP4A9/11). Therefore, there is a potential of drug interaction with kava-kava especially for drugs metabolized by cytochrome P 450 system, but actual systemic studies with human subjects to demonstrate such drug interactions are very limited *(89)*. A recent study involving six healthy human volunteers who consumed traditional aqueous extract of kava indicated that caffeine metabolic ratio increased twofold from 0.3 with consumption of kava to 0.6 at 30 days after the subjects stopped using kava. The later value corresponds to metabolic ratios in healthy subjects. The authors concluded that kava drinking inhibits CYP1A2 *(90)*.

Kava has known sedative effect and it is speculated that kava may interact with central nervous system depressants such as benzodiazepines, alcohol and barbiturates. There is a case report describing interaction of kava with alprazolam. A 54-year-old patient taking alprazolam, cimetidine and terazosin started self-medication with kava for 3 days and was hospitalized. The authors suggested that both kavaloactones and alprazolam have additive effect because both act on the same GABA receptors. Moreover, kavalactones are potent inhibitor of CYP3A4 which metabolizes alprazolam *(91)*. Although in mouse model, kava was shown to have additive effect on alcohol, a clinical study using human subjects indicated that kava did not alter safety-related performance in volunteers taking alcohol *(92)*. Another recent study indicates that kava is unable to inhibit alcohol dehydrogenase activity in vitro *(93)*.

## 10. FOOD–DRUG INTERACTIONS

Food–drug interactions may be pharmacokinetic or pharmacodynamic in nature. Certain foods alter activity of drug metabolic enzymes, and especially CYP3A4 appears to be the key enzyme in food–drug interaction *(94)*. It has also long been recognized that intake of food and fluid can alter the extent of drug absorption. This alteration may be related to alteration of physiological factors in the gut such as gastric pH, gastric emptying time, intestinal motility, hepatic portal blood flow or bile flow rate. Moreover, direct interaction of food with drug may also alter bioavailability such as adsorption of a drug in insoluble dietary component, complex formation of a drug with a metal ion or partitioning of a drug in dietary fat. Food may be able to alter performance of modified released oral formulation *(95)*. It has been recognized that smoking, intake of charcoal-broiled food or cruciferous vegetables induce the metabolism of multiple drugs, whereas grapefruit juice increases bioavailability of many drugs. Energy deficiency and low intake of a protein may cause about 20–40% decrease in clearance of theophylline and phenazone, while elimination of these drugs may be accelerated in the presence of protein-rich diet *(96)*. Fegan et al. reported increased clearance of propranolol and theophylline in the presence of high protein/low carbohydrate diet compared to a low protein/high carbohydrate diet with 6 volunteers. When the diet was switched from a low protein/high carbohydrate to a high protein/low carbohydrate, the clearance of propranolol was increased by an average of 74%, and clearance of theophylline was increased by an average of 32% *(97)*.

### *10.1. Grapefruit Juice and Drug Interactions*

It was reported in 1991 that a single glass of grapefruit juice caused a twofold to threefold increase in the plasma concentration of felodipine, a calcium channel blocker, after oral intake of a 5-mg tablet, but a similar amount of orange juice showed no effect *(98)*. Subsequent investigations demonstrated that pharmacokinetics of approximately 40 other drugs are also affected by intake of grapefruit juice *(99)*. The main mechanism for enhanced bioavailability of drugs after intake of grapefruit juice is the inhibition of CYP3A4 in the small intestine. Grapefruit juice causes significant increases in the bioavailability of drugs after oral dosing but does not alter pharmacokinetic parameters of the same drug after intravenous administration. Therefore, it appears that grapefruit juice inhibits intestinal CYP3A4 but has no significant effect on liver CYP3A4 *(100,101)*. Multiple drug-resistant (MDR) transporters play an important role in the disposition of many drugs. P-glycoprotein is a major MDR transporter that decreases the fraction of drug absorbed by carrying the drug back to the intestinal lumen from enterocytes. Although few studies showed activation of P-glycoprotein by grapefruit juice, most studies reported significant inhibition of P-glycoprotein by components of grapefruit juice *(101,102)*. Furanocoumarins found in the grapefruit juice are probably responsible for interactions between grapefruit juice and drugs. The major furanocoumarin present in grapefruit juice is bergamottin which showed time-and concentration-dependent inactivation of cytochrome P 450 enzymes in vitro *(103)*. Moreover metabolite of bergamottin (6′,7′ dihydrobergamottin) and a number of other furanocoumarin derivatives may also be involved in inhibiting CYP3A4 and CYP1B1 *(104)*. Flavonoids such as naringin and naringenin may also

play a role. Concentrations of such compounds also vary significantly between different sources of grapefruit juice. De Casto et al. *(105)* reported that concentrations of naringin may vary from 174 to 1492 μmol/L among various grapefruit juices whereas concentrations of bergamottin varied from 1.0 to 36.6 μmol/L. Therefore, the magnitude of grapefruit juice–drug interactions may also be variable. Paine et al. *(106)* reported that furanocoumarin-free grapefruit juice showed no interaction with felodipine, thus establishing that furanocoumarins are responsible for interactions between felodipine and grapefruit juice.

Time of ingestion of grapefruit juice also affects interaction between grapefruit juice and drugs. In most cases, ingestion of a single glass (~250 ml) of regular strength grapefruit juice is enough to produce the effect of increased bioavailability of drugs. Lundahl et al. *(107)* observed no further change in pharmacokinetics of felodipine following 14 days of daily intake of grapefruit juice compared to the effects observed after the first glass. There is no need to take grapefruit juice along with a drug in order to observe the interaction. The bioavailability of lovastatin has been reported to double even when the drug was taken 12 h after intake of grapefruit juice *(108)*. However, an interval of more than 24 h usually prevents a clinically significant interaction between a drug and grapefruit juice *(109)*.

The oral formulation of cyclosporine (Sandimmun) has a variable absorption. The development of cyclosporine microemulsion (Sandimmun Neoral) resulted in increased bioavailability. Bistrup et al. *(110)* using eight stable renal transplant recipients demonstrated that grapefruit juice increased median AUC of cyclosporine (following oral administration of Sandimmun Neoral) by 38% whereas no change was observed in maximum cyclosporine concentration in blood or half-life of cyclosporine. Furonocoumarins containing drinks such as Sundrop Citrus Soda (registered trademark used under license of Dr Pepper/Seven Up Inc, 2002) may also produce grapefruit juice like effect with cyclosporine. A 32-year-old lung transplant recipient showed a trough cyclosporine concentration of 358 ng/mL 2 weeks after being discharged from the hospital. On the next four visits spanning 24 days, elevated cyclosporine concentrations were observed in two occasions. These values of 676 and 761 ng/mL were substantially higher that two other cyclosporine concentration of 319 and 374 ng/mL during the same time period. Furthermore, there was no change in dosage of cyclosporine. The higher cyclosporine concentrations correlated with consumption of Sundrop Citrus Soda during breakfast by the patient, which contains furocoumarin *(111)*. Delayed effect of grapefruit juice on pharmacokinetcs and pharmacodynamics of tracrolimus has been reported in a living donor liver transplant recipient. The patient demonstrated a considerable increase in trough blood concentration of tacrolimus after concomitant ingestion of grapefruit juice (250 ml) four times a day for 3 days. The trough blood concentrations were not affected during or immediately after repeated intake of grapefruit juice. However, almost 1 week after final ingestion of grapefruit juice, the trough tacrolimus concentration increased to 47.4 ng/mL from a preingestion tacrolimus concentration of 4.7 ng/mL *(112)*.

Interactions between grapefruit juice and antiepileptic drugs have been reported. In a randomized crossover study involving 10 patients with epilepsy, grapefruit juice (300 ml) increased the trough concentration of carbamzaepine (4.51 μg/mL in the control group vs. 6.28 μg/mL in the grapefruit juice group). The steady-state

carbamazepine concentrations were also significantly increased in patients who ingested grapefruit juice and carbamazpine in comparison to the control group *(113)*. In contrast, grapefruit juice has no effect on the pharmacokinetics and bioavailability of another anticonvulsant drug phenytoin *(114)*.

Saquinavir, a HIV protease inhibitor, has poor bioavailability due to extensive metabolism in the intestine initiated by CYP3A4. Oral bioavailability of saquinavir increased by a factor of 2 following ingestion of 400 ml of grapefruit juice without affecting clearance. This effect was absent when the drug was delivered by intravenous administration *(115)*. In contrast, grapefruit juice slightly decreased maximum serum concentration of another HIV protease inhibitor amprenavir (7.11 ng/mL in the presence of grapefruit juice vs. 9.10 ng/mL in the control), but the difference was not clinically significant *(116)*. Grapefruit juice did not have any significant effect on bioavailability and pharmacokinetics of indinavir *(117)*.

Interactions between grapefruit juice and various calcium channel antagonists have been extensively studied in the past. The most striking effect was observed with felodipine where increases up to 430% in maximum serum concentration and increases up to 300% in AUC had been observed in the presence of grapefruit juice. A further decrease in diastolic blood pressure was also observed when felodipinen was taken with grapefruit juice as well as adverse effects such as increased heart rate and orthostatic hypotension *(98)*. Similar interactions with grapefruit juice were also observed with nisoldipine and nicardipine. Increased bioavailability of nitrendipine (100% increases), pranidipine (73% increases) and nimodipine (50% increases) were also observed in the presence of grapefruit juice *(100,101)*. A significant increase in bioavailability of verapamil in the presence of grapefruit juice has also been reported *(118)*.

Grapefruit juice increases bioavailability of several benzodiazepines including diazepam, triazolam and midazolam but has no effect on alprazolam even after repeated intake *(101)*. Ozdemir et al. *(119)* reported a threefold increases in AUC of diazepam due to intake of grapefruit juice. Grapefruit juice also interacts with cholesterol lowering drugs. Simvastatin, a substrate for CYP3A4, is extensively metabolized during first pass. Grapefruit juice (200 ml once a day for 3 days) increased the AUC (0–24 h) of simvastatin by 3.6-fold and simvastatin acid by 3.3-fold. The peak concentrations were also increased significantly, and only one glass of grapefruit juice taken daily is capable of producing such effects *(120)*. On the other hand, when subjects ingested double strength grapefruit juice three times a day (200 ml) for 3 days, the peak serum concentrations and the AUC were increased by 12.0-fold and 13.5-fold respectively compared to the control. When simvastatin was taken 24 h after ingestion of grapefruit juice, the effect of grapefruit juice on AUC of simvastatin was only 10% of the effect observed during concomitant intake of simvastatin with grapefruit juice *(109)*. Grapefruit juice increased the AUC (0–24 h) of atorvastatin acid by 83% whereas the AUC of pitavastatin was increased by only 13% *(121)*.

Digoxin is a substrate of P-glycoprotein. In a study involving 12 healthy volunteers, grapefruit juice had no significant effect on maximum plasma drug concentration of digoxin or the AUC (0–48 h) *(122)*. However in another study involving 12 volunteers, administration of cisapride with grapefruit juice (250 ml) increased the AUC of cisapride by 151% and maximum plasma concentration by 168% *(123)*. Grapefruit

juice has modest effect in increasing the AUC (0–48 h) of itraconazole by 17% and reduces oral clearance of itraconazole by 14% *(124)*.

Although most reports indicate increased bioavailability of drugs in the presence of grapefruit juice or no clinically significant interaction, Dresser et al. reported significant reduction in bioavailability of fexofenadine, a non-sedating antihistamine. This drug does not undergo any significant intestinal or hepatic metabolism. Recent developments indicate that the family of drug uptake transporters known as organic anion transporting polypeptides (OATPs) play an important role in disposition of certain drugs. In the small intestine, OATPs facilitate absorption of certain medications, and inhibition of this transport system may cause reduced bioavailability. Grapefruit juice (300 or 1200 ml) produced lower mean maximum plasma concentration and AUC of fexofenadine compared to when the drug was taken with the same volume of water. The mean maximum plasma concentration of fexofenadine was 436 ng/mL when the drug was taken with 300 ml of water compared to the mean maximum plasma concentration of 233 ng/mL when the medication was administered with 300 ml of grapefruit juice. The reduction of maximum plasma concentration was more striking in the presence of 1200 ml of grapefruit juice. Similar reductions were also observed in AUC of fexofenadine. Because fexofenadine is a zwitter ion, it has high solubility in aqueous medium over a wide pH range, and it is unlikely that acidic pH of grapefruit juice could reduce the solubility significantly. Therefore, authors postulated that ingredients of grapefruit juice have prolonged inhibitory effects on inherent activity of intestinal OATP-A activity, thus causing a clinically significant effect of reduced bioavailability of fexofenadine *(125)*. Common interactions between drugs and fruit juices are given in table 3.

### *10.2. Interaction of Drugs with Pomelo Juice, Pomegranate Juice, Orange Juice and Cranberry Juice*

Pomelo, closely related to grapefruit, interacts with cyclosporine. In a study involving 12 healthy male volunteers, 200 mg dose of cyclosporine was administered with 240 ml of pomelo juice. The average maximum concentration of cyclosporine in blood was 1494 ng/mL when cyclosporine was administered with pomelo juice compared to an average concentration of 1311 ng/mL when cyclosporine was administered along with water. However, intake of cyclosporine along with cranberry juice had no effect on bioavailability of cyclosporine. Authors concluded that pomelo juice increased bioavailability of cyclosporine possibly by inhibiting CYP3A4, P-glycoprotein activity or both *(126)*. Pomelo juice also increases the blood level of tacrolimus. Egashira et al. reported a case where a 44-year-old male with a renal transplant showed therapeutic concentrations of tacrolimus 3 months after transplant. His tacrolimus concentration varied between 8 and 10 ng/mL, and in one occasion, he showed a blood tacrolimus concentration of 25.2 ng/mL. There was no change of tacrolimus dose. The patient consumed about 100 gm of pomelo grown in his garden. Pomelo contains furanocoumarins and was considered responsible for increased bioavailability of tacrolimus in this patient *(127)*.

Components of pomegranate juice are potent inhibitors of CYP3A4. Hidaka et al. demonstrated that incubation of pomegranate juice (5% by vol) with human liver microsome resulted in 1.8% residual activity of CYP3A for converting carbamazepine

**Table 3**
**Common Fruit Juice–Drug Interactions**

| *Fruit juice* | *Interacting drug* | *Comments* |
|---|---|---|
| Grapefruit juice | Felodipine | A 430% increase in maximum plasma level of felodipine |
| | Nitrendipin | Bioavailability increased by 100% |
| | Pranidipine | Bioavailability increased by 73% |
| | Nimodipine | Bioavailability increased by 50% |
| | Cyclosporine | Increased blood level of cyclosporine |
| | Tacrolimus | Increased of trough concentration delayed by 1 week |
| | Diazepam | Increased plasma concentration |
| | Traizolam/midazolam | Increased plasma concentration |
| | Carbamazepine | Increased plasma concentration |
| | Saquinavir | Increased plasma concentration |
| | Simvastatin | AUC increased by 3.6-fold |
| | Atorvastatin | AUC increased by 83% |
| | Pitavastatin | AUC increased by only 13% |
| | Fexofenadine | Plasma level reduced from 436 ng/mL (300 ml of water) to 233 ng/mL (300 ml of grapefruit juice) |
| | Digoxin | No clinically significant interaction |
| Pomelo juice | Cyclosporine | Increased concentration of cyclosporine |
| | Tacrolimus | Increased blood level of tacrolimus |
| Pomegranate juice | Carbamazepine | Increased AUC of carbamazepine by 1.5-fold |
| Cranberry juice | Warfarin | INR >50 in a patient due to interaction of warfarin with cranberry juice. The patient died. Increased INR in two other patients |
| Orange juice | Pravastatin | Increased AUC of pravastatin |
| | Simvastatin | No clinically significant interaction |
| | Celiproolo | AUC reduced by 83% |
| Seville orange juice | Felodipine | AUC of felodipine increased by 76% |

AUC, area under the curve; INR, international normalization ratio.

to carbamazepine 10, 11-epoxide. The residual activity of CYP3A after 30-min incubation with pomegranate juice was 47.5% compared to 38.3% residual activity when treated with grapefruit juice. Moreover in comparison with water, the AUC of carbamazepine was approximately 1.5-fold higher with pomegranate juice (2 ml) that was orally given 1 h before oral administration of carbamazepine in rats *(128)*.

Cranberry juice is a potent inhibitor of human and rat CYP3A, and oxidation of nifedipine can be decreased in vitro in the presence of cranberry juice. Moreover, in vivo experiments with rats demonstrated that AUC of nifedipine was 1.6-fold higher when 2 ml of cranberry juice was intradudenally introduced 30 min prior to the intraduodenal administration of nifedipine (30 mg/kg). These data suggest that cranberry juice inhibits the function of enteric CYP3A *(129)*. Interaction between cranberry juice and warfarin has also been reported. After a chest infection, a man had a poor appetite and had nothing but cranberry juice for 2 weeks as well as his regular drugs (digoxin, phenytoin and warfarin). Six weeks after starting with cranberry juice, he was admitted in the hospital with an INR of over 50. He died of gastrointestinal and pericardial hemorrhage. Cranberry juice contains flavonoids that can inhibit cytochrome P 450, and warfarin is predominately metabolized by CYP2C19. Authors concluded that patients taking warfarin should not consume cranberry juice *(130)*. Another case report indicated elevated INR in a patient on warfarin 2 weeks after the patient started drinking cranberry juice. Subsequent symptoms included postoperative bleeding problem *(131)*.

Orange juice increased AUC (0–4 h) of pravastatin (a 3-hydroxy-3-methyl glutaryl CoA reductase inhibitor) in healthy volunteers when administered orally. Orange juice also increased AUC of pravastatin in rats when the drug was given orally but demonstrated no effect when pravastatin was administered intravenously. However, orange juice had no effect on bioavailability of simvastatin another 3-hydroxy-3-methyl glutaryl CoA reductase inhibitor *(132)*. Orange juice also enhances aluminum absorption from antacid preparation. In a study involving 15 normal subjects when antacid "Aludrox" was taken with orange juice, there was an approximately 10-fold increase in 24 h urinary excretion of aluminum. However, milk had no effect on aluminum absorption *(133)*. Several published reports also indicated reduced bioavailability of several drugs when taken orally along with orange juice. Kamath et al. reported that orange juice and apple juice significantly reduced oral bioavailability of fexofenadine in rats (31 and 22%, respectively). This may be related to inhibition of OATPs *(134)*. It was discussed earlier that grapefruit juice also reduced oral bioavailability of fexofenadine *(134)*. Orange juice substantially reduced the bioavailability of celiprolol, a β-adrenergic receptor blocking agent used in the treatment of hypertension. In a study involving 10 healthy volunteers, orange juice (200 ml) reduced the mean peak plasma concentration of celiprolol by 89% (330.0 ng/mL in control and 35.5 ng/mL in subjects taking orange juice). The AUC was also reduced by 83% due to intake of orange juice. The authors concluded that this interaction is likely to have clinical importance *(135)*. Another report indicated that orange juice reduced bioavailability of clofazimine (used in treatment of multidrug-resistant *Mycobacterium tuberculosis* and leprosy) in humans. Aluminum-based antacids also reduced bioavailability of clofazimine, whereas a fatty meal increased the bioavailability *(136)*.

Sour orange also known as bitter or Seville orange is different from a sweet orange. Malhotra et al. reported that Seville orange juice increased AUC of felodipine by 76%,

whereas grapefruit juice increased the AUC by 93%. The concentration of bergamottin and 6,7-dihydoxybergamottin were 5 and 36 μmol/L in Seville orange juice, whereas concentrations of bergamottin and 6,7-dihydoxybergamottin were 16 and 23 μmol/L in grapefruit juice. The authors concluded that Seville orange juice and grapefruit juice interact with felodipine by a common mechanism *(137)*.

### *10.3. Drug Interactions with Piperine, Major Constituent of Black Pepper*

Piperine, a major constituent of black and long pepper, has been reported to act as bioavailability enhancer of several drugs by inhibiting drug metabolism and by increasing oral absorption. Bharadwaj et al. reported that piperine inhibits both the drug transported P-glycoprotein and the major drug metabolizing enzyme CYP3A4. Therefore, dietary piperine could affect plasma concentrations of drugs that are substrates of P-glycoprotein or CYP3A4 enzyme especially if administered orally *(138)*. Animal study using rat model has demonstrated that piperine enhanced bioavailability of beta-lactam antibiotics amoxicilline and cefotaxime *(139)*. Piperine also enhanced the effect of pentobarbital-induced sleeping time in rats due to higher blood and brain levels of pentobarbital in the presence of piperine *(140)*.

Administration of piperine significantly increased plasma rifampicin concentrations in patients with pulmonary tuberculosis *(141)*. Rifampicin is a substrate of P-glycoprotein, and inhibition of P-glycoprotein by piperrine is a possible mechanism. In a study involving six human subjects, piperine (20 mg daily dose) increased both maximum serum concentration and AUC of propranolol. Comparable effects were also observed with theophylline *(142)*. Velpandian et al. reported increased AUC of phenytoin in rats in the presence of piperine. Similar effect was also observed when phenytoin was administered in rats intravenously indicating that piperine also blocked metabolism of phenytoin. Similar effect was also observed with human subjects *(143)*. In another study, 20 mg dose of piperine increased AUC and maximum plasma concentrations of phenytoin compared to controls. The elimination rate constant was also affected *(144)*.

## 11. CONCLUSIONS

The use of herbal supplements in the United States is steadily growing and raises concern about safety, efficacy and how they affect patient care. The direct risks of using herbal supplements include hypertension, prolonged bleeding and the potential for drug–herb interactions. These potential drug interactions are of particular concern in patients undergoing anesthesia *(145)*. Interaction between a drug and herbal supplement may even cause death *(81)*. Moreover, interactions between fruit juices and drugs also have important clinical significance. Such interactions may also lead to unusually low or high concentration of a therapeutic drug in a patient who demonstrated stable therapeutic level of the same drug before. Clinical laboratory professionals need to be aware of such interactions. In many cases, patients consider herbal supplements as natural and thus safe and do not inform their clinicians about their self-medication with such herbal supplements. Therefore, if a laboratory professional is suspicious of an unusual concentration of a therapeutic drug as a result of a potential drug–herb interaction, the individual should alert the ordering physician.

## REFERENCES


1. Mahady GB. Global harmonization of herbal health claims. J Nutr 2001; 131: 1120S–1123S.
2. Honda K, Jacobson JS. Use of complementary and alternative medicine among United States adults: The influence of personality, coping strategies, and social support. Prev Med 2005; 40: 46–53.
3. Kelly JP, Kaufman DW, Kelley K, Rosenberg L, Mitchell AA. Use of herbal/natural supplements according to racial/ethnic group. J Altern Complement Med 2006; 12: 555–561.
4. Gulla J, Singer AJ, Gaspari R. Herbal use in ED patients. Acad Emerg Med 2001; 8: 450.
5. Haller CA, Benowitz NL. Adverse cardiovascular and central nervous system events associated with dietary supplements containing ephedra alkaloids. N Engl J Med 2000; 343: 1833–1838.
6. Gow PJ, Connelly NJ, Hill RL, Crowley P, Angus PW. Fatal fulminant hepatic failure induced by a natural therapy containing kava. Med J Aust 2003; 178: 442–442.
7. Klepser TB, Klepser ME. Unsafe and potentially safe herbal therapies. Am J Health Syst Pharm 1999; 56: 125–141.
8. Rousseaux GC, Schachter H. Regulatory issues concerning the safety, efficacy and quality of herbal remedies. Birth Defects Res B Dev Reprod Toxicol 2003; 68: 505–510.
9. Brownie S. The development of the US and Australian dietary supplement regulations: What are the implications for product quality? Complement Ther Med 2005; 13: 191–198.
10. Cho M, Ye X, Dobs A, Cofrancesco J. Prevalence of complementary and alternative medicine use among HIV patients for perceived lipodystrophy. J Altern Complement Med 2006; 12: 475–482.
11. Schwarz JT, Cupp MJ. St John's Wort in Toxicology and Clinical Pharmacology of Herbal Products (Cupp MJ, ed.). Totowa: Humana Press, 2000; pp 67–78.
12. Kubin A, Wierrani F, Burner U, Alth G, Grunberger W. Hypericin-the facts about a controversial agent. Curr Pharm Des 2005; 11: 233–253.
13. Bove GM. Acute neuropathy after exposure to sun in a patient treated with St. John's wort. Lancet 1998; 352: 1121–1122.
14. O'Breasail AM, Argouarch A. Hypomania and St. John's wort. Can J Psychiatry 1998; 43: 746–747.
15. Demiroglu YZ, Yeter TT, Boga C, Ozdogu H, et al. Bone marrow necrosis: A rare complication of herbal treatment with Hypericum perforatum (St. John's wort). Acta Medica (Hradec Kralove) 2005; 48: 91–94.
16. Pal D, Mitra AK. MDR and CYP3A4-mediated drug-herbal interactions. Life Sci 2006; 78: 2131–2145.
17. Landrum-Michalets E. Update: Clinically significant cytochrome P450 drug interactions. Pharmacotherapy 1998; 18: 84–112.
18. Krusekopf S, Roots I. St. John's wort and its constituent hyperforin concordantly regulate expression of genes encoding enzymes involved in basic cellular pathways. Pharmacogenet Genomics 2005; 15: 817–829.
19. Hu Z, Yang X, Ho PC, Chan SY, et al. Herb-drug interactions: A literature review. Drugs 2005; 65: 1239–1282.
20. Wentworth JM, Agostini M, Love J, Schwabe JW, Chatterjee VK. St John's wort, a herbal antidepressant. Activates the steroid X receptor. J Endocrinol 2000; 166: R11–R16.
21. Raffa R. Screen of receptor and uptake site activity of hypericin components of St John's wort reveal σ receptor binding. Life Sci 1998: 62: PL265–PL270.
22. Madabushi R, Frank B, Drewelow B, Derendorf H, Butterweck V. Hyperforin in St. John's wort drug interactions. Eur J Clin Pharmacol 2006; 62: 225–233.
23. Martin-Facklam M, Rieger K, Riedel KD, Burhenne J, et al. Undeclared exposure of St. John's wort in hospitalized patients. Br J Clin Pharmacol 2004; 58: 437–441.
24. Zhou S, Chan E, Pan SQ, Huang M, Lee EJ. Pharmacokinetic interactions of drugs with St. John's wort. J Psychopharmacol 2004; 18: 262–276.
25. Nebel A, Schneider BJ, Kroll DJ. Potential metabolic interaction between St. John's wort and theophylline. Ann Pharmacother 1999; 33: 502.
26. Karyekar CS, Eddington ND, Dowling TC. Effect of St. John's wort extract on intestinal expression of cytochrome P4501A2: Studies with LS180 cells. J Postgrad Med 2002; 48: 97–100.

27. Morimoto T, Kotegawa T, Tsutsumi K, Ohtani Y, et al. Effect of St. John's wort on the pharmacokinetics of theophylline in healthy volunteers. J Clin Pharmacol 2004; 44: 95–101.
28. Barone GW, Gurley BJ, Ketel BL, Abul-Ezz SR. Herbal supplements; a potential for drug interactions in transplant recipients. Transplantation 2001; 71: 239–241.
29. Izzo AA. Herb-drug interactions: An overview of the clinical evidence. Fundam Clin Pharmacol 2005; 19: 1–16.
30. Bauer S, Stromer E, Johne A, Kruger H, et al. Altercation of cyclosporine A pharmacokinetics and metabolism during treatment with St. John's wort in renal transplant patients. Br J Clin Pharmacol 2003; 55: 203–211.
31. Alscher DM, Klotz U. Drug interaction of herbal tea containing St. John's wort with cyclosporine [Letter]. Transpl Int 2003; 16: 543–544.
32. Mai I, Bauer S, Perloff ES, Johne A, et al. Hyperforin content determines the magnitude of the St. John's wort-cyclosporine drug interaction. Clin Pharmacol Ther 2004; 76: 330–340.
33. Hebert MF, Park JM, Chen YL, Akhtar S, Larson AM. Effects of St John's wort (Hypericum perforatum) on tacrolimus pharmacokinetics in healthy volunteers. J Clin Pharmacol 2004; 44: 89–94.
34. Bolley R, Zulke C, Kammerl M, Fischereder M, Kramer BK. Tracrolimus induced nephrotoxicity unmasked by induction of CYP3A4 system with St. John's wort. [Letter]. Transplantation 2002; 73: 1009.
35. Mai I, Stormer E, Bauer S, Kruger H, et al. Impact of St. John's wort treatment on the pharmacokinetics of tacrolimus and mycophenolic acid in renal transplant patients. Nephrol Dial Transplant 2003; 18: 819–822.
36. Johne A, Brockmoller J, Bauer S, Maurer A, et al. Pharmacokinetic interaction of digoxin with an herbal extract from St John's wort (Hypericum perforatum). Clin Pharmacol Ther 1999; 66: 338–345.
37. Durr D, Stieger B, Kullak-Ublick GA, Rentsck KM, et al. St John's wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. Clin Pharmacol Ther 2000; 68: 598–604.
38. Muller SC, Uehleke B, Woehling H, Petzsch M, et al. Effect of St. John's wort dose and preparation on the pharmacokinetics of digoxin. Clin Pharmacol Ther 2004; 75: 546–557.
39. van den Bout-van den Beukel CJ, Koopmans PP, van der Ven AJ, De Smet PA, Burtger DM. Possible drug metabolism interactions of medicinal herbs with antiretroviral agents. Drug Metab Rev 2006; 38: 477–514.
40. Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM, Fallon J. Indinavir concentrations and St. John's wort. Lancet 2000; 355: 547–548.
41. Busti AJ, Hall RJ, Margolis DM. Atazanavir for the treatment of human immunodeficiency virus infection. Pharmacotherapy 2004; 24: 1732–1747.
42. Cvetkovic RS, Goa KL. Lopinavir/ritonavir: A review of its use in the management of HIV infection. Drugs 2003; 63: 769–802.
43. Johne A, Schmider J, Brockmoller J, Stadelman AM, et al. Decreased plasma levels of amitriptyline and its metabolites on comedication with an extract from St. John's wort (Hypericum porforatum). J Clin Psychopharmacol 2002; 22: 46–54.
44. Wang Z, Gorski JC, Hamman MA, Huang SM, et al. The effects of St. John's wort (Hypericum perforatum) on human cytochrome P450 activity. Clin Pharmacol Ther 2001; 70: 317–326.
45. Wang Z, Hamman MA, Huang SM, Lesko LJ, Hall SD. Effect of St. John's wort on the pharmacokinetics of fexofenadine. Clin Pharmacol Ther 2002; 71: 411–420.
46. Eich-Hochli D, Oppliger R, Golay KP, Baumann P, Eap CB. Methadone maintenance treatment and St. John's wort - a case study. Pharmacopsychiatry 2003; 36: 35–37.
47. Smith P. The influence of St. John's wort on the pharmacokinetics and protein binding of imatinib mesylate. Pharmacotherapy 2004; 24: 1hbox1508–1514.
48. Mathijssen RH, Verweij J, de Bruijn P, Loos WJ, Sparreboom A. Effects of St. John's wort on irinotecan metabolism. J Natl Cancer Inst 2002; 94: 1247–1249.
49. Thummel KE, Wilkinson GR. In vitro and in vivo drug interactions involving human CYP3A. Annu Rev Pharmacol Toxicol 1998; 38: 389–430.
50. Guengerich FP. Oxidation of 17-ethynylestradiaol by human liver cytochrome P 450. Mol Pharmacol 1998; 33: 500–508.

51. Hill SD, Wang Z, Huang SM, Hamman MA, et al. The interaction between St. John's wort and oral contraceptives. Clin Pharmacol Ther 2003; 74: 525–535.
52. Murphy PA, Kern SE, Stanczyk FZ, Westhoff CL. Interaction of St. John's wort with oral contraceptives: Effects on the pharmacokinetics of norethindrone and ethinyl estradiol, ovarian activity and breakthrough bleeding. Contraception 2005; 71: 4102–4108.
53. Sugimoto K, Ohmori M, Tsuruoka S, Nishiki K, et al. Different effect of St. John's wort on the pharmacokinetics of simvastatin and pravastatin. Clin Pharmacol Ther 2001; 70:518–524.
54. Wang LS, Zhou G, Zhu B, Wu J, et al. St. John's wort induces both cytochrome P450 3A4 catalyzed sulfoxidation and 2 C19 dependent hydroxylation of omeprazole. Clin Pharmacol Ther 2004; 75: 191–197.
55. Tannergren C, Engman H, Knutson L, Hedeland M, et al. St John's wort decreases the bioavailability of R and S-verapamil through induction of the first pass metabolism. Clin Pharmacol Ther 2004; 75: 298–309.
56. Burstein AH, Horton RL, Dunn T, Alfaro RM, et al. Lack of effect of St. John's wort on carbamazepine pharmacokinetics in healthy volunteers. Clin Pharmacol Ther 2000; 68: 605–612.
57. Szegedi A, Kohnen R, Dienel A, Kieser M. Acute treatment of moderate to severe depression with hypericum extract WS 5570 (St. John's wort): Randomized controlled double blind non-inferiority trial versus paroxetine. BMJ 2005; 330 (7490): 503
58. Gordon JB. SSRIs and St. John's wort: Possible toxicity? [Letter]. Am Fam Physician 1998; 57: 950–953.
59. Draves AH, Walker SE. Analysis of hypercin and pseudohypercin content of commercially available St. John's wort preparation. Can J Clin Pharmacol 2003; 10: 114–118.
60. Arold G, Donath F, Maurer A, Diefenbach K, et al. No relevant interaction with alprazolam, caffeine, tolbutamide and digoxin by treatment with a low hyperforin St. John's wort extract. Planta Med 2005; 71: 331–337.
61. Ang CY, Hu L, Heinze TM, Cui Y, et al. Instability of St. John's wort (Hypericum perforatul L) and degradation of hyperforin in aqueous solutions and functional beverage. J Agric Food Chem 2004; 52: 6156–6164.
62. Bauer S, Stromer E, Graubaum HJ, Roots I. Determination of hyperforin, hypercin and pseudohypercin in human plasma using high performance liquid chromatography analysis with fluorescence and ultraviolet detection. J Chromatogr B Biomed Sci Appl 2001; 765: 29–35.
63. Pirker R, Huck CW, Bonn GK. Simultaneous determination of hypercin and hyperforin in human plasma using liquid-liquid extraction, high performance liquid chromatography and liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2002; 777: 147–153.
64. Mauri P, Pietta P. High performance liquid chromatography/electrospray mass spectrometry of Hypericum perforatum extracts. Rapid Commun Mass Spectrom 2000; 14: 95–99.
65. Ruckert U, Eggenreich K, Wintersteiger R, Wurglics M, et al. Development of a high performance liquid chromatographic method with electrochemical detection for the determination of hyperforin. J Chromatogr A 2004; 1041: 181–185.
66. Greenblatt DJ, von Moltke LL. Interaction of warfarin with drugs, natural substances and food. J Clin Pharmacol 2005; 45: 127–132.
67. Jiang X, Williams KM, Liauw WS, Ammit AJ, et al. Effect of St. John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects. Br J Clin Pharmacol 2004; 57: 592–599.
68. Heck AM, DeWitt BA, Lukes AL. Potential interactions between alternative therapies and warfarin. Am J Health Syst Pharm 2000; 57: 1221–1227.
69. Lambert JP, Cormier A. Potential interaction between warfarin and boldo-fenugreek. Pharmacotherapy 2002; 21: 509–512.
70. Tam LS, Chan Tym Leung WK, Critchley JA. Warfarin interaction with Chinese traditional medicines: Danshen and methyl salicylate medicated oil. Aust N Z J Med 1995; 25: 238.
71. Yu CM, Chan JC, Sanderson JE. Chinese herbs and warfarin potentiation by danshen. J Intern Med 1997; 25: 337–339.
72. Sunter WH. Warfarin and garlic. Pharm J 1992; 246: 772.

73. Piscitelli SC, Brustein AH, Welden N, Gallicano KD, Falloon J. The effect of garlic supplements on the pharmacokinetics of saquinavir. Clin Infect Dis 2002; 34: 234–238.
74. Macan H, Uykimpang R, Alconcel M, Takasu J, et al. Aged garlic extract may be safe for patients on warfarin therapy. J Nutr 2006; 136 (3 Suppl) 793S–795S.
75. Lee FC, Ko JH, Park JK, Lee JS. Effects of Panax ginseng on blood alcohol clearance in man. Clin Exp Pharmacol Physiol 1987; 14: 543–546.
76. Koo MW. Effects of ginseng on ethanol induced sedation in mice. Life Sci 1999; 64: 153–160.
77. Jones BD, Runikis AM. Interaction of ginseng with phenelzine. J Clin Psychopharmacol 1987; 3: 201–202.
78. Yin OQ, Tomlinson B, Waye MM, Chow AH, Chow MS. Pharmacogenetics and herb-drug interactions: Experience with ginkgo. Pharmacogenetics 2004; 14: 841–850.
79. Mauro VF, Mauro LS, Kleshinski JF, Khuder SA, et al. Impact of ginkgo biloba on the pharmacokinetics of digoxin. Am J Ther 2003; 10: 247–252.
80. Yang CY, Chao PD, Hou YC, Tsai SY, et al. Marked decrease of cyclosporine bioavailability caused by coadministration of ginkgo and onion. Food Chem Toxicol 2006; 44: 1572–1578.
81. Kupiec T, Raj V. Fetal seizures due to potential herb-drug interactions with ginkgo biloba. J Anal Toxicol 2005; 29: 755–758.
82. Granger AS. Ginkgo biloba precipitating epileptic seizures. Age Ageing 2001; 30: 523–525.
83. Kubota Y, Kobayashi K, Tanaka N, Nakamura K, et al. Interaction of ginkgo biloba extract (GBE) with hypotensive agent nicardipine in rats. In Vivo 2003; 17: 409–412.
84. Sugiyama T, Kubota Y, Shinozuka K, Yamada S, et al. Ginkgo biloba extract modifies hypoglycemic action of tolbutamide via hepatic cytochrome P450 mediated mechanism in aged rats. Life Sci 2004; 75: 1113–1132.
85. Escher M, Desmeules J. Hepatitis associated with kava, a herbal remedy. BMJ 2001; 322: 139.
86. Humberston CL, Akhtar J, Krenzelok EP. Acute hepatitis induced by kava-kava. J Toxicol Clin Toxicol 2003; 41: 109–113.
87. Stickel F, Baumuller HM, Seitz K, Vasilakis D, et al. Hepatitis induced by kava (Piper methysticum rhizoma). J Hepatol 2003; 39: 62–67.
88. Clouatre DL. Kava Kava: Examining new reports of toxicity. Toxicol Lett 2004; 150: 85–96.
89. Anke J, Ramzan I. Pharmacokinetic and pharmacodynamic drug interactions with Kava (Piper methysticum Forst.f). J Ethnopharmacol 2004; 93: 153–160.
90. Russmann S, Lauterburg BH, Barguil Y, Choblet E, et al. Traditional aqueous kava extracts inhibit P450 1A2 in humans: Protective effect against environmental carcinogens? [Letter]. Clin Pharmacol Ther 2005; 77: 453–454.
91. Almeida JC, Grimsley EW. Coma from the health food store: Interaction between Kava and alprazolam. Ann Intern Med 1996; 125: 940–941.
92. Herberg KW. Effect of kava special extract WS 1490 combined with ethyl alcohol on safety relevant performance parameters [Article in German]. Blutalkohol 1993; 30: 96–105.
93. Anke J, Fu S, Razman I. Kava lactones fail to inhibit alcohol degydrogenase in vitro. Phytomedicine 2006; 13: 192–195.
94. Fujita K. Food drug interactions via human cytochrome P450 3A (CYP3A). Drug Metabol Drug Interact 2004; 20: 195–217.
95. Evans AM. Influence of dietary components on the gastrointestinal metabolism and transport of drugs. Ther Drug Monit 2000; 22: 131–136.
96. Walter-Sack I, Klotz U. Influence of diet and nutritional status on drug metabolism. Clin Pharmacokinet 1996; 31: 47–64.
97. Fegan TC, Walle T, Oexmann MJ, Walle UK, et al. Increased clearance of propranolol and theophylline by high-protein compared with high carbohydrate diet. Clin Pharmacol Ther 1987; 41: 402–406.
98. Bailey DG, Spence JD, Munoz C, Arnold JM. Interaction of citrus juices with felodipine and nifedipine. Lancet 1991; 337: 268–269.
99. Saito M, Hirata-Koizumi M, Matsumoto M, Urano T, Hasegawa R. Undesirable effects of citrus juice on the pharmacokinetics of drugs: Focus on recent studies. Drug Saf 2005; 28: 677–694.

100. Uno T, Ohkubo T, Sugawara K, Higashiyama A, et al. Effects of grapefruit juice on the stereoselective disposition of nicardipine in humans: Evidence for dominant presystematic elimination at the gut site. Eur J Clin Pharmacol 2000; 56: 643–649.
101. Dahan A, Altman H. Food-drug interaction: Grapefruit juice augments drug bioavailability-mechanism, extent and relevance. Eur J Clin Nutr 2004; 58: 1–9.
102. Tian R, Koyabu N, Takanaga H, Matsuo H, et al. Effects of grapefruit juice and orange juice on the intestinal efflux of P-glycoprotein substrates. Pharm Res 2002; 19: 802–809.
103. He K, Iyer KR, Hayes RN, Sinz MW, et al. Inactivation of cytochrome P 450 3A4 by bergamottin, a component of grapefruit juice. Chem Res Toxicol 1998; 11: 252–259.
104. Girennavar B, Poulose SM, Jayaprakasha GK, Bhat NG, Patil BS. Furocpumarines from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes. Bioorg Med Chem 2006; 14: 2602–2612.
105. De Casto WV, Mertens-Talcott S, Rubner A, Butterweck V, Dernedorf H. Variation of flavonoids and furanocoumarines in grapefruit juice: A potential source of variability in grapefruit juice-drug interaction studies. J Agric Food Chem 2006; 54: 249–255.
106. Paine MF, Widmer WW, Hart HL, Pusek SN. A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice-felodipine interaction. Am J Clin Nutr 2006; 83: 1097–1105.
107. Lundahl U, Regardh GC, Edger B, Johnsson G. The interaction effect of grapefruit juice is maximal after the first glass. Eur J Clin Pharmacol 1998; 54: 75–81.
108. Rogers JD, Zhou J, Liu L, Amin RD, et al. Grapefruit juice has minimal effect on plasma concentrations of lovastatin derived 3-hydroxy 3-methylglutaryl coenzyme A reductase inhibitors. Clin Pharmacol Ther 1999; 66: 358–366.
109. Lilja JJ, Kivisto KT, Neuvonen PJ. Duration of effect of grapefruit juice on the pharmacokinetics of the CYP3A4 substrate simvastatin. Clin Pharmacol Ther 2000; 68: 384–390.
110. Bistrup C, Nielsen FT, Jeppesen UE, Dieperink H. Effect of grapefruit juice on Sandimmun Neoral absorption among stable renal allograft recipients. Nephrol Dial Transplant 2001; 16: 373–377.
111. Johnston PE, Milstone A. Probable interaction of bergamotting and cyclosporine in a lung transplant recipient. [Letter to the editor] Transplantation 2005; 27: 746.
112. Fukatsu S, Fukudo M, Masuda S, Yano I, et al. Delayed effect of grapefruit juice on pharmacokinetics and pharmacodynamics of tacrolimus in a living donar transplant recipient. Drug Metab Pharmacokinet 2006; 21: 122–125.
113. Garg SK, Kumar N, Bhargava VK, Prabhakar SK. Effect of grapefruit juice on carbamazepine bioavailability in patients with epilepsy. Clin Pharmacol Ther 1998; 64: 286–288.
114. Kumar N, Garg SK, Prabhakar S. Lack of pharmacokinetic interaction between grapefruit juice and phenytoin in healthy male volunteers and epileptic patients. Methods Find Exp Clin Pharmacol 1999; 21: 629–632.
115. Kupferschmidt HH, Fattinger KE, Ha HR, Follath F, Krahenbuhl S. Grapefruit juice enhances the bioavailability of HIV protease inhibitor saquinavir in man. Br J Clin Pharmacol 1998; 45: 355–359.
116. Demarles D, Gillotin G, Bonaventure-Paci S, Vincent I, et al. Single dose pharmacokinetics of amprenavir co administered with grapefruit juice. Antimicrob Agents Chemother 2002; 46: 1589–1590.
117. Penzak SR, Acosta EP, Turner M, Edward DJ, et al. Effect of Seville orange juice and grapefruit juice on indinavir pharmacokinetics. J Clin Pharmacol 2002; 42: 1165–1170.
118. Fuhr U, Muller-Peltzer H, Lopez-Rojas P, Junemann M, et al. Effects of grapefruit juice and smoking on verapamil concentration in steady state. Eur J Clin Pharmacol 2002; 58: 45–53.
119. Ozdemir M, Aktan Y, Boydag BS, Cingi MI, et al. Interaction between grapefruit juice and diazepam in man. Eur J Drug Metab Pharmacokinet 1998; 23: 55–59.
120. Lilja JJ, Neuvonen M, Neuvonen PJ. Effects of regular consumption of grapefruit juice on the pharmacokinetics of simvastatain. Br J Clin Pharmacol 2004; 58: 56–60.
121. Ando H, Tsuruoka S, Yanagihara H, Sugimoto K, et al. Effects of grapefruit juice on the pharmacokinetics of pitavastatin and atorvastatin. Br J Clin Pharmacol 2005; 60: 494–497.
122. Becquemont L, Verstuyft C, Kerb R, Brinkmann U, et al. Effect of grapefruit juice on digoxin pharmacokinetics in humans. Clin Pharmacol Ther 2001; 70: 311–316.

123. Offman EM, Freeman DJ, Dresser GK, Munoz C, et al. Red wine-cisapride interaction: Comparison with grapefruit juice. Clin Pharmacol Ther 2001; 70: 17–23.
124. Gubbins PO, McConnell SA, Gurley BJ, Fincher TK, et al. Influence of grapefruit juice on the systematic availability of itraconazole oral solution in healthy adult volunteers. Pharmacotherapy 2004; 24: 460–467.
125. Dresser GK, Kim RB, Bailey DG. Effect of grapefruit juice volume on the reduction of fexofenadine bioavailability: Possible role of anion transporting polypeptides. Clin Pharmacol Ther 2005; 77: 170–177.
126. Grenier J, Fradetta C, Morelli G, Merritt GJ, et al. Pomelo juice but not cranberry juice affects the pharmacokinetics of cyclosporine in humans. Clin Pharmacol Ther 2006; 79: 255–262.
127. Egashira K, Fukuda E, Onga T, Yogi Y, et al. Poemelo-induced increase in the blood level of tacrolimus in a renal transplant patient [Brief Report]. Transplantation 2003; 75: 1057.
128. Hidaka M, Okumura M, Fujita K, Ogikubo T, et al. Effects of pomegranate juice on human cytochrome P450 3A (CYP3A) and carbamazepine pharmacokinetics in rats. Drug Metab Dispos 2005; 33: 644–648.
129. Uesawa Y, Mohri K. Effects of cranberry juice on nifedipine pharmacokinetics in rats. J Pharm Pharmacol 2006; 58: 1067–1072.
130. Suvarna R, Pirmohamed M, Henderson L. Possible interaction between warfarin and cranberry juice. BMJ 2003; 327: 1454.
131. Grant P. Warfarin and cranberry juice: An interaction? J Heart Valve Dis 2004; 13: 25–26.
132. Koitabashi Y, Kumai T, Matsumoto N, Watanabe M, et al. Orange juice increased bioavailability of paravastin, 3-hydroxy-3-methylglutaryl CoA reductase inhibitor in rats and healthy human subjects. Life Sci 2006; 78: 2852–2859.
133. Fairweather-Tait S, Hickson K, McGaw B, Reid M. Orange juice enhances aluminum absorption from antacid preparation. Eur J Clin Nutr 1994; 48: 71–73.
134. Kamath AV, Yao M, Zhang Y, Chong S. Effect of fruit juices on the oral bioavailability of fexofenadine. J Pharm Sci 2005; 94: 233–239.
135. Lilja JJ, Juntti-Patinen L, Neuvonen PJ. Orange juice substantially reduced the bioavailability of the beta-adrenergic blocking agent celiprolol. Clin Pharmacol Ther 2004; 75: 184–190.
136. Nix DE, Adam RD, Auclair B, Krueger TS, et al. Pharmacokinetics and relative bioavailability of clofazimine in relation to food, orange juice and antacid. Tuberculosis (Edinb) 2004; 84: 365–373.
137. Malhotra S, Bailey DG, Paine MF, Watkins PB. Seville orange juice-felodipine interaction: Comparison with dilute grapefruit juice and involvement of furocoumarins. Clin Pharmacol Ther 2001; 69: 14–23.
138. Bhardwaj RK, Glaser H, Becquemont L, Klotz U, et al. Piperine, a major constituent of black pepper inhibits human P-glycoprotein and CYP3A4. J Pharmacol Exp Ther 2002; 302: 645–650.
139. Hiwale AR, Dhuley JN, Naik SR. Effect of co-administration of piperine on pharmacokinetics of beta lactum antibiotics in rats. Indian J Exp Biol 2002; 40: 277–281.
140. Majumdar AM, Dhuley JN, Deshmukh VK, Raman PH, et al. Effect of piperine on pentobarbitone induced hypnosis in rats. Indian J Exp Biol 1990; 28: 486–487.
141. Zutshi RK, Singh R, Zutshi U, Johri RK, Atal CK. Influence of piperine on rifampicin blood levels in patients of pulmonary tuberculosis. J Assoc Physicians India 1985; 33: 223–224.
142. Bano G, Raina RK, Zutshi U, Bedi KL, et al. Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers. Eur J Clin Pharmacol 1991; 41: 615–617.
143. Velpandian T, Jasuja R, Bhardwaj RK, Jaiswal J, Gupta SK. Piperine in food: Interference in the pharmacokinetics of phenytoin. Eur J Drug Metab Pharmacokinet 2001; 26: 241–247.
144. Pattanaik S, Hota D, Prabhakar S, Kharbanda P, Pandhi P. Effect of piperine on the steady state pharmacokinetics of phenytoin in patients with epilepsy. Phytother Res 2006; 20: 683–686.
145. Messina BA. Herbal supplements: Facts and myths-talking to your patients about herbal supplements. J Perianesth Nurs 2006; 21: 268–278.

# 14 Toxic Element Testing with Clinical Specimens

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***Contents***



**Summary**

Elements are the basis of all things. However, human exposure to significant amounts of some elements can lead to adverse health effects including death. Laboratory testing can help identify unrecognized exposures as well as monitor-associated decontamination efforts. Regular testing is also important to identify exposures in populations that are at high risk for exposure to a specific toxic element. For example, aluminum is important to monitor in dialysis patients. Lead is important to monitor in children that live in areas in which environmental lead contamination is prevalent. Mercury is important to monitor in dental workers and individuals for whom predatory fish frequents the diet. Arsenic and cadmium are important to monitor in certain industrial settings. Iron is important to monitor for individuals at risk for iron overload. The quality of laboratory results depends on collection of the appropriate specimen and efforts to minimize external contamination of the specimen with the element of interest. Interpretation of results should also consider the time between specimen collection and exposure, whether the exposure is acute or chronic, the specific elemental form involved, and the clinical status of the patient. Pre-analytical, analytical, and post-analytical factors important to the investigation of known or unknown toxic elemental exposures are discussed in this chapter, particularly relating to aluminum, arsenic, cadmium, iron, lead, and mercury.

**Key Words:** Aluminum; arsenic; cadmium; iron; lead; mercury; metals.

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## 1. INTRODUCTION

Toxic elements, often thought of as heavy metals, are essential or non-essential elements that can contribute to or cause adverse health effects, including death. Exposure may occur as a result of an acute incident, such as an industrial accident or poisoning, or chronically, such as through long-term use of contaminated well water. Symptoms of exposure can be non-specific and may mimic an adverse drug reaction or disease. Thus, detection of toxic element exposure depends on laboratory testing. It is important to identify exposure to toxic elements because many symptoms or pathological processes resulting from exposure can be stopped or reversed by removing of the toxic element (decontamination). This chapter addresses pre-analytical and analytical considerations in testing for toxic elements, as well as interpretation of results, from a non-forensic perspective. Six common toxic elements for which laboratory testing is routinely performed are discussed in detail: aluminum, arsenic, cadmium, iron, lead, and mercury.

## 2. SPECIMENS

Toxic elements, much like drugs, have characteristic absorption, distribution, metabolism, and elimination kinetics that must be considered when selecting the best specimen for testing. If the toxic element is known, selection of the most appropriate specimen will be based on the kinetics of that element, coupled with the circumstances surrounding the exposure. For example, the best specimen for detecting arsenic testing is usually urine, because most forms of arsenic are readily excreted from the body. If, however, the exposure to arsenic occurred very acutely (<8 hr prior to presentation), then blood may be the more appropriate specimen. If the exposure to arsenic occurred more than 3 weeks prior to presentation, then perhaps hair or nail testing would be most appropriate. The use of whole blood samples is preferred for cadmium and lead because they are greater than 90% bound to cellular proteins. Serum or plasma samples are preferred in the analysis of elements that bind to serum proteins, such as aluminum. However, cadmium, lead, and aluminum are found in urine and are often tested in urine to screen for exposure in an occupational setting, due to the ease of collection. The preferred specimens, sources, and clinical symptoms of excess exposure of common toxic elements are summarized in Table 1.

When exposure to toxic elements is suspected clinically, but the specific toxic element involved is not known, testing becomes somewhat analogous to drug screening. In this case, urine is generally the preferred specimen, and testing is performed to detect multiple toxic elements. If a specific element is identified in the screening, then future testing can be selected based on the situation and timing of exposure versus presentation, as well as the toxicokinetics of the particular toxic element. Lead exposure, for example, may be detected in urine or a capillary blood specimen, but should be confirmed with a venous blood specimen. For arsenic and mercury, toxic elements that exist in multiple forms or species, second-line testing may include fractionation to identify which form(s) are responsible for the exposure and from that information better assess the toxic potential of the exposure, likely sources of exposure, kinetics of elimination, and, if necessary, best means of monitoring decontamination.

**Table 1**
**Summary of Sources, Clinical Effects Related to Deficiency or Toxicity, and Suggested Specimen Types for Analysis of Common Toxic Elements *(1,5,25,37)***

| *Toxic element* | *Signs and symptoms related to toxicity* | *Possible sources* | *Preferred specimen(s)* |
|---|---|---|---|
| Aluminum (Al)[a] | *Acute (Systemic)*: Constipation, decreased calcium and iron absorption<br>*Acute (Inhalation)*: Difficulty breathing, pulmonary fibrosis, aluminosis<br>*Chronic*: Anemia, osteopenia, abnormally low PTH, dementia, hallucinations, seizures, EEG changes, speech impairment, encephalopathy | *Medicinal*: dialysis fluids, parenteral nutrition, antacids, phosphate binders<br>*Environmental*: cookware, particularly ceramic or glass<br>*Occupational*: metal dust, welding fumes, paints | Serum, urine |
| Arsenic (As)[a,b] | *Acute*: Nausea, gastroenteritis, skin flushing, hyperpigmentation<br>*Chronic*: Renal damage, hematuria, proteinuria, hypertension arrhythmias, delirium, Encephalopathy, muscle weakness, peripheral neuropathy, pulmonary edema, respiratory distress syndrome | *Inorganic*: Groundwater, soil, insecticides, wood preservatives<br>*Organic*: Diet seafood, fish, rice, milk, meat | Urine |
| Cadmium (Cd)[a,b] | *Acute (inhalation)*: Difficulty breathing; pulmonary edema, pneumonitis, emphysema, lung tumors<br>*Chronic*: renal damage, hematuria, proteinuria, osteomalacia, eye irritation, loss of smell, hypertension, anemia, yellowing of teeth | *Occupational*: semiconductors, electroplating, paint, silver solder, nickel and lead smelting<br>*Environmental*: mine tailings, tobacco smoke, batteries<br>*Diet*: rice, wheat, oysters, mussels | Whole blood, urine |
| Iron (Fe)[a] | *Acute*: Gastroenteritis, hemolysis<br>*Chronic*: Skin color changes (bronzing), hepatic cirrhosis, diabetes, cardiomyopathies, Endocrine deficiencies | *Medicinal*: blood transfusions, pre-natal vitamins, supplements<br>*Diet:* liver, meat, egg yolks, legumes, red wine, spinach<br>*Occupational:* steel processing, mining, arc welding | Serum, liver biopsy (chronic) |

*(Continued)*

**Table 1**
***(Continued)***

| *Toxic element* | *Signs and symptoms related to toxicity* | *Possible sources* | *Preferred specimen(s)* |
|---|---|---|---|
| Lead (Pb)[a,b] | *Acute*: Thirst, abdominal pain, muscle weakness, severe anemia, renal damage, proteinuria, hematuria *Chronic*: Hypertension, gastroenteritis, muscle weakness, encephalopathy, learning impairment, low IQ, hypochromic microcytic anemia, porphyria, renal damage, proteinuria, hematuria, reproductive disorders | *Occupational*: Smelting *Environmental*: mine tailings, lead paint, glazed pottery, leaded crystal glassware, tobacco smoke, batteries, plumbing, automobile exhaust | *Whole blood* |
| Mercury (Hg)[a,b] | *Acute*: Central nervous system damage, gastroenteritis, tubular nephritis, renal failure *Chronic*: Tremor, ataxia, sensory impairment, Tubular necrosis, proteinuria, Nephrotic syndrome | *Diet*: predatory fish (tuna, swordfish, shark), shellfish *Occupational*: mining smelting, paper and plastic production, dentistry, electrical *Environmental*: dental amalgams, thermometers, latex paints, granite | Whole blood, urine |

[a] Threshold limit values (TLV) represented as a time-weighted average (TWA) are published by the American Conference of Governmental Industrial Hygienists (ACGIH).
[b] Biological Exposure Indices (BEI) are published by the ACGIH.

Elevated toxic element concentrations in a specimen should always be confirmed with secondary testing to ensure that the elevated result was not due to external contamination. Sources of external contamination include collection materials, the specimen donor, the environment, transport containers, or assay reagents. As a result, certified trace element-free blood collection containers are available. Royal-blue top blood collection tubes, available with or without anticoagulant, are certified as trace element-free for most clinically important elements. Tan top tubes [ethylene diamine tetra acetate (EDTA) anticoagulant] are certified as lead-free, so can, in addition to the royal blue tubes, be used for lead testing. Laboratories that perform trace element testing are often physically isolated from other laboratory areas, and pay particular attention to sources of external contamination such as labware materials, dust control, and airflow dynamics. Certified trace metal-free reagents are typically used, or non-certified materials are validated to meet laboratory-determined criteria. Indices to identify environmental contamination on a daily basis are also employed.

Falsely low results could occur as well, due to volatilization and/or adherence to collection containers. It is important that sample preparation tubes and other labware be

evaluated for susceptibility to adsorption by elements of interest. Strengths, limitations, and unique aspects of specimens used for toxic element detection and quantification are discussed below.

### *2.1. Urine*

Most toxic elements are eliminated at least in part, in the urine. For this reason, and due to the relative ease of collection, urine is commonly utilized for toxic element screening for persons with normal renal function. Urine is the specimen of choice for detecting exposure to aluminum, arsenic, cadmium, and mercury. For these elements, the extent of exposure is related to urine concentrations. Spot or random urine specimens are adequate for screening purposes, but because elimination of toxic elements can be multi-phasic, a 24-h urine collection is important for diagnostic purposes. Elemental concentrations in urine are often normalized to creatinine, a metabolic end product of creatine phosphate that is excreted at a relatively constant rate if individuals have normal renal function, to improve utility of serial specimens from the same patient or comparison of results between patients. Several reference intervals and toxic thresholds are based on creatinine-normalized values of toxic elements in urine *(1–5)*. Urine can also be used to monitor elimination of toxic elements, particularly in conjunction with chelation therapy, a common decontamination strategy.

Falsely elevated results are often consequential to external contamination. The collection site is a considerable source of contamination, and as such, urine specimens should be collected away from the workplace or other areas that are likely to be contaminated. Guidelines on personal hygiene should also be followed, and smoking should be avoided throughout the collection and pre-analytical processes to minimize external contamination. The collection container is a potential source of external contamination as well. Rubber and colored dyes used in the manufacture of catheters or collection containers may contain cadmium or other elements that can leech into the sample. Even clear plastics contain some elements that may lead to a falsely elevated result (see Table 2). Unless tested to qualify as trace element free, preservative substances added to the urine collection should be considered a potential contamination source as well and should be avoided if possible *(6–9)*.

Falsely low results may be consequential to loss of elements by adsorption to vial surfaces, volatilization, or precipitation. The use of urine preservatives such as hydrochloric or nitric acids has been used historically to prevent loss due to adhesion. However, the use of preservatives does not appear to be necessary for most elements if specimens are refrigerated and stored for up to 65 days *(6)*. Both mercury and lead are considered "sticky" and are therefore vulnerable to "loss" by adhesion to the specimen container or other materials. Mercury loss is also possible through volatilization, possibly due to formation of volatile nitrates when nitric acid is used as a preservative or as part of the analytical setup. As a result, mercury testing methods often employ hydrochloric acid instead of nitric acid, or gold amalgamation with inductively-coupled plasma mass spectrometry (ICP-MS) or atomic absorption spectroscopy (AAS) *(10,11)*. Figure 1 illustrates the loss of mercury from urine collected on day "zero" and tested at regular intervals for 65 days. In addition to elemental loss due to adhesion or volatilization, falsely low results may also occur consequential to the formation of

**Table 2**
**Examples of Possible Sources of Contamination that may Contribute to Falsely Elevated Toxic Element Results *(6,9,10,12)***

| *Toxic element* | *Possible sources* |
|---|---|
| Aluminum | Plastic (PS, PC), serum or plasma separator gels, EDTA (blood anticoagulant), boric acid (urine preservative), acids and reagents, ceiling tiles, insulation, HEPA filters, skin |
| Arsenic | Boric acid and potassium pyrosulfate (urine preservatives), acids and reagents, skin |
| Cadmium | Yellow or red-colored plastics, acids and reagents, skin |
| Iron | Plastic (PS), PVC tubing, Teflon, acids and reagents |
| Lead | Plastic (ETFE), heparin, acids and reagents, skin |
| Mercury | Analytical carryover |

ETFE, ethylenetetrafluoroethylene; PC, polycarbonate; PS, polystyrene; PVC, polyvinyl chloride.

precipitate. Visible precipitate is common in urine, particularly after it has been refrigerated or when urine is obtained from a patient with an active urinary tract infection. Variability in analytical results has been shown with specimens containing arsenic or mercury, two toxic elements that concentrate in urine precipitates. If the laboratory removes the precipitate, such as through centrifugation of the specimen, results will be falsely low. If the specimens are not well mixed, inconsistent and erroneous results may be observed *(6,10–12)*.

### *2.2. Whole Blood, Serum, and Plasma*

Whole blood is the specimen of choice for lead and cadmium detection due to the high proportion (>90%) of these elements that binds to cellular proteins and localize to erythrocytes. Serum or plasma is the specimen of choice for detecting aluminum toxicity in dialysis patients and to detect iron overload. Sources of external contamination associated with whole blood collection include the blood collection tube and the skin, particularly with collection of capillary specimens. In fact, the vast majority of capillary blood specimens that generate elevated lead results do not confirm as elevated when a second specimen collected with non-trace element-free tubes (venous blood) is tested. Stainless steel needles used to perform venipuncture have not been shown to contribute to falsely elevated toxic element concentrations, but there have been some reports of elemental contamination from evacuated blood collection tubes. Such contamination could arise from the material used to construct the tube or rubber stopper, or from preservatives within the tubes *(10,12)*.

The most common clinically significant contamination documented and observed is with aluminum, a common component of anticoagulant preservatives, serum separator gels, and rubber stoppers used in both standard and non-trace element-free blood collection tubes. Thus, aluminum results can be falsely elevated, even with trace element-free blood collection tubes, particularly if the serum is in contact with the rubber stopper during storage or transport. Aluminum contamination is also observed after the serum is transferred from the original collection tube to another tube that is not trace element free. An example of this phenomenon is shown in Fig. 2 wherein

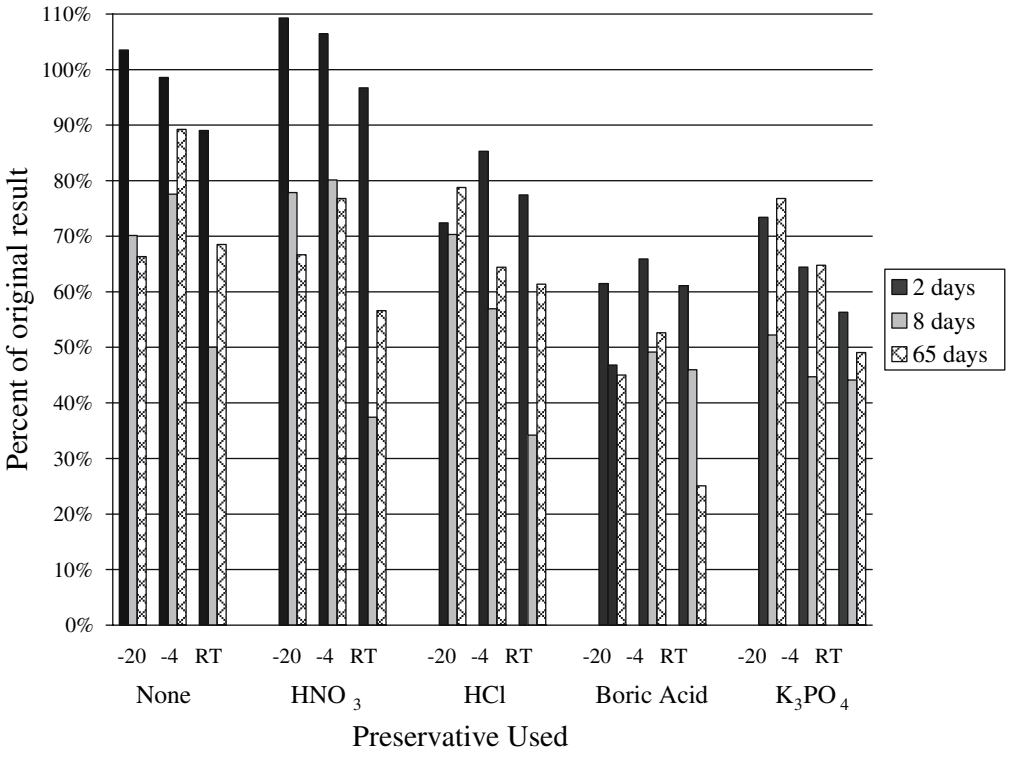


**Fig. 1.** Loss of mercury in urine specimens over time, considering storage temperature and common preservatives. Mercury testing performed with human urine specimens ($n = 15$), spiked to 25 μg/L. Samples were tested to verify accuracy of the spike, were stored under several conditions, and then tested again after 2, 8, and 65 days to evaluate the recovery. The percent of original result was plotted for specimens stored at three different temperatures: room temperature, RT; refrigerated, −4°C; and frozen, −20°C. Also considered, at each temperature, were commonly used urine preservatives including nitric acid ($HNO_3$, 0.12 mol/L final concentration), hydrochloric acid (HCl, 0.12 mol/L), boric acid (5 mg/mL), potassium pyrosulfate ($K_3PO_4$, 8 mg/mL), or no preservative. Bars represent the mean value of triplicate analyses. Loss of mercury in urine specimens over time occurs without regard to storage temperature or common preservatives.

results from serum specimens that were split between two tubes were compared. This exemplifies that elevated aluminum results should always be confirmed with a second specimen before clinical action is taken *(7,8,10,13)*.

Falsely low results may occur with blood, serum, or plasma when elements adsorb to the container in which a specimen is stored. This is a particular concern with lead and mercury in specimens that are transferred to tubes other than the primary trace element-free blood collection tube.

### *2.3. Hair and Nails*

For arsenic, and possibly other elements that are rapidly eliminated, hair and nails represent non-invasive and appropriate specimen opportunities for detecting past exposures. The concept of hair and nails as specimens for elemental analysis is based on the fact that elements are transported via the blood to cells that produce hair and

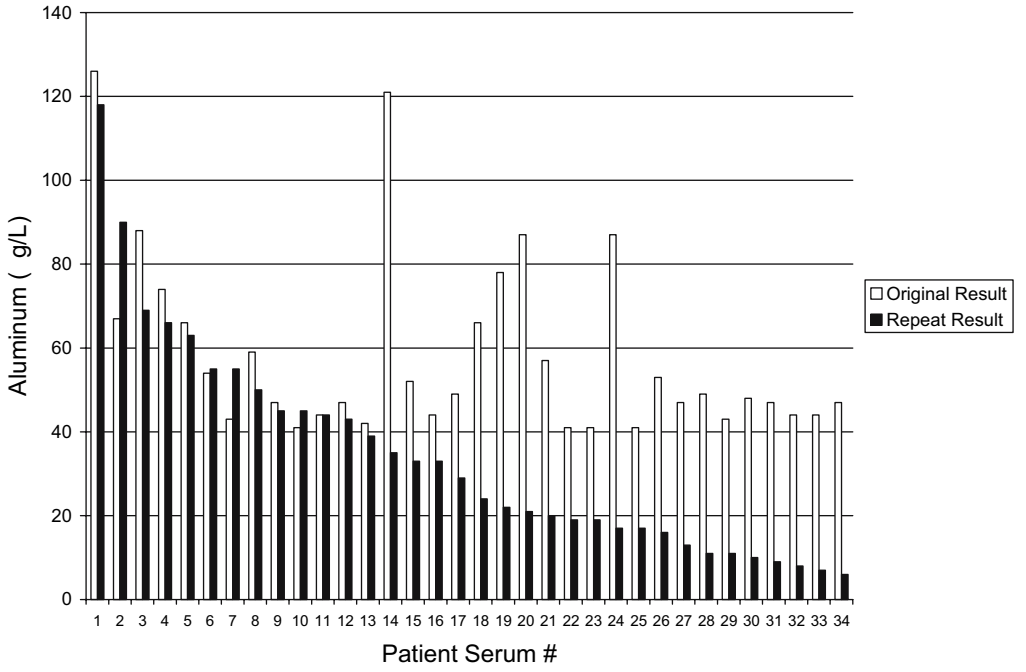


**Fig. 2.** Aluminum results obtained with split serum specimens stored in two different plastic tubes. Serum specimens collected from dialysis patients were split and stored in two plastic tubes. Specimens for which the concentration of aluminum exceeded 40 μg/L were tested again using the second serum aliquot ($n = 34$). The repeat testing results confirmed elevated aluminum concentration in only 12 specimens (35%). While four specimens generated higher results with the repeat testing than the original testing, results for both specimens were greater than the 40 μg/L threshold defined here. The most likely cause for the inconsistency in the two results is external contamination in the first plastic tube used or processes relating to analysis of the original specimen tube. These data strongly support the need for elevated serum aluminum results to be confirmed with a second specimen before action is taken.

nails. Elements circulating in the blood bind to or are trapped by constituents of hair and nails, such as keratin, and are carried with the hair and nails as they grow. As such, hair and nails provide a chronological history of exposure. Hair on the scalp grows at a rate of approximately 1 cm per month; fingernails grow at a rate of approximately 1 cm every 100 days. Actual growth rates vary per person based on season, nutrition, genetics, age, cosmetics, and other factors. Distribution and extent of deposition of toxic elements in hair and nails varies within and between individuals. It is not surprising that reference intervals for hair and nails are broad or poorly established, and the validity of these specimen types is controversial. Not surprisingly, interpretation of quantitative results for most elements measured in hair and nails is challenging.

However, qualitative detection of chronic or historical exposures to toxic elements with hair and/or nail testing has proved useful. For example, the presence of toxic elements in hair or nails that is consistent with results from other biological matrices and/or consistent with clinical findings provides additional support for the diagnosis and extent of exposure. Indeed, hair is considered a preferred specimen for diagnosis of

chronic poisoning with arsenic, wherein inorganic arsenic is preferentially incorporated. The pattern of exposure can be estimated based on analysis of hair segments, but the extent of incorporation may vary between individuals. High exposures to cadmium and lead can be detected in hair and nails, but blood is a preferred specimen. The organic form of mercury, methylmercury, is detected in hair, but urine is considered a better specimen of mercury exposure and inorganic mercury body burden. Aluminum exposure has no correlation with hair concentrations *(5,14,15)*.

The quality and utility of hair testing results also depends on the quality of the specimen and pre-analytical processing. External contamination of hair or nail specimens can occur easily because toxic elements in the environment adhere to the surface of the hair and nails. Environmental sources of contamination may include cosmetics, soaps and hair products, cigarette smoke, air-borne dust, and workplace fumes. It is very difficult to distinguish between exogenously versus endogenously deposited toxic elements in hair. The stringency of washing the hair in the laboratory prior to analysis varies from no wash to aggressive washes with organic solvents and/or strong acids. For this reason, segmental analysis wherein the patient can serve as his or her own pre-exposure or post-exposure control is most useful.

### *2.4. Soft Tissue*

Biopsy specimens of soft tissues, particularly liver, are useful for assessing the body burden of some elements. Reference intervals for soft tissues have been established for many elements using autopsy specimens obtained from accident victims *(16–18)*. Much of the knowledge regarding toxic element accumulation and elimination kinetics has been learned from these studies. For example, accumulation of cadmium is higher in smokers than in non-smokers and, due to the slow elimination kinetics, increases with age. Age-related accumulation is also recognized for mercury and iron.

Chronic iron overload is routinely evaluated with liver biopsy specimens, particularly to support the diagnosis of hemochromatosis. However, multiple specimens may be required as distribution of iron is not homogenous *(19)*. Tissue quality should be verified histologically to ensure the quality of the hepatic tissue that is analyzed, and results should be expressed based on age of the patient. The hepatic iron index (μmol iron per year of life, per mg of tissue) is quite useful for distinguishing iron accumulation due to hemochromatosis from that which occurs with age or mild liver disease, such as is observed with chronic alcohol use and hepatitis *(20)*.

Like other specimens collected for elemental analysis, procedures followed for collection of soft tissue should be sensitive to sources of external contamination. Blocked tissue should not be stained prior to quantitative elemental analysis because many histological stains and reagents contain toxic elements that could contribute to falsely elevated results. The preferred specimen is a fresh needle biopsy core submitted to the laboratory in a trace metal-free tube, without any transport media or preservatives. Because these specimens are typically dried prior to analysis (to obtain a dry weight for which results can be expressed), transport media is not necessary. In fact, storing liver biopsy tissue in saline was shown to generate falsely low iron results because the unbound forms of the iron diffuse from the tissue into the saline. However, concentrations of many elements in paraffin-embedded and formalin-fixed tissue are comparable to fresh tissue *(6,21,22)*.

### *2.5. Bone*

For elements that accumulate in bone and have long elimination kinetics, bone is a good specimen for evaluating body burden. Although both lead and aluminum accumulate in the bone, bone biopsies are neither practical nor required for diagnosis of lead or aluminum poisoning. Evaluating bone concentrations of lead with non-invasive techniques (e.g., X-ray fluorescence) may be useful for evaluating body burden and for monitoring efficacy of chelation-based decontamination therapies.

## 3. LABORATORY METHODS

While several analytical methods are available for trace element analysis and quantification, the overall best, in terms of accuracy, specificity, dynamic range, multi-element capability, and precision, is ICP-MS. This technology, used in clinical laboratories since the 1980s, employs a very high temperature (~6000–10,000 K) plasma to ionize a sample and mass spectrometry to detect specific isotopes. ICP-MS is, however, more expensive and technically difficult to perform than other techniques, and the possibility for interferences from isobaric, doubly charged, and polyatomic species exists. Interferences can be overcome by adjusting instrument settings and/or incorporating a collision or reaction cell *(23,24)*.

AAS, analogous to spectrophotometry, is a common alternative method used for the detection of trace elements vaporized to gas-phase atoms in a flame or graphite furnace. The atoms absorb ultraviolet or visible light, leading to higher electronic energy levels. Each element absorbs a characteristic wavelength of light as the sample is passed through the flame or furnace, and the elemental concentration is determined from the amount of absorption. AAS has been replaced by ICP-MS in many laboratories because AAS generally measures only one element at a time whereas ICP-MS can be used to measure multiple elements simultaneously. ICP-MS can also accommodate a much wider dynamic range and is less subject to analytical interferences than AAS. An exception, however, is mercury, for which AAS performs very well and is widely used *(25–27)*.

In order to facilitate population screening of blood lead, portability or field testing is desirable. As a result, anodic stripping voltametry, a technique that can be performed point-of-care using a capillary blood sample and a hand-held or small bench top device (LeadCare®), is commonly used. Briefly, lead in a blood lysate is plated onto an electrode. A stripping current is then applied to the electrode, the amount of which is directly proportional to the amount of lead in the sample. This method has shown excellent correlation with other methods such as AAS and ICP-MS for low concentrations of lead *(28,29)*.

Serum iron concentrations are typically measured by releasing iron ($Fe^{+3}$) from transferrin by acid and reaction of the iron to a chromogenic compound such as ferrozine following reduction to ($Fe^{+2}$). In addition, the total iron binding capacity can be obtained by saturating the iron-binding sites on transferring with exogenous $Fe^{+3}$ prior to analysis. Ferritin is another commonly used indicator and is typically measured by immunoassay *(25,30)*. Elevated iron thought to be associated with chronic accumulation is confirmed and monitored with periodic liver biopsies for which quantitative iron concentrations can be determined by staining techniques and/or ICP-MS.

## 4. INTERPRETATION OF TOXIC ELEMENT TESTING RESULTS

Clinical interpretation of toxic element results requires consideration of the reason for testing, the relationship between time(s) of exposure and time of specimen collection, the extent (dose) of exposure, whether the exposure was acute or has been chronic, the elemental form or species involved in the exposure, the route of exposure, the kinetics of the element in question, and interpretive guidelines such as thresholds for toxicity and case reports of fatalities. Reference intervals are widely available for most elements but are dependent on the geographical location from which the people studied live because elemental concentrations and diet vary from place to place. However, toxic thresholds for the elements are in general much higher than the upper limit of common reference intervals. Biological exposure indices are published by the American Conference of Governmental Industrial Hygienists (ACGIH) that provide additional interpretive value, particularly related to occupational exposures. Table 1 details the preferred specimen, common sources of exposure, and clinical symptoms of excess of six toxic elements most frequently analyzed in the clinical laboratory: aluminum, arsenic, cadmium, iron, lead, and mercury. Additional characteristics unique to these elements are detailed below.

### *4.1. Aluminum (Al)*

Aluminum is the most abundant metallic element on Earth, making exposure very common. However, aluminum toxicity is not a significant risk for most individuals, because aluminum is not well absorbed after oral ingestion and is readily eliminated by the kidneys. Individuals experiencing renal failure are more prone to accumulate aluminum, and aluminum is not eliminated by dialysis. Furthermore, dialysis patients are highly susceptible to aluminum accumulation and toxicity because dialysate fluids may contain aluminum and because aluminum-based phosphate binders are commonly used *(31,32)*. The concentration of aluminum in dialysate fluids is now highly regulated, and dialysis patients are routinely monitored using serum to detect aluminum accumulation.

Excess aluminum is deposited in the bone, where it replaces calcium, and also in the brain. It is not surprising that symptoms associated with aluminum toxicity include speech impairment, seizures, dementia, visual and auditory hallucinations, osteopenia, depression of parathyroid hormone (PTH) production, and anemia. The so-called Dialysis Encephalopathy Syndrome, described in the 1970s, is potentially fatal, but is not common today. Potentially toxic aluminum exposure can also occur through total parenteral nutrition or in occupational settings. Pulmonary aluminosis (Shaver's disease), not common today, is precipitated by chronic inhalation of aluminum dust and fumes. Although aluminum has been implicated in Alzheimer's disease, the relationship of aluminum with this disease remains controversial and unproven *(5,33)*.

Serum is the specimen of choice for routine monitoring of dialysis patients *(34)*. Urine concentrations of aluminum are useful for detecting exposures in occupational settings. Due to the very high risk of external contamination with specimens collected for aluminum quantification (see Section 2.2), any elevated aluminum result should be confirmed with a second specimen collected with a trace element-free tube.

### 4.2. Arsenic (As)

Signs and symptoms of acute arsenic poisoning are primarily gastrointestinal, whereas chronic exposure leads to muscle weakness, neurological effects, and renal damage. Arsenic is one of the few elements that undergoes biotransformation in vivo. The toxic forms of arsenic are the trivalent [As(III)] and pentavalent [As(V)] inorganic forms. Although the use of arsenic is not widespread in industrial processes, the inorganic toxicants can be found at significant levels in groundwater, in wood preservatives, and also in some insecticides. Upon absorption, these forms are metabolized in the liver to much less toxic forms (monomethylarsine and dimethylarsine) and excreted over the course of 1–3 weeks. More commonly seen are the relatively non-toxic organic forms such as arsenobetaine and arsenocholine. These organic species are found at substantial levels in many foods, especially shellfish and predatory fish, and are rapidly cleared (within 1–2 days) through the urine. The structures of the relatively common inorganic and organic forms of arsenic are given in Fig 3. The concentration of organic forms is included and is indistinguishable from the inorganic forms in laboratory results that do not separate inorganic and organic forms. This inclusion in the "total" arsenic concentration leads to significant confusion as to the clinical significance of an elevated total arsenic concentration. Thus, elevated arsenic results require speciation or fractionation to interpret.

Urine is the sample of choice for arsenic testing because it is sensitive to detection of toxic forms for up to 3 weeks following an exposure. The extremely brief half-life of inorganic arsenic in serum and whole blood (4–6 h) disfavors the use of these sample types except in very acute exposures or research settings. When a total arsenic concentration is determined, foods such as fish and seafood that are likely to contain the non-toxic organic arsenic should be avoided for at least 72 h prior to sample collection. Dietary restriction is not necessary if the analytical method discriminates between inorganic and organic forms. Chromatographic techniques such as high performance liquid chromatography can be readily employed in conjunction with ICP-MS to discriminate the concentrations of inorganic and organic forms of arsenic in biological samples. A liquid-liquid or other extraction method may also separate inorganic and organic forms prior to analysis by ICP-MS. Storage of urine samples for up to 2 months does not appear to affect the quality of speciation results *(35)*.

For chronic exposure to arsenic, hair, and/or nails may be the specimens of choice. The high affinity of arsenic for keratin in the hair and nails enables these samples to be used to document historical arsenic exposure. In nails, visible evidence of arsenic exposure may be manifested by the appearance of white transverse striae (Mees' lines) after a few weeks. Formation of Mees' lines does not always occur, however, so the absence of such lines does not exclude exposure *(5,36,37)*.

### 4.3. Cadmium (Cd)

Cadmium exposure is largely an industrial/occupational hazard, with inhalation as the most common route. Common sources include organic-based spray paints, fumes from electroplating processes, and cigarette smoking. Metal fumes can induce congestion in the lungs that appears similar to pulmonary emphysema and may promote tumor formation. Additionally, osteomalacia and bone decalcification or Itai-Itai (literally Ouch-Ouch) disease have been documented in cases of chronic exposure *(38)*.

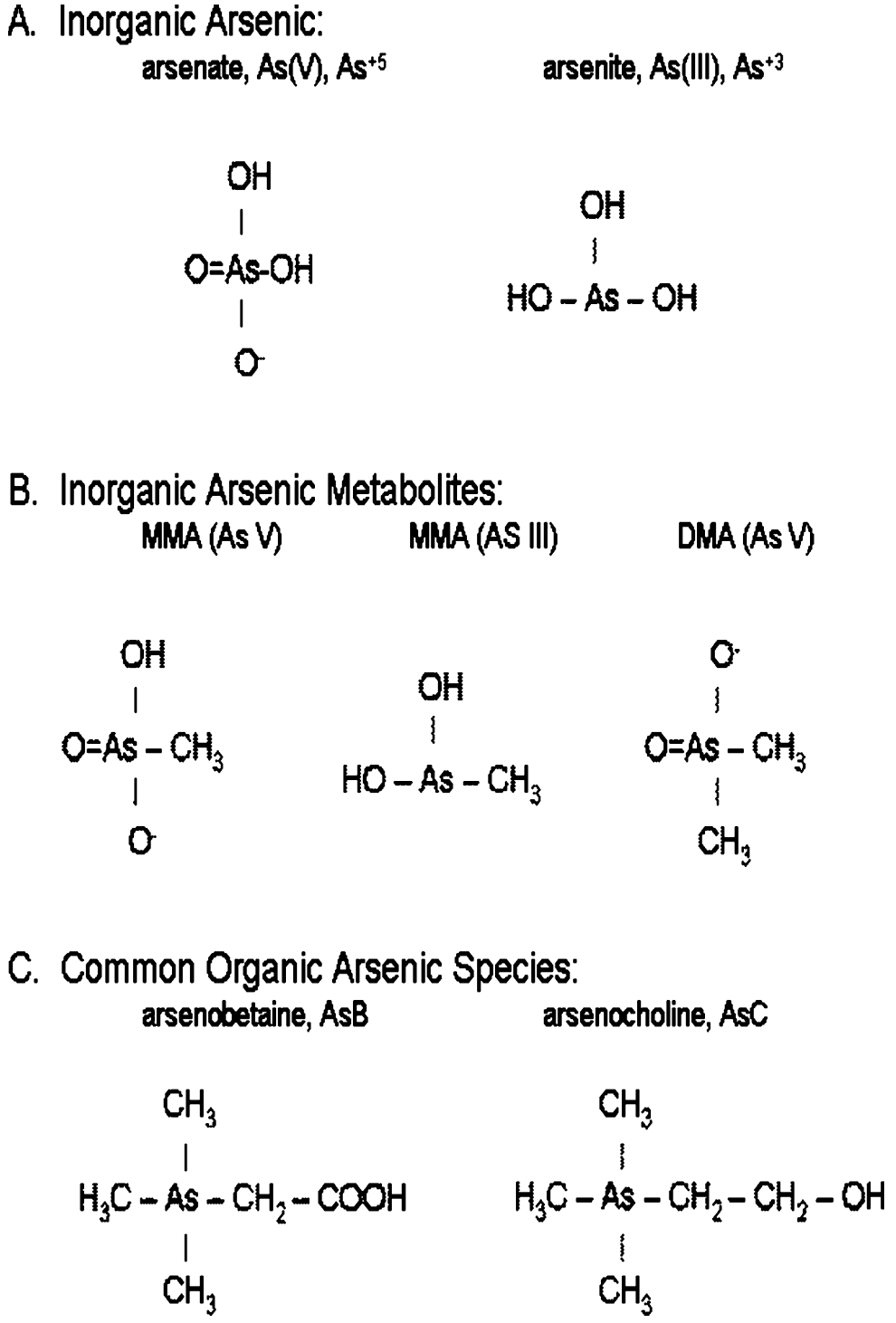


**Fig. 3.** Arsenic species. Names and chemical structures for common arsenic species and metabolites found in human urine.

Cadmium is also deposited in the liver and renal cortex and may interfere with zinc and copper binding to the metallothionein, leading to renal dysfunction and proteinuria.

Whole blood and urine are the most commonly used specimens for the investigation of cadmium exposure. Cadmium resides primarily in the erythrocytes and is excreted slowly in the urine, with an elimination half-life as long as 10 years. Urinary and whole blood cadmium is concentrations are higher in smokers and also increase with age *(5,37,39)*.

### *4.4. Iron (Fe)*

Iron is an essential but potentially toxic element that is stored via a number of different body compartments including hemoglobin and myoglobin in erythrocytes, other proteins including ferritin and hemosiderin in serum and body tissues, as well as in the labile free form. Iron is absorbed into the upper intestinal tract; total body iron stasis is mainly achieved by regulation of iron uptake. Normally, less than 10% of iron in the diet is absorbed. Iron varies diurnally with lower serum iron concentrations observed in the afternoon and evening. Pre-menstrual serum iron levels in women are significantly higher (10–50%) than post-menstrual levels serum iron levels. With acute overdose, a relatively common form of unintentional poisoning in children, iron leads to intestinal corrosion and hemorrhage associated with vomiting, diarrhea, abdominal pain, hypertension, and blood loss. With chronic accumulations, liver disease is pronounced.

The specimen of choice to initially identify a potentially toxic exposure to iron is serum. Situations that commonly lead to elevated serum iron concentrations include overdose of iron supplements or pre-natal vitamins, hereditary or acquired hemochromatosis, and hepatitis. The value of laboratory testing to identify an acute ingestion of iron is dependent on the time that has passed since the ingestion and the dose. Acute ingestions may also be investigated with abdominal imaging techniques *(40,41)*. Chronic iron overload leads to increased serum iron and transferrin levels. A liver biopsy is often used to confirm overload and help determine the related cause. Specifically, normalization of hepatic iron concentrations for age (hepatic iron index) may help distinguish between the various forms of iron overload *(20)*. In hereditary hemochromatosis, iron absorption is enhanced and elimination is impaired, leading to accumulation in tissues and organ impairment. While about 0.3% of populations of northern European descent are homozygous for defective mutations (including C282Y) in the *HFE* gene, only a fraction of individuals manifest clinical complications. Men are much more susceptible than women perhaps due to the protective effects of menstruation. Genetic testing for mutations in the *HFE* gene and hepatic iron quantification with a liver biopsy are used to diagnose this disorder. Generally the hepatic iron index in hereditary hemochromatosis exceeds 1.9. Acquired hemochromatosis, indicated by a hepatic iron index greater than 1.0 but less than 1.9, is often consequential to disorders such as ß-thalassemia major, wherein decreased erythropoesis can lead to increase iron stores. The increased dietary iron absorption and blood transfusions often associated with this disorder can exacerbate increased iron load. Alcoholics with cirrhosis and patients with hepatitis also have been observed to have increased tissue iron storage as well *(42,43)*. The distribution of iron as well as potentially other elements is variable,

so results that are inconsistent with clinical findings should be confirmed by a second specimen *(19)*.

### *4.5. Lead (Pb)*

Human exposure to lead has declined as awareness of the toxic effects of lead has increased and use of leaded gasoline and lead-based paints has declined. The median blood lead concentration in children dropped 7-fold between 1978 and 1999. However, many environmental sources in certain geographical areas still contribute to lead poisoning. Following inhalation or absorption, lead is deposited in all tissues and is excreted in the urine and bile. The half-life of lead in blood is 1–2 months while the half-life of lead in other tissues such as bone is as much as 10–20 years. There are several mechanisms of lead toxicity. For example, lead inhibits the enzyme amino levulinic acid dehydratase that promotes the production of heme from porphyrin and leads to anemia. There is also a decreased incorporation of iron into protoporphyrin IX resulting in a proportional increase in dysfunctional zinc protoporphyrin. Lead binds readily to sulfhydryl groups, leading to protein dysfunction in a wide variety of tissues, including the central nervous system *(5,37)*.

The critical populations to monitor for lead poisoning are children under 6 years of age and adults at risk of occupational exposure. Guidelines for testing and laboratory performance expectations have been published by several organizations worldwide. In the United States, guidelines published by the CDC for children and by OSHA for adults are observed *(44)*. Children are particularly vulnerable to lead poisoning because they absorb a relatively high percentage of lead (up to 50% more than adults) and exhibit behaviors such as hand–mouth exploration of dirt, paint chips, toys, and other likely lead sources. Central nervous system damage in cases of lead toxicity results in intellectual impairment in children. Inverse correlations between IQ and blood lead concentrations in children have been demonstrated at relatively low levels. Whole blood (generally capillary blood for screening and venous for confirmation) is the specimen of choice for the diagnosis and monitoring of lead poisoning, although X-ray fluorescence is gaining popularity as a non-invasive tool for assessing body lead burden *(45–47)*.

### *4.6. Mercury (Hg)*

Mercury is commonly encountered in our environment that receives much public attention. While elemental mercury ($Hg^0$) is relatively non-toxic, the ionized inorganic species ($Hg^{2+}$) is toxic, and organic species such as methyl mercury ($CH_3Hg^+$) are highly toxic. Elemental mercury can be converted into ionized inorganic or organic mercury by microorganisms that are commonly found in bodies of water and digestive systems. The toxic methyl mercury species accumulates in predatory fish, perhaps the most likely source of mercury exposure. Unborn children are exposed to extremely high concentrations of methyl mercury in utero, relative to the mother's blood, via placental transfer. As a result, the FDA has advised that pregnant women avoid eating excessive tuna and other predatory fish that may contain high levels of methyl mercury. Limiting intake not only prevents acute exposures, but also minimizes the accumulation of toxic levels over time. Dental amalgams release small amounts of elemental mercury as

well, particularly when installed, removed, or with gum chewing, yet the incidence of toxicity associated with dental amalgams is rare. Dentists and dental workers are at higher risk of exposure while working with the amalgams; hygiene guidelines have been proposed to minimize this occupational exposure. Although mercury exposure in vaccine preparations has been proposed as a contributing factor in the increase of the incidence of autism, this hypothesis has not been substantiated *(48–50)*.

Acute exposure to toxic mercury forms can cause gastroenteritis and tubular nephritis, as well as severe and potentially irreversible damage to the central nervous system. Chronic exposure often manifests itself as a broad range of emotional disturbances, tremor, sensory impairment, tubular necrosis, and nephrotic syndrome. Whole blood or urine specimens are useful to assess mercury levels; the total body burden has a half-life of 30–60 days. Care must be taken to insure that mercury in specimens is not lost through volatilization or adherence to specimen containers *(51,52)*.

## 5. TREATMENT OF TOXIC ELEMENT POISONING

Removal of the poisoned individual from the environmental source (or vice versa) responsible for the toxic metal contamination and supportive care are generally all that is required for decontamination. In some cases, however, chelation therapy using organic compounds to bind and remove metals from the body may be warranted. Because chelation therapy may pose life-threatening side effects, due to the simultaneous chelation of essential elements such as calcium, care must be taken to manage the administration of chelation therapy *(53–56)*. In any case, monitoring the elimination of toxic element exposure and confirmation of effective decontamination are useful when the appropriate laboratory specimen is utilized. For lead and iron, blood is a good specimen for monitoring decontamination, whereas urine is preferred for monitoring decontamination of mercury and most other toxic elements.

Chelation therapy to treat children with blood lead concentrations of 20–44 μg/dL showed no benefit *(57)*, but is recommended for children with blood lead levels ≥45 μg/dL using the compound dimercaptosuccinic acid (DMSA). Chelation therapy should be considered for adults with blood lead levels above 60 μg/dL and is recommended when blood levels exceed 80 μg/dL. When blood levels exceed 70 μg/dL, both DMSA and EDTA may be utilized. Dimercaprol (British Anti-Lewisite, BAL) is also occasionally utilized. Blood lead levels should be monitored not only to insure the subject has been removed from exposure, but also to identify the potential rebound in blood lead levels that could occur as lead from body stores are redistributed *(58)*. In cases of iron overload, both therapeutic phlebotomy and iron chelation therapy have been utilized. Treatment with the chelators deferoxamine and deferiprone has been shown to be effective in reducing tissue injury especially in those who suffer from transfusional iron overload *(59,60)*.

In cases of poisoning with other metals, the role of chelation therapy is less well established. Chelation of arsenic with BAL, D-penicillamine, 2–3 dimercaptopropane sulfate, or DMSA can serve to accelerate the reduction of acute symptoms, but does little to ameliorate the effects of chronic exposure *(61)*. Aggressive chelation treatment of mercury poisoning may accelerate its removal from the body. Both arsenic and mercury decontamination can be monitored with urine. However, the efficacy of such treatment is controversial and may not outweigh the risks to the patient. The need

for chelation therapy should be evaluated case by case *(62,63)*. Chelation therapy for cadmium exposure is most useful for acute exposures and should be modestly pursued due to the overload to the kidneys that results from liberating body stores with the chelator *(64)*. For aluminum toxicity, the most effective treatment appears to be removal of sources of aluminum exposure, although treatment with deferoxamine has been utilized.

## 6. OTHER TOXIC ELEMENTS

Although the elements discussed here represent some of the most commonly investigated toxic elements, other toxic elements may be important to consider, based on the clinical situation. These include the toxic elements associated with therapeutics such as lithium (psychiatric indications), antimony (oncological indications), bromide (neurological indications), iodine (endocrinology and ophthalmic indications), gold (arthritis), and bismuth (gastrointestinal indications). Unconventional therapy with elements such as silver may also lead to toxicity that is diagnosed with elemental testing. Also of potential toxicological interest are essential elements that accumulate with disease, such as copper (Wilson Disease) or iron (hemochromatosis). Excessive supplementation with essential elements such as manganese, magnesium, and chromium may contribute to adverse health effects as well. Finally, more esoteric elements, exposure to which may be associated with an industrial or military setting, hobby, or contaminated geographical location, include nickel, molybdenum, vanadium, beryllium, cobalt, platinum, tellurium, thallium, uranium, and potentially others. Some clinical and forensic laboratories provide testing to detect these various elements. However, in many cases, the relationship between exposure levels, the toxicokinetics, and resulting clinical symptoms and/or concentrations in biological fluids is not well understood.

## 7. CONCLUSIONS

Laboratory testing is critical to the identification and monitoring of toxic elemental exposures. Toxic elements are much like drugs in that they exhibit characteristic absorption, distribution, metabolism, and elimination properties. As such, the utility of laboratory results requires consideration of the circumstances surrounding an exposure and controlled collection of the most appropriate specimen(s). Unlike drugs, however, external contamination is common to elemental testing, particularly with aluminum and lead. Thus, elevated results should be confirmed with a second specimen. Elemental loss of mercury is also common and should be considered when faced with results that are inconsistent with the clinical scenario. Specialized testing may also be required. For arsenic, an elevation in total concentrations cannot be interpreted without additional testing to fractionate or speciate the specimen to determine whether the elevated total result is due to toxic inorganic forms or relatively non-toxic organic forms of arsenic. Panels designed to detect a wide variety of potentially toxic elements are available clinically. Once confirmed, a toxic elemental exposure can be managed to minimize clinical consequences. Such management should include laboratory testing to monitor decontamination as well as identify any rebound exposures resulting from redistribution of tissue stores.

## REFERENCES


1. ACGIH. TLVS and BEIS: Based on the Documentation of the Threshold Limit Values for Chemical Substances and Physical Agents & Biological Exposure Indices. Cincinnati, OH: ACGIH, 2006.
2. Ewers U, Krause C, Schulz C, Wilhelm M. Reference values and human biological monitoring values for environmental toxins. Report on the work and recommendations of the Commission on Human Biological Monitoring of the German Federal Environmental Agency. Int Arch Occup Environ Health 1999;72:255–260.
3. Herber RF. Review of trace element concentrations in biological specimens according to the TRACY protocol. Int Arch Occup Environ Health 1999;72:279–283.
4. Komaromy-Hiller G, Ash KO, Costa R, Howerton K. Comparison of representative ranges based on U.S. patient population and literature reference intervals for urinary trace elements. Clin Chim Acta 2000;296:71–90.
5. Lauwerys RR, Hoet P. Industrial Chemical Exposure Guidelines for Biological Monitoring. 3rd ed. Lewis Publishers, Boca Raton, FL, USA 2001.
6. Bornhorst JA, Hunt JW, Urry FM, McMillin GA. Comparison of sample preservation methods for clinical trace element analysis by inductively coupled plasma mass spectrometry. Am J Clin Pathol 2005;123:578–583.
7. Frank EL, Hughes MP, Bankson DD, Roberts WL. Effects of anticoagulants and contemporary blood collection containers on aluminum, copper, and zinc results. Clin Chem 2001;47:1109–1112.
8. Subramanian KS. Storage and preservation of blood and urine for trace element analysis. A review. Biol Trace Elem Res 1995;49:187–210.
9. Moody JR, Lindstrom RM. Selection and cleaning of plastic containers for storage of trace element samples. Anal Chem 1977;49:2264–2267.
10. Moyer TP, Mussmann GV, Nixon DE. Blood-collection device for trace and ultra-trace metal specimens evaluated. Clin Chem 1991;37:709–714.
11. Nuttall KL, Gordon WH, Ash KO. Inductively coupled plasma mass spectrometry for trace element analysis in the clinical laboratory. Ann Clin Lab Sci 1995;25:264–271.
12. Aitio A, Jarvisalo J. Biological monitoring of occupational exposure to toxic chemicals. Collection, processing, and storage of specimens. Ann Clin Lab Sci 1985;15:121–139.
13. Rodushkin I, Odman F. Assessment of the contamination from devices used for sampling and storage of whole blood and serum for element analysis. J Trace Elem Med Biol 2001;15:40–45.
14. Shamberger RJ. Validity of hair mineral testing. Biol Trace Elem Res 2002;87:1–28.
15. Nuttall KL. Interpreting hair mercury levels in individual patients. Ann Clin Lab Sci 2006;36:248–261.
16. Garcia F, Ortega A, Domingo JL, Corbella J. Accumulation of metals in autopsy tissues of subjects living in Tarragona County, Spain. J Environ Sci Health A Tox Hazard Subst Environ Eng 2001;36:1767–1786.
17. Lyon TD, Fell GS, Halls DJ, Clark J, McKenna F. Determination of nine inorganic elements in human autopsy tissue. J Trace Elem Electrolytes Health Dis 1989;3:109–118.
18. Caroli S, Alimonti A, Coni E, Petrucci F, Senofonte O, Violante N. The assessment of reference values for elements in human biological tissues and fluids: a systematic review. Crit Rev Anal Chem 1994;24:363–398.
19. Emond MJ, Bronner MP, Carlson TH, Lin M, Labbe RF, Kowdley KV. Quantitative study of the variability of hepatic iron concentrations. Clin Chem 1999;45:340–346.
20. Bassett ML, Halliday JW, Powell LW. Value of hepatic iron measurements in early hemochromatosis and determination of the critical iron level associated with fibrosis. Hepatology 1986;6:24–29.
21. Olynyk JK, O'Neill R, Britton RS, Bacon BR. Determination of hepatic iron concentration in fresh and paraffin-embedded tissue: diagnostic implications. Gastroenterology 1994;106:674–677.
22. Beilby JP, Prins AW, Swanson NR. Determination of hepatic iron concentration in fresh and paraffin-embedded tissue. Clin Chem 1999;45:573–574.
23. Ash KO, Komaromy-Hiller G. Analysis of clinical specimens using inductively coupled plasma mass spectrometry. In: Steve HY, Wong IS, ed. Handbook of Analytical Therapeutic Drug Monitoring & Toxicology. CRC Press, Boca Raton, FL, USA 1997:107–125.
24. Komaromy-Hiller G. Flame, flameless, and plasma spectroscopy. Anal Chem 1999;71:338R–342R.

25. Burtis CA, Ashwood ER, Bruns DE, eds. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 4th ed. USA: Elsevier Saunders, 2006.
26. Ertas OS, Tezel H. A validated cold vapour-AAS method for determining mercury in human red blood cells. J Pharm Biomed Anal 2004;36:893–897.
27. Kalamegham R, Ash KO. A simple ICP-MS procedure for the determination of total mercury in whole blood and urine. J Clin Lab Anal 1992;6:190–193.
28. Pineau A, Fauconneau B, Rafael M, Viallefont A, Guillard O. Determination of lead in whole blood: comparison of the LeadCare blood lead testing system with Zeeman longitudinal electrothermal atomic absorption spectrometry. J Trace Elem Med Biol 2002;16:113–117.
29. Zentner LE, Rondo PH, Latorre Mdo R. Blood lead concentrations in maternal and cord blood evaluated by two analytic methods. Arch Environ Occup Health 2005;60:47–50.
30. Tietz NW, Rinker AD, Morrison SR. When is a serum iron really a serum iron? The status of serum iron measurements. Clin Chem 1994;40:546–551.
31. Cannata-Andia JB, Fernandez-Martin JL. The clinical impact of aluminium overload in renal failure. Nephrol Dial Transplant 2002;17 Suppl 2:9–12.
32. Drueke TB. Intestinal absorption of aluminium in renal failure. Nephrol Dial Transplant 2002;17 Suppl 2:13–16.
33. Campbell A. The potential role of aluminium in Alzheimer's disease. Nephrol Dial Transplant 2002;17 Suppl 2:17–20.
34. McCarthy JT, Milliner DS, Kurtz SB, Johnson WJ, Moyer TP. Interpretation of serum aluminum values in dialysis patients. Am J Clin Pathol 1986;86:629–636.
35. Feldmann J, Lai VW, Cullen WR, Ma M, Lu X, Le XC. Sample preparation and storage can change arsenic speciation in human urine. Clin Chem 1999;45:1988–1997.
36. Yoshida T, Yamauchi H, Fan Sun G. Chronic health effects in people exposed to arsenic via the drinking water: dose-response relationships in review. Toxicol Appl Pharmacol 2004;198:243–252.
37. Rosenstock L, Cullen MP, Brodkin CA, Redlich CA. Textbook of Clinical Occupational and Environmental Medicine. Philadelphia, PA: Elsevier Saunders, 2005.
38. Inaba T, Kobayashi E, Suwazono Y, Uetani M, Oishi M, Nakagawa H, Nogawa K. Estimation of cumulative cadmium intake causing Itai-Itai disease. Toxicol Lett 2005;159:192–201.
39. Jin T, Lu J, Nordberg M. Toxicokinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. Neurotoxicology 1998;19:529–535.
40. Berkovitch M, Livne A, Lushkov G, Barr J, Tauber T, Eshel G, et al. Acute iron intoxication: significant differences between sexes. Vet Hum Toxicol 1997;39:265–267.
41. Singhi SC, Baranwal AK, M J. Acute iron poisoning: clinical picture, intensive care needs and outcome. Indian Pediatr 2003;40:1177–1182.
42. Lyon E, Frank EL. Hereditary hemochromatosis since discovery of the HFE gene. Clin Chem 2001;47:1147–1156.
43. Weinberg ED. Iron loading and disease surveillance. Emerg Infect Dis 1999;5:346–352.
44. Parsons PJ, Reilly AA, Esernio-Jenssen D, Werk LN, Mofenson HC, Stanton NV, Matte TD. Evaluation of blood lead proficiency testing: comparison of open and blind paradigms. Clin Chem 2001;47:322–330.
45. Schutz A, Olsson M, Jensen A, Gerhardsson L, Borjesson J, Mattsson S, Skerfving S. Lead in finger bone, whole blood, plasma and urine in lead-smelter workers: extended exposure range. Int Arch Occup Environ Health 2005;78:35–43.
46. Farias P, Echavarria M, Hernandez-Avila M, Villanueva C, Amarasiriwardena C, Hernandez L, et al. Bone, blood and semen lead in men with environmental and moderate occupational exposure. Int J Environ Health Res 2005;15:21–31.
47. Popovic M, McNeill FE, Chettle DR, Webber CE, Lee CV, Kaye WE. Impact of occupational exposure on lead levels in women. Environ Health Perspect 2005;113:478–484.
48. Clarkson TW, Magos L, Myers GJ. The toxicology of mercury – current exposures and clinical manifestations. N Engl J Med 2003;349:1731–1737.
49. Nierenberg DW, Nordgren RE, Chang MB, Siegler RW, Blayney MB, Hochberg F, et al. Delayed cerebellar disease and death after accidental exposure to dimethylmercury. N Engl J Med 1998;338:1672–1676.

50. Counter SA, Buchanan LH. Mercury exposure in children: a review. Toxicol Appl Pharmacol 2004;198:209–230.
51. Nuttall KL. Interpreting mercury in blood and urine of individual patients. Ann Clin Lab Sci 2004;34:235–250.
52. Gochfeld M. Cases of mercury exposure, bioavailability, and absorption. Ecotoxicol Environ Saf 2003;56:174–179.
53. Andersen O, Aaseth J. Molecular mechanisms of in vivo metal chelation: implications for clinical treatment of metal intoxications. Environ Health Perspect 2002;110 Suppl 5:887–890.
54. Brown MJ, Willis T, Omalu B, Leiker R. Deaths resulting from hypocalcemia after administration of edetate disodium: 2003–2005. Pediatrics 2006;118:e534–e536.
55. Kalia K, Flora SJ. Strategies for safe and effective therapeutic measures for chronic arsenic and lead poisoning. J Occup Health 2005;47:1–21.
56. Blanusa M, Varnai VM, Piasek M, Kostial K. Chelators as antidotes of metal toxicity: therapeutic and experimental aspects. Curr Med Chem 2005;12:2771–2794.
57. Rogan WJ, Ware JH. Exposure to lead in children – how low is low enough? N Engl J Med 2003;348:1515–1516.
58. Moline JM, Landrigan PJ. Lead. In: Rosenstock L, Cullen MR, Brodkin CA, Redlich CA, eds. Textbook of Clinical Occupational and Environmental Medicine, 2nd ed: Elsevier Saunders Philadelphia, PA, USA 2005:967–978.
59. Franchini M. Hereditary iron overload: update on pathophysiology, diagnosis, and treatment. Am J Hematol 2006;81:202–209.
60. Hershko CM, Link GM, Konijn AM, Cabantchik ZI. Iron chelation therapy. Curr Hematol Rep 2005;4:110–116.
61. Franzblau A. Arsenic. In: Rosenstock L, Cullen MR, Brodkin CA, Redlich CA, eds. Textbook of Clinical Occupational and Environmental Medicine, 2nd ed: Elsevier Saunders, Philadelphia, PA, USA 2005:946–949.
62. Risher JF, Amler SN. Mercury exposure: evaluation and intervention the inappropriate use of chelating agents in the diagnosis and treatment of putative mercury poisoning. Neurotoxicology 2005;26:691–699.
63. Anonymous. Safety and efficacy of succimer in toddlers with blood lead levels of 20–44 microg/dL. Treatment of Lead-Exposed Children (TLC) Trial Group. Pediatr Res 2000;48:593–599.
64. Goyer RA, Cherian MG, Jones MM, Reigart JR. Role of chelating agents for prevention, intervention, and treatment of exposures to toxic metals. Environ Health Perspect 1995;103:1048–1052.

# 15 Alcohol Testing

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***Contents***



**Summary**

Ethanol, commonly also referred to as alcohol, is widely used as well as abused, and measurement of alcohol is a common test in clinical toxicology and forensic laboratories. Although blood alcohol measurement is most commonly used, alcohol can also be measured in breath, urine, saliva and vitreous humor. Whole blood alcohol levels are lower than serum alcohol levels, and the value depends on the hematocrit. Although enzymatic assays based on capability of alcohol dehydrogenase to convert alcohol to acetaldehyde is widely used for rapid determination of serum or plasma alcohol concentrations, values may be elevated in postmortem serum as well as in patients with highly elevated lactate and lactate dehydrogenase level in blood. Gas chromatographic methods widely used for determination of forensic alcohol are considered as the reference method, and there are also few reports of using gas chromatography combined with mass spectrometry for determination of alcohol concentrations in biological matrix.

**Key Words:** Alcohol testing; enzyme assay; lactate; LD; gas chromatography; isopropanol; methanol; acetone.

## 1. INTRODUCTION

Ethanol is often considered to be the most used and abused chemical substance. As a result, measurement of ethanol is one of the most frequently performed toxicological tests. Ethanol analysis is important for both clinical and forensic purposes. Alcohol is a depressant of the central nervous system (CNS), and when taken in sufficient quantities, death may result from respiratory depression. Rapid and accurate analysis in the clinical setting is extremely important for patient care. For forensic purposes, ethanol is measured for workplace drug testing, investigation of driving impairment

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and accident investigation, and for postmortem evaluation. As the legal ramifications of impairment due to ethanol consumption can be very serious, accurate measurement of ethanol using methods and handling procedures that are defensible in a court of law is critical.

Although alcohol is often used in reference to ethanol only, the term also includes other alcohols such as methanol, isopropanol, and acetone that might also be present; the latter is a metabolite of isopropanol. Laboratories should alert users as to whether the alcohol method in use detects only ethanol or whether all alcohols are detected.

The effects of ethanol on the CNS vary according to the blood ethanol concentration. Low concentrations (<50 mg/dL) are typically associated with decreased inhibitions and mild euphoria; higher concentrations (>100 mg/dL) generally result in increased disorientation and loss of coordination, whereas markedly increased levels (>400 mg/dL) may result in coma or death. The combination of ethanol with drugs that are CNS depressants can result in the above effects being observed at much lower ethanol concentrations. Food ingestion also influences the rate at which ethanol is absorbed. In fasting individuals, acute ingestion of ethanol results in peak blood ethanol concentrations within 0.5–2.0 h. In nonfasting individuals, peak ethanol concentrations may be achieved in anywhere from 1 to 6 h.

The metabolism of ethanol occurs primarily in the liver by hepatic alcohol dehydrogenase (ADH). Ethanol is converted to acetaldehyde by ADH and then to acetic acid by aldehyde dehydrogenase. Gastric ADH also plays a role in the metabolism of ethanol. Chronic ethanol users show lower activities of gastric ADH compared with nonusers *(1)*. The lower levels of gastric ADH activity in chronic ethanol users result in greater bioavailability of ethanol and higher blood ethanol concentrations.

## 2. METHANOL

Methanol or isopropanol is usually not detectable in blood or other body fluids. These alcohols are sometimes found in postmortem specimens as a contaminant of embalming fluid and the embalming process. Methanol, sometimes referred to as wood alcohol, is often used as a solvent in cleaning products, paint stripper, industrial solvents, and as a component of canned fuel. Methanol is slowly metabolized by the liver at approximately one-tenth the rate of ethanol. Approximately 90–95% of methanol is metabolized in the liver and 5–10% is excreted unchanged through the lungs and kidneys.

Methanol itself is relatively nontoxic; however, it is metabolized to compounds that result in acidosis, blindness, and possibly death. In 2002, there were 2610 reported methanol exposures, with 18 fatalities *(2)*. The lethal dose of methanol has been estimated to be 1–2 mL/kg *(3)*. Methanol is metabolized by ADH to formaldehyde, and the formaldehyde is further metabolized to formic acid by aldehyde dehydrogenase. Formic acid is responsible for the profound anion gap metabolic acidosis and ocular toxicity that develops. Serum formate concentrations correlate more closely with the severity of acidosis and ocular toxicity compared with serum methanol concentrations. Therefore, measurement of formate to assess toxicity is preferable. Historically, treatment of methanol poisoning, as well as ethylene glycol poisoning, was accomplished through administration of ethanol. Ethanol competes with methanol for metabolism by ADH. More recent treatment modalities utilize 4-methylpyrazole (4-MP), which is a competitive inhibitor of ADH that blocks the metabolism of

methanol to its active metabolite. Hemodialysis may also be required to remove formate that has already accumulated before initiation of treatment.

### *2.1. Isopropanol*

Isopropanol is commonly found in readily available rubbing alcohol (70% isopropanol), antifreeze, skin lotions, and some home-cleaning products. It has up to three times the potency of ethanol and causes hypotension and cardiac and respiratory depression more readily than ethanol. Peak levels of isopropanol occur approximately 30 min following ingestion. Death from ingestion of isopropanol is uncommon. Serum isopropanol concentrations >50 mg/dL are associated with signs of intoxication, whereas concentrations >150 mg/dL are associated with coma *(4)*.

Up to half the ingested isopropanol is excreted unchanged by the kidney, whereas 50–80% is converted in the liver to acetone. Acetone also exhibits CNS depression effect. Acetone is excreted primarily by the kidneys, with some excretion through the lungs. The elimination half-life of isopropanol is 4–6 h whereas that of acetone is 16–20 h. The prolonged CNS depression seen with isopropanol ingestion is partially related to CNS-depressant effects of acetone itself. Isopropanol or acetone may by followed when monitoring patients.

## 3. SPECIMEN

Several body fluids, such as venous blood, capillary blood, serum or plasma, urine (fresh void), vitreous humor, tear fluid, cerebrospinal fluid (lumbar fluid), saliva, sweat, and breath, are suitable for determination of ethanol in living subjects. Serum, plasma, or urine may be used if ethanol is assayed using enzymatic methods. When gas chromatography (GC) analysis is used, any body fluid or tissue is suitable for analysis. Anticoagulants do not interfere with either method.

### *3.1. Blood*

Alcohol dissolves in an aqueous environment, and because the water content of serum (~98%) is greater than that of whole blood (~86%), serum ethanol results are always higher than those obtained with whole blood. The serum : whole blood ethanol ratio is 1.14 (1.09–1.18) and varies slightly with hematocrit *(5)*. Although most states have enacted laws that define intoxication while driving a motor vehicle based on whole blood ethanol levels, some states do not specify the specimen type. Therefore, laboratories that perform alcohol analysis must report the type of sample required.

Whole blood specimens should be drawn in a grey-top (potassium oxalate/sodium fluoride) Vacutainer. Preferably, the venipuncture site should be cleaned with an alcohol-free disinfectant, such as aqueous benzalkonium chloride (zephiran) or povidone iodine. Samples must be well capped and preferably refrigerated to prevent loss of ethanol. No ethanol was lost from whole blood specimens stored at 0–3°C or at room temperature (22–29°C) for up to 14 days *(6)*. For longer storage or for nonsterile postmortem specimens, sodium fluoride can be used as a preservative to prevent increases in ethanol concentration because of fermentation.

Measurement of blood alcohol concentration is important for law enforcement as it is the measure for the determination of driving under the influence. Examples of

punishable blood alcohol concentration limits include 80 mg/dL (0.08 gram percent) in the USA, UK, and Canada; 0.20 mg/g in Sweden; and 0.50 mg/g in most EU countries *(7)*. However, these values have no relevance to clinical management because there is no consensus on the concentration that defines clinical intoxication.

### *3.2. Breath*

In capillary alveolar blood, ethanol equilibrates rapidly with alveolar air in a ratio of approximately 2100:1 (blood : breath). Breath ethanol expressed as g/210 L is approximately equivalent to g/dL whole blood ethanol. Traffic laws in the USA state that "alcohol concentration shall mean either grams of alcohol per 100 mL of blood or grams per 210 liters of breath" *(8)*. Before breath analysis, a waiting period of 15 min is required to allow for clearance of any residual alcohol from very recent drinking, use of alcohol-containing mouthwash, or vomiting alcohol-rich gastric fluid.

The current Clinical Laboratory Improvement Act of 1988 does not regulate breath alcohol testing because a discrete sample is not collected or analyzed separately. A taskforce of the AACC Therapeutic Drug Monitoring and Clinical Toxicology Division has proposed several recommendations for a breath alcohol QA/QC program (Table 1) *(9)*.

### *3.3. Saliva*

Use of saliva is a noninvasive, convenient, and rapid way to obtain a sample for ethanol analysis. There is also the advantage that ethanol concentration is 9% higher in saliva (water content 99%) than in whole blood (water content 85% w/v) *(10)*. In one scheme of saliva ethanol testing with the Q.E.D. Saliva Alcohol Test (STC Diagnostics, Bethlehem, PA), sample is obtained by absorbing saliva on a swab, which is then inserted into the test cartridge. Despite the convenience of such a specimen, it is to be noted that for individuals with anticholinergic symptomology, such as dry

**Table 1**
**Proposed Quality Assurance Guidelines for Clinical Breath Alcohol Testing *(9)***

| *Guideline* | *Degree of Consensus* |
|---|---|
| 1. Clinical breath alcohol testing is point-of-care (POC) and must meet the same QA/QC requirements as any POC test. As a part of the laboratory ongoing QA effort, a program must be in place to monitor and evaluate policy, protocols, and the total testing process so that breath alcohol results are accurate and reliable. The clinical laboratory should be involved in the design, implementation, and monitoring of the QA program | A |
| 2. The laboratory should be involved in the selection, validation, and deployment of the breath alcohol devices used | A |
| 3. Alcohol concentrations should be reported in units clearly defined by the laboratory, with a notation as to the sample matrix that was tested (serum or plasma, urine, whole blood, breath) and methodology | A |

mouth associated with tricyclic antidepressant overdose, or alcoholics with impaired salivary flow, a saliva-based alcohol test may not be suitable.

### 3.4. Urine

For ethanol analysis, random urine samples can be collected with no preservative requirement. Although urine ethanol is suggested to be approximately 1.3 times higher than that in blood in the post-absorptive phase *(11)*, urine alcohol concentration represents an average of blood alcohol concentration during the period in which urine gets collected in the bladder. Therefore, a representative urine sample can be collected by first emptying the bladder and then collecting the urine after 20–30 min. Urine alcohol testing may be performed in conjunction with urine testing for drugs of abuse.

### 3.5. Vitreous humor

Vitreous humor is the clear solution that fills the space between the lens and the retina of the eyeball. Vitreous humor is anatomically remote from the gut and thus is less likely to be contaminated by spread of bacteria. This is important in testing of postmortem specimens, especially if the corpse has decomposed or been exposed to severe trauma. Vitreous humor is also important when a pure blood specimen is difficult to obtain because of trauma or if there was a suspicion that bacteria may have compromised a blood sample. When compared with blood alcohol levels, vitreous alcohol can frequently indicate the phase of body alcohol (absorptive or excretory) *(12)*. Vitreous alcohol is now accepted in most courts for determining whether an individual was impaired by alcohol.

### 3.6. Postmortem Specimens

For postmortem analysis, ethanol is usually assayed in femoral blood, heart blood, bladder urine, vitreous humor, CSF (cisternal), bile, synovial fluid, brain, skeletal muscle, or liver *(13)*. There could be other sources for postmortem ethanol. Many microorganisms are known to be responsible for ethanol formation in postmortem tissues, which is a frequent complication affecting interpretation of postmortem ethanol results. *Candida albicans* is most often reported to be responsible for ethanol formation in postmortem tissues using glucose as a substrate. *C. albicans* is located ubiquitously throughout the body particularly in the mouth and on the skin *(14)*. Almost 100 different species of bacteria, yeast, and fungi have been reported to produce postmortem ethanol *(15)*. Under optimal conditions, large quantities of ethanol can be generated by microorganism within hours of death thus complicating interpretation of ethanol levels in postmortem specimens. No significant increase in ethanol concentration was observed when postmortem specimens were homogenized in 1% sodium fluoride and stored at 4 or 25°C *(16)*.

#### 3.6.1. Biomarkers of Antemortem Ethanol Ingestion and Postmortem Ethanol Synthesis

To distinguish between antemortem ingestion and postmortem ethanol synthesis, several biomarkers of ethanol synthesis have been introduced.

**3.6.1.1. Nonoxidative Metabolites of Ethanol.** Phosphatidylethanol and esters between ethanol and short-chain fatty acids have been recognized as markers of antemortem ethanol ingestion *(16,17)*. These metabolites can be measured in blood using sensitive methods, such as GC-MS, and are excreted in urine with half-lives longer than ethanol *(18)*.

**3.6.1.2. Serotonin Metabolites.** Two urinary metabolites of serotonin, 5-hydroxytryptophol (5HTOL) and 5-hydroxyindoleacetic acid (5HIAA), have been investigated *(19)*. These analytes can be measured using gas chromatography-mass spectrometry (GC-MS). A urinary ratio of 5HTOL/5HIAA >15 suggests that ethanol has undergone metabolism, and a positive blood ethanol result stems from antemortem ingestion *(19,20)*.

**3.6.1.3. Low-Molecular-Weight Volatiles.** Microbial synthesis of ethanol from different substrates also yields other low-molecular-weight volatiles such as isopropanol, *n*-propanol, isoamyl alcohol, acetaldehyde, and propionic acid *(21)*. Isobutyric acid and *n*-butanol have been proposed as reliable indicators of putrefaction, and if detected in blood, they indicate that blood ethanol result is uncertain *(21)*.

**3.6.1.4. Ethyl Glucuronide.** Minute amounts of ethyl glucuronide (EtG) are produced during enzymatic metabolism of ethanol. EtG can disclose recent drinking about 6–10 h after ethanol is no longer measurable *(22)*. Meanwhile, EtG does not seem to be produced by yeast or bacteria from glucose. Therefore, if ethanol is produced in the body after death, no detectable EtG should be expected in the samples analyzed. EtG levels were found to decrease in urine specimens contaminated with *Escherichia coli*, probably because of cleavage by β-glucuronidase *(23)*. These bacterial actions can be stopped by adding sodium fluoride that prevents the formation of polysaccharides by the bacteria and prevents bacterial growth *(22)*.

## 4. METHODS FOR MEASUREMENT OF ETHANOL

Methods for the measurement of ethanol range from nonspecific and semiquantitative techniques such as osmolality and diffusion methods, to assays that are quantitative and specific for each particular alcohol. The earliest methods for ethanol analysis were colorimetric techniques based on the oxidation of ethanol by potassium dichromate in an acidic medium. This method detects all volatile-reducing agents and therefore is not specific for ethanol.

### *4.1. Estimation of Alcohol by Osmolal Gap Assessment*

Another method for estimating ethanol, and other alcohol concentrations, is based on the increase in serum osmolality following alcohol ingestion. One important caveat of this technique is that osmolality should be measured using a freezing point depression technique. Use of a vapor pressure depression osmometer will result in underestimation of the serum osmolality. This occurs because alcohol, being a volatile substance, leads to an increase in the vapor pressure above the solution; nonvolatile solutes should normally decrease the vapor pressure above the solution. Measurement of the difference between measured osmolality, determined by freezing point depression, and osmolality

calculated using sodium, urea, and glucose measured in the same sample, can be used to estimate the alcohol content of the sample. Each milligram of ethanol, methanol, or isopropanol present in the sample leads to a specific increase in the serum osmolality. The relationship between alcohol concentration and the corresponding increase in serum osmolality is as follows:

$$1\,\text{mg/dL ethanol} = 0.22\,\text{mOsm/kg increase}$$

$$1\,\text{mg/dL methanol} = 0.34\,\text{mOsm/kg increase}$$

$$1\,\text{mg/dL isopropanol} = 0.17\,\text{mOsm/kg increase}$$

Discrepancies in the ability of these relationships to predict the presence of ethanol may be due to the presence of another volatile. However, some investigators have found that this technique may result in overestimation of alcohol concentrations by up to 30% *(24)*. Thus, although this technique may be useful for estimating the presence of volatiles, they should not be relied upon exclusively for assessing the concentration of the volatile that is present. The gap may also be increased because of the presence of nonalcohol compounds such as mannitol and low-molecular-weight hydrocarbons. Current recommendations indicate that very high osmolal gaps ($>50$ mOsM/kg) should be further investigated to determine the presence of an alcohol or other agent responsible for the increase in measured osmolality *(25)*.

### *4.2. Enzymatic Ethanol Methods*

Enzymatic measurement of ethanol is the most common method in use in clinical laboratories for quantitation of ethanol. These methods utilize ADH to oxidize ethanol to acetaldehyde with concomitant reduction of $NAD^+$ to NADH, the latter being measured directly at 340 nm.

$$NAD^+ + \text{ethanol} \xrightarrow{\text{ADH}} \text{NADH}\ (340\,\text{nm}) + \text{acetaldehyde} + H^+$$

In addition to measuring the NADH produced in the above reaction, the NADH that is produced may also be utilized in secondary reactions involving colorimetric, fluorometric, or electrochemical detection methods. One such colorimetric method couples the NADH that is produced to the reduction of iodonitrotetrazolium dye by diaphorase. The reduced iodonitrotetrazolium dye can be readily measured at 500 nm.

$$NAD^+ + \text{ethanol} \xrightarrow{\text{ADH}} \text{NADH} + \text{acetaldehyde} + H^+$$

$$\text{NADH + iodonitrotetrazolium dye} \rightarrow NAD^+ \text{ + reduced iodonitrotetrazolium dye (500 nm)}$$

Another detection scheme that has been used is termed radiative energy attenuation *(26,27)*. This fluorometric method is based on the degree of inhibition of fluorescence of fluorescein dye following the production of a colored dye. Thiazolyl blue dye (MTT) reacts with NADH and is reduced resulting in a colored compound with an absorbance at 565 nm. The color of the reduced dye reduces the amount of light interacting with the fluorescein, thereby decreasing its fluorescence. The decrease in

fluorescence intensity of the fluorescein dye present in the reagent is inversely related to ethanol present in the sample.

$$NAD^+ + \text{ethanol} \xrightarrow{ADH} NADH + \text{acetaldehyde} + H^+$$

$$NADH + MMT \xrightarrow{\text{Fluorescein}} NAD^+ + \text{reduced MMT}$$

The enzymatic ethanol methods are generally accurate and provide good agreement with chromatographic methods.

### *4.3. Electrochemical/Infrared Detection (Breath Ethanol Analysis)*

Measurement of ethanol in expired breath is an easy and noninvasive means for assessing ethanol. Small amounts of unmetabolized ethanol are expired with every breath, the amount of which is proportional to the whole blood ethanol concentration. The relationship between whole blood ethanol and ethanol in alveolar air is based on the underlying assumption that the ethanol present in 1 mL of whole blood will equilibrate with 2100 ml of alveolar air. Various factors can influence the exchange ratio of ethanol in blood with that in alveolar air. These factors include lung volume, body temperature, breath temperature, and hematocrit. Body size also plays a role with smaller individuals showing greater expired blood ethanol levels. In addition, the relative accuracy of breath ethanol decreases with increasing blood alcohol concentrations. Although the low false-positive rate of the breath ethanol test is acceptable for legal purposes, measurement of blood alcohol is more appropriate for clinical use in emergency settings, because breath tests can underestimate the degree of toxicity *(28)*.

Instruments to measure the ethanol content in expired breath are usually based on electrochemical detection. Ethanol present in expired air is oxidized by a fuel cell resulting in the production of free electrons. The current generated by these free electrons is directly proportional to the amount of ethanol oxidized by the fuel cell. Infrared detection of breath ethanol is the most common method in use. The amount of infrared energy lost because of absorption by ethanol is proportional to ethanol concentration in breath. Up to five different wavelengths may be used, resulting in high specificity for ethanol *(29)*.

### *4.4. Gas Chromatography*

GC is the reference method for alcohol testing. Serum, plasma, whole blood, urine, or vitreous fluid may be used. This method is specific for ethanol and is also able to identify other monohydroxy alcohols at the same time (Fig.1). Sample may be introduced directly onto the column (direct injection method) or by headspace analysis where the airspace above the sample is allowed to equilibrate with ethanol in the sample, and the air sample is subsequently analyzed. The direct injection technique offers the advantage of more rapid analysis; however, fouling of the column and plugging of the inlet and syringe can occur. Ethanol and other alcohols are separated using chromatography, and each alcohol is identified by its retention time (Fig.1). Quantitation is accomplished by comparing the peak height ratio of the sample to an internal standard with the peak height ratio of the calibration standard to an internal

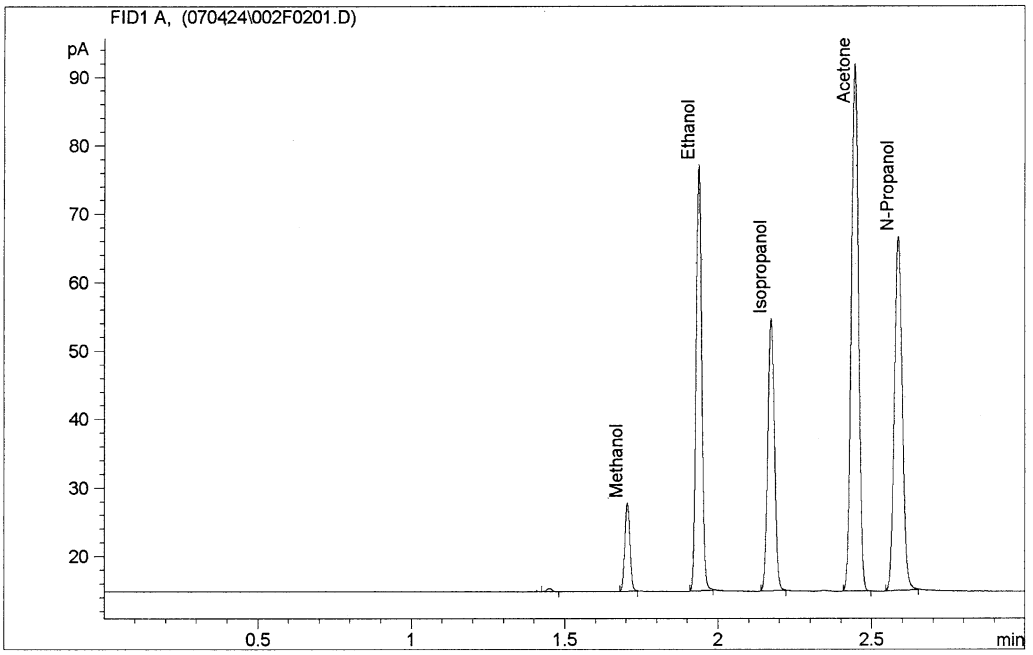


**Fig. 1.** Chromatogram of an alcohol standard containing 0.395% ethanol. *N*-propanol was used as the internal standard. The specimen injected in the column was 1.0 mL headspace sample of a blood alcohol mixture, with an isothermal oven temperature, 70°C equilibrium temp, and flow rate of 20 mL per minute. Retention time of each alcohol is on the horizontal axis.

standard. The internal standard typically used is *n*-propanol (Fig.1). Another advantage of GC is the capability of this technique for simultaneous analysis of other volatiles such as diethylene glycol, ethylene glycol, methanol, acetone, and isopropyl alcohol along with ethanol. There are numerous methods reported in the literature. For example, Williams et al. *(30)* used a capillary column and GC methods for simultaneous quantitation of diethyl glycol, ethylene glycol, methanol, isopropanol, acetone along with ethanol. After removal of serum proteins from the specimen by ultrafiltration, 1 ml of ultrafiltrate was manually injected into the GC column. The authors used *n*-propanol as an internal standard for analysis of alcohols and acetone and 1,3-butanediol for analysis of glycols *(30)*.

### *4.5. Gas Chromatography-Mass Spectrometry*

GC/MS has also been utilized for determination of alcohol concentration in serum. Dean et al. *(31)* reported a method for simultaneous determination of ethanol and ethyl–d5 alcohol in serum using stable isotope GC/MS. Wasfi et al. *(32)* developed a sensitive and specific method using static headspace GC combined with MS for quantitative determination of ethanol in biological fluids using *n*-propanol as an internal standard. The GC was performed in an isothermal mode with a run time of only 2.6 min, and the quantification was performed using a mass spectrometer operated in scan mode

abstracting a quantitative ion (m/z 31) and a qualifier ion (m/z 46) for ethanol and for the internal standard (m/z 31 and 60, respectively). The method was linear for a concentration range of 5–200 mg/dL, and no interference was observed from methanol, acetaldehyde, acetone, or endogenous materials *(32)*. Maeda et al. *(33)* also used an automated headspace GC combined with mass spectrometry for analysis of postmortem ethanol concentrations in pericardial fluid and bone marrow aspirate.

## 5. INTERFERENCES IN ETHANOL ANALYSIS

### *5.1. Enzymatic Assays*

Although ADH is reasonably specific for ethanol, interferences from isopropanol, methanol, and ethylene glycol have been reported. Acetone is not a ADH substrate. ADH does, however, show varying degrees of cross-reactivity with isopropanol (6%), methanol (3%), ethylene glycol (4%), and *n*-propanol (1%) *(29)*. Disinfection of skin with isopropanol before blood collection does not affect ethanol concentrations if the isopropanol is allowed to completely dry before phlebotomy. Use of ethanol to disinfect the skin has been shown to result in increases in measured blood ethanol concentrations *(34)*.

Falsely increased ethanol concentrations have also been found in patients with increased lactic acid and lactate dehydrogenase (LD) when ethanol is measured using enzymatic assays. This is because LD catalyzes the conversion of lactate to pyruvate and $NAD^+$ to NADH, thus generating a false signal even in the absence of any blood alcohol. However, the concentrations of lactate and LD necessary for such falsely increased results need to be at least 10 times the upper reference interval limit before an effect is seen *(35)*. The EMIT serum alcohol assay (Behring, San Jose, CA) has been reported to produce false-positive results in postmortem samples because of increased concentrations of lactate and LD. This phenomenon was also observed in living subjects with high concentrations of lactate and LD. A patient with lactic acidosis may have high serum LD concentration because of cellular breakdown. End-stage liver disease, liver transplant (biliary atresia), Duchenne muscular dystrophy, and chronic myelogenous leukemia may also lead to high LD and lactate in living patients that may cause false-positive ethanol results by immunoassays. Nine et al. *(36)* observed a correlation between increasing lactate and LD concentration and false-positive ethanol results. This interference was most noticeable with the EMIT assay for alcohol and less remarkable with Abbott and Roche assays. With EMIT assay, false-positive ethanol started at LD activity of 682 U/L and lactate concentration of 14 mmol/L. The threshold was much higher for Abbott and Roche assays. Interestingly, authors observed apparent disappearance of this interference in the EMIT assay with high levels of lactate and LDH. This may be related to depletion of the NAD coenzyme *(36)*.

Lactate concentrations tend to increase in trauma patients and Dunne et al. reported that 27% of 15,179 patients they studied had positive alcohol screen (mean alcohol: 141 mg/dL, 1 SD: 95 mg/dL, range: 10–508 mg/dL) and lactate correlated with magnitude of injury *(37)*. Therefore, measurement of alcohol using immunoassays in hospital laboratories may be of concern. However, Winek et al. *(38)* compared alcohol concentration obtained by using an immunoassay (Dimension, Dade Behring) and GC in trauma patients and observed no false positive using immunoassays. Alcohol

concentrations obtained by using immunoassays correlated well with GC values, and only in six specimens (out of 27) the differences between GC and immunoassay values exceeded 10%, and the highest difference was 22%. Authors concluded that immunoassay method can be used in hospital laboratory for determination of alcohol concentrations in trauma patients *(38)*.

The interference of LD and lactate in enzyme assays for alcohol can be eliminated by taking advantage of the high molecular weight of LD, and low molecular weight of ethanol. LD is absent in the protein-free ultrafiltrate, but alcohol is not bound to serum proteins and is present in the protein-free ultrafiltrate. Although lactate is present in the ultrafiltrate, lactate alone cannot cause this interference in the absence of LD. Measuring alcohol concentrations in protein-free ultrafiltrate (prepared by centrifugation of sera for 20 min at $1500 \times g$ using Centrifree Micropartition System, MW cutoff of filter: 30,000 Da) can completely eliminate interference of lactate and LD in alcohol determination using immunoassays *(39)*.

Serum or plasma is the most common specimen for ethanol analysis using ADH-based methods. Although whole blood may be used directly with some methods, others require a precipitation step before analysis to avoid interference from hemoglobin *(40)*.

### *5.2. Uropathogens Causing False-Negative Urinary EtG*

Following ethanol ingestion, the majority of ethanol (95–98%) is eliminated in the liver by conversion first to acetaldehyde by ADH and then to acetic acid by aldehyde dehydrogenase. A very small fraction of ingested ethanol (<0.1%) is converted to EtG and ethyl sulfate (EtS) by uridine–diphosphate–glucuronyltransferase and sulfotransferase, respectively *(41,42)*. Because both EtG and EtS have longer elimination times than ethanol *(42)*, a positive result of EtG and/or EtS indicates that the person was recently drinking ethanol, even if ethanol concentration is no longer detected.

EtG, but not EtS, is cleaved by β-glucuronidase, an enzyme that is found in most strains of *E. coli (23)*. *E. coli* is the predominant pathogen responsible for almost 80% of urinary tract infections *(43)*. Helander and Dahl *(44)* reported that EtG may not be stable if urine specimens are infected with microorganisms possessing β-glucuronidase activity. However, EtS was found to be stale to bacterial hydrolysis. Therefore, refrigeration or freezing of specimens or use of fluoride preservative is necessary to prevent bacterial growth and reduce EtG hydrolysis.

## 6. CONCLUSION

Despite limitations, enzyme-based immunoassays are used for rapid determination of alcohol concentrations in clinical laboratories, but for forensic application, GC or GC/MS should be the method of choice. Breath analysis is a rapid way to identify a subject with alcohol intoxication, but this test also suffers from limitation. Alcohol can also be measured indirectly by using osmolar gap; but direct measurement of alcohol in serum or whole blood is the state of art practice. For legal alcohol, caution should be taken with regard to methodology and chain of custody.

## REFERENCES


1. Freeza M, diPadova C, Pozzato G, Terpin M, Baraona E, Lieber CS. Higher blood alcohol levels in women: The role of decreased alcohol dehydrogenase activity and first pass metabolism. *N Engl J Med* 1990;322:95–99.
2. Watson WA, Litovitz TL, Klein-Schwartz W, et al. 2002: Annual report of the American association of poison control centers toxic exposure surveillance system. *Am J Emerg Med* 2003;21:353–421.
3. Jacobson D, McMartin KE. Methanol and ethylene glycol poisonings. Mechanism of toxicity, clinical course, diagnosis and treatment. *Med Toxicol* 1986;1:309–334.
4. Warden CR. Alcohols. In: *Emergency Medicine*. Abhababian RV, ed. *The Core Curriculum*. Philadelphia: Lippincott-Raven, 1998:1021–1023.
5. Winek CL, Carfagna M. Comparison of plasma, serum, and whole blood ethanol concentrations. *J Anal Toxicol* 1987;11:267–268.
6. Winek CL, Paul LJ. Effect of short term storage conditions on alcohol concentrations in blood from living human subjects. *Clin Chem* 1983;29:1959–1983.
7. Jones AW. Medicolegal alcohol determinations – breath- or blood- alcohol concentrations? *Forensic Sci Rev* 2000;12:23–47.
8. Mason MF, Dubowski KM. Breath as a specimen for analysis for ethanol and other low molecular weight alcohols. In: *Medicolegal Aspects of Alcohols*. Garriott JC, ed. Tucson, AZ: Lawyers & Judges Publishing Co., 1996: pp. 171–180.
9. Kwong TC, Jenny RW, Jortnai SA, Pinder RD. Clinical breathe alcohol testing. Proposed quality assurance guidelines. Washington DC; American Association for Clinical Chemistry TDM Toxicol Division, 2000.
10. Jones AW. Pharmacokinetics of ethanol in saliva: comparison with blood and breath alcohol profiles, subjective feelings of intoxication, and diminished performance. *Clin Chem* 1993;39:1837–1844.
11. Caplan YH. Blood, urine, and other fluid and tissue specimens for alcohol analyses. In: *Medicolegal Aspects of Alcohols*. Garriott JC, ed. Tucson, AZ: Lawyers & Judges Publishing Co., 1996: pp. 137–150.
12. Coe JL. Postmortem chemistry update. Emphasis on forensic application. *Am J Forensic Med Pathol* 1993;14:91–117.
13. Kugelberg FC, Jones AW. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. *Forensic Sci Intl* 2006; (E-Pub ahead of print).
14. Chang J, Kollman SE. The effect of temperature on the formation of ethanol by Candida albicans in blood. *J Forensic Sci* 1989;34:105–109.
15. O'Neal CL, Poklis A. Postmortem production of ethanol and factors that influence interpretation: a critical review. *Am J Forensic Med Pathol* 1996;17:8–20.
16. Hansson P, Varga A, Krantz P, Alling C. Phosphatidylethanol in post-mortem blood as a marker of previous heavy drinking. *Int J Legal Med* 2001;115:158–161.
17. Refaai MA, Nguyen PN, Steffensen TS, Evans RJ, Cluette-Brown JE, Laposata M. Liver and adipose tissue fatty acid ethyl esters obtained at autopsy are postmortem markers for premortem ethanol intake. *Clin Chem* 2002;48:77–83.
18. Kulig CC, Beresford TP, Everson GT. Rapid, accurate, and sensitive fatty acid ethyl ester determination by gas chromatography-mass spectrometry. *J Lab Clin Med* 2006;147:133–138.
19. Helander A, Beck O, Jones AW. Distinguishing ingested ethanol from microbial formation by analysis of urinary 5-hydroxytryptophol and 5-hydroxyindoleacetic acid. *J Forensic Sci* 1995;40:95–98.
20. Johnson RD, Lewis RJ, Canfield DV, Dubowski KM, Blank CL. Utilizing the urinary 5-HTOL/5-HIAA ratio to determine ethanol origin in civil aviation accident victims. *J Forensic Sci* 2005;50:670–675.
21. Statheropoulos M, Spiliopoulou C, Agapiou A. A study of volatile organic compounds evolved from the decaying human body. *Forensic Sci Int* 2005;153:147–155.
22. Lough PS, Fehn R. Efficacy of 1% sodium fluoride as a preservative in urine samples containing glucose and Candida albicans. *J Forensic Sci* 1993;38:266–271.

23. Kilian M, Bulow P. Rapid identification of Enterobacteriaceae. II. Use of a beta-glucuronidase detecting agar medium (PGUA agar) for the identification of E. coli in primary cultures of urine samples. *Acta Pathol Microbiol Scand [B]* 1979;87:271–276.
24. Bhagat CI, Beilby JP, Garcia-Webb P, Dusci LJ. Errors in estimating ethanol concentration in plasma by using the "osmolal gap". *Clin Chem* 1985;31:647–648.
25. Wu AHB, Broussard LA, Hoffman RS, et al. National academy of clinical biochemistry laboratory medicine practice guidelines: recommendation for the use of laboratory tests to support the impaired and overdosed patient from the emergency department. *Clin Chem* 2003;49:357–379.
26. Yost DA, Boehnlein L, Schaffer M. A novel assay to determine ethanol in whole blood on the Abbott TDX. *Clin Chem* 1984;30:1029A.
27. Cary PL, Whitter PD, Johnson CA. Abbott radiative energy attenuation method for quantifying ethanol evaluated and compared with gas-liquid chromatography and the Du Pont aca. *Clin Chem* 1984;30:1867–1870.
28. Currier GW, Trenton AJ, Walsh PG. Innovations: emergency psychiatry: relative accuracy of breath and serum alcohol readings in the psychiatric emergency service. *Psychiatr Serv* 2006;57:34–36.
29. Jones AW, Pounder DJ. Measuring blood alcohol concentrations for clinical and forensic purposes. In: *Drug Abuse Handbook*. Karch SB, ed. Boca Raton, FL: CRC Press, 1998.
30. Williams RH, Shah SM, Maggiore JA, Erickson TB. Simultaneous detection and quantitation of diethylene glycol, ethylene glycol, and the toxic alcohols in serum using capillary column gas chromatography. *J Anal Toxicol* 2000;24:621–626.
31. Dean RA, Thomasson HR, Dumaual N, et al. Simultaneous measurement of ethanol and ethyl d5-alcohol by stable isotope gas chromatography-mass spectrometry. *Clin Chem* 1996;42:367–342.
32. Wasfi IA, Al-Awadhi AH, Al-Hatali ZN, et al. Rapid and selective static headspace gas chromatography-mass spectrometric method for the analysis of ethanol and abused inhalants in blood. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;799:331–336.
33. Maeda H, Zhu BL, Ishikawa T, et al. Evaluation of postmortem ethanol concentrations in pericardial fluid and bone marrow aspirate. *Forensic Sci Int* 2006;161:141–143.
34. Peek GJ, Marsh A, Keating J, Ward RJ, Peters TJ. The effects of swabbing the skin on apparent blood ethanol concentration. *Alcohol Alcoholism* 1990;25:639–640.
35. Winek CL, Wahba WW. A response to "serum ethanol determination: comparison of lactate and lactate dehydrogenase interference in three enzymatic assays. *J Anal Toxicol* 1996;20:211.
36. Nine JS, Moraca M, Virji MA, Rao KN. Serum-ethanol determination: comparison of lactate and lactate dehydrogenase interference in three enzymatic assays. *J Anal Toxicol* 1995;19:192–196.
37. Dunne JR, Tracy JK, Scalea TM, Napolitano L. Lactate and base deficit in trauma: Does alcohol or drug use impair predictive accuracy? *J Trauma* 2005;58:959–966.
38. Winek CL, Wahba WW, Windisch R, Winek CL. Serum alcohol concentrations in trauma patients determined by immunoassays versus gas chromatography. *Forensic Sci Int* 2004;139:1–3.
39. Thompson WC, Malhotra D, Schammel DP, et al. False-positive ethanol in clinical and postmortem sera by enzymatic assay: elimination of interference by measuring alcohol in protein-free ultrafiltrate. *Clin Chem* 1994;40:1594–1595.
40. Gadsden RH, Taylor EH, Steindel SJ, et al. Ethanol in biological fluids by enzymatic analysis. In: *Selected Methods of Emergency Toxicology*. Frings CS, Faulkner WR, eds. Vol. 11. Selected Methods of Clinical Chemistry, Washington DC, AACC press, 1986, pp. 63–65.
41. Dahl H, Stephanson N, Beck O, Helander A. Comparison of urinary excretion characteristics of ethanol and ethyl glucuronide. *J Anal Toxicol* 2002;26:201–204.
42. Helander A, Beck O. Mass spectrometric identification of ethyl sulfate as an ethanol metabolite in humans. *Clin Chem* 2004;50:936–937.
43. Ronald A. The etiology of urinary tract infection: traditional and emerging pathogens. *Am J Med* 2002;113 (Suppl 1A):14S–19S.
44. Helander A, Dahl H. Urinary tract infection: a risk factor for false-negative urinary ethyl glucuronide but not ethyl sulfate in the detection of recent alcohol consumption. *Clin Chem* 2005;51:1728–1730.

# 16 Introduction to Drugs of Abuse Testing

*Tai C. Kwong, PhD*

***Contents***



**Summary**

Testing for drugs of abuse for clinical or forensic purposes has become a significant service of a toxicology laboratory. This chapter will summarize the basic practice of the various technical processes of drug testing, from specimen collection to analysis and reporting. Although urine remains the primary test specimen, the advantages and disadvantages of performing testing on alternate specimen matrices (hair, oral fluid, and sweat) will be discussed. The importance of initial test immunospecificity on test accuracy will be examined for the common drug groups. The need for confirmation will be discussed and the basic principles of mass spectrometry including gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, and tandem mass spectrometry will be presented.

**Key Words:** Abused drugs; alternative specimens; GC-MS; immunoassays; urine testing.

## 1. INTRODUCTION

The abuse of drugs, both medications and illicit drugs, is widespread. The risk to public safety, loss in workplace productivity, and impact on the health and welfare of the drug abusers have resulted in many government, business, public health, medical, and educational initiatives to combat drug abuse. Drug testing is used increasingly as a deterrent to illicit drug use and as an objective means to document drug abuse among patients, job applicants, employees, athletes, and students.

The traditional clinical toxicology service has been to support the emergency department for the diagnosis and treatment of suspected drug overdose patients. This

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role in recent years has been largely supplanted by demands from other clinical services for managing the medical consequences of drug abuse; pediatrics for testing newborns suspected of in utero drug exposure, adolescence medicine for drug dependency; obstetrics for pregnant addicts at risk for drug-related complications; organ transplant for assessment of candidates; pain management for monitoring patients on narcotic medications for compliance and for deterrence against diversion of drug for street sale; psychiatry and addiction medicine for monitoring drug treatment/rehabilitation outcome.

Drug testing in the workplace for nonmedical purposes (e.g., pre-employment and random workplace testing) is forensic testing, requiring detailed documentation of the chain of custody of test specimens and aliquots from collection to analysis and the use of the highest standards of analytical methodologies. The focus of this chapter is on clinical drugs of abuse testing, but forensic testing will be mentioned whenever it is relevant to the discussion of clinical testing.

## 2. SPECIMEN MATRICES

Most drug testing activities are based on urine although alternate specimen matrices—hair, oral fluid, sweat, and meconium—are gaining in popularity in specific testing situations. Urine as a drug testing specimen has many distinct advantages, but also possesses some disadvantages *(1,2)*. The advantages of using urine specimens are the ease of collection and the relatively high drug concentrations making detection and quantitation possible with relatively inexpensive instrumentation. Therefore, the cost of testing is relatively low. Moreover, there is vast field experience gained from many years of urine drug testing to draw on for analytical methodologies and the interpretation of results. The disadvantages of urine testing are that urine drug concentrations are not related to drug effects and that the windows of detection of drugs/metabolites of 1–7 days reflect recent use. Furthermore, urine specimens can be easily adulterated or tampered with, and the vigorous collection protocols designed to deter adulteration and tampering are intrusive and raise the issue of invasion of privacy.

The current urine cutoffs mandated by the Department of Health and Human Services, Substance Abuse and Mental Health Services Administration (SAMSHA), and the Department of Transportation (DOT) are listed in Table 1.

### *2.1. Hair*

The use of hair for drugs of abuse testing is recent despite its long established use in metal testing *(3)*. The drug or metabolite concentrations in hair are relatively low, and analysis requires analytical techniques that are far more challenging than those used for urine testing. Parent drugs are generally present in higher concentrations than those of metabolites, thus rendering the urine immunoassays designed to detect metabolites inadequate for hair testing. Sensitive and parent drug-specific radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are used as initial screening tests in hair testing. Confirmation testing requires more sensitive methodologies and generally involves the use of more advanced mass spectrometers, particularly those using tandem mass spectrometry [gas chromatography mass spectrometry-mass spectrometry (GC-MS-MS) or liquid chromatography mass spectrometry-mass spectrometry (LC-MS-MS)]. Proper sample preparation is particularly critical: careful washing to remove

**Table 1**
**Cutoff Concentrations Mandated by Federal Drug Testing Programs***

| *Drug or drug class* | *Immunoassay (ng/ml)* | *GC-MS confirmation* | *(ng/ml)* |
|---|---|---|---|
| Amphetamines | 1000 | Amphetamine | 500 |
| | | Methamphetamine | 500[a] |
| Cannabinoids | 50 | THC-COOH[b] | 15 |
| Cocaine metabolites | 300 | Benzoylecgonine | 150 |
| Opiates | 2000 | Morphine | 2000 |
| | | Codeine | 2000 |
| | | 6-Acetylmorphine | 10[c] |
| Phencyclidine | 25 | Phencyclidine | 25 |

* Department of Health and Human Services, Substance Abuse Mental Health Services Administration (SAMHSA). Federal Regist 1988; 53:11970; Federal Regist 1994;59:29908; Federal Regist 1997; 62:51118. Department of Transportation (DOT). Federal Regist 2000; 65:79462.

[a] Amphetamine must be present ≥200 ng/ml.

[b] THC-COOH, 11-nor-delta 9-tetrahydrocannabinol-9-carboxylic acid.

[c] Test for 6-acetlmorphine when morphine concentration >2000 ng/ml.

external contamination and digestion (chemical or enzyme) or pulverization of hair prior to solid phase extraction. The advantages of using hair are specimen collection (of head hair) is noninvasive; detection window is for longer term drug use (up to 90 days); specimen adulteration and tampering is more difficult; hair specimen is stable, allowing long-term storage; shipment or transportation of specimen is easier.

Because hair grows at a fairly constant rate of approximately 0.3–0.4 mm/day, it is theoretically possible to perform analysis on hair segments to yield a retrospective, long-term measure of drug use *(4)*. The disadvantages of hair testing are that it cannot detect recent drug use; sample preparation and analysis are technically challenging; there is a limited knowledge of drug disposition into hair including variations which are dependent on differences in texture and color pigmentation of hair *(5)*. Hair testing for clinical purposes is limited to testing of newborns for the diagnosis of in utero drug exposure *(6)*.

The cutoff values for hair testing in the proposed revisions of the SAMHSA guidelines are listed in Table 2.

### *2.2. Oral Fluid*

Oral fluid has been used in clinical laboratories for therapeutic drug monitoring. Most drugs get into saliva by diffusion or ultrafiltration *(7)*. The resultant saliva drug concentrations are partly dependent on saliva pH, which changes and becomes more alkaline as flow is stimulated for collection. The detection window for drugs and metabolites in oral fluid is relatively short. Therefore, the detection of a drug in saliva indicates recent use. Drug concentrations in saliva are generally proportional to those found in plasma, and it is possible to relate drug concentration in saliva to pharmacological effects *(7)*. Oral fluid is used both clinically and forensically for ethanol testing using point of care (POC) devices that have been cleared by the FDA and approved by the National Highway Traffic Safety Administration for inclusion in its

**Table 2**
**SAMHSA Cutoff Values in Proposed Revisions to Mandatory Guidelines[a]**

| | *Hair (pg/mg)* | *Oral fluid (ng/ml)* | *Sweat (ng/patch)* | *Urine (ng/ml)* |
|---|---|---|---|---|
| Initial test | | | | |
| THC metabolites | 1 | 4[b] | 4 | 50[c] |
| Cocaine metabolites | 500 | 20 | 25 | 150 |
| Opiates[d] | 200 | 40 | 25 | 2000 |
| Phencyclidine | 300 | 10 | 20 | 25 |
| Amphetamines[e] | 500 | 50 | 25 | 500 |
| MDMA | 500 | 50 | 25 | 500 |
| Confirmation test | | | | |
| THC Parent drug | | | 2 | 1 |
| THC metabolite | 0.05[c] | | | 15[c] |
| Cocaine | 500[f] | 8[g] | 25[g] | |
| Cocaine metabolite | 50[f] | 8[g] | 25[g] | 100[n] |
| Opiates | | | 25[h] | |
| Morphine | 200 | 40 | | 2000 |
| Codeine | 200 | 40 | | 2000 |
| 6-Acetylmorphine | 200[i] | 4 | | 10[j] |
| Phencyclidine | 300 | 10 | 20 | 25 |
| Amphetamine | 300 | 50 | 25 | 250 |
| Methamphetamine | 300[k] | 50[l] | 25[l] | 250[m] |
| MDMA | 300 | 50 | 25 | 250 |
| MDA | 300 | 50 | 25 | 250 |
| MDEA | 300 | 50 | 25 | 250 |

MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine.
[a] Federal Register 2004;69:19673.
[b] THC Parent drug and metabolite.
[c] $\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH).
[d] Initial test 6-acetylmorphine permitted at cutoffs of 200 pg/mg (hair), 4 ng/ml (oral fluid), 25 ng/patch (sweat), and 10 ng/ml (urine).
[e] Methamphetamine is the target analyte.
[f] Cocaine ≥ cutoff and BZE/Cocaine ≥0.05 or cocaethylene ≥50 pg/mg or norcocaine ≥50 pg/mg.
[g] Cocaine or benzoylecgonine.
[h] Morphine, codeine, or 6-acetylmorphine.
[i] Specimen must also contain morphine ≥200 ng/ml.
[j] If both initial test kits were used to screen concurrently, may report 6-AM alone.
[k] Specimen must also contain amphetamine ≥50 pg/mg.
[l] Specimen must also contain amphetamine ≥ limit of detection.
[m] Specimen must also contain amphetamine ≥100 ng/ml.
[n] Benzoylecgonine.

Conforming Product List *(8)*. Oral fluid testing has been used extensively in insurance testing for cotinine and HIV. Several commercial products for collection of oral fluid for testing drugs of abuse have been evaluated *(9,10)*. Sensitive immunoassays, many of which are ELISA, for detection of parent drugs (typically the principle analytes) are now available. Oral fluid testing, when used at point of collection, has potential for use in forensic roadside drug testing and parole/probation testing and in clinical

settings such as occupational medicine clinics, drug treatment programs, and hospital emergency departments.

The advantages of oral fluid testing for drugs of abuse include easy and noninvasive collection, the possibility of relating drug concentration to pharmacological effects and the option of POC testing *(2)*. The disadvantages are the relatively short detection window and contamination of oral cavity resulting in high drug concentration if drug is administered by ingestion or smoking. Moreover, there is no proficiency testing program.

The cutoff values for oral fluid testing in the proposed revisions of the SAMHSA guidelines are listed in Table 2.

### *2.3. Sweat*

Sweat testing for drugs of abuse requires a specially designed collection device that typically consists of an absorbent patch that can be worn on the skin for an extended period of time, but cannot be reattached once it is removed. The device is nonocclusive in that it allows water vapor and other volatile compounds to evaporate while trapping larger drug molecules. The device is also impermeable to external contamination. Sweat collection is noninvasive but at the end of the collection period (e.g., 1 week), the device is sent to a specialized laboratory for testing. The principle analytes in sweat are the parent drugs. Sweat testing provides a cumulative and prospective, rather than retrospective, measure of drug exposure once the device is applied. Thus, sweat testing is a sensitive method for detecting drug use and its use is most applicable in monitoring drug abstinence among individuals enrolled in drug treatment programs or in criminal justice probation/parole programs *(11,12)*. The results, however, at least in detection of cocaine use, may be affected by the collection period and by the site of sweat collection, and loss of cocaine may occur from skin collection patches *(13)*. Other disadvantages of sweat testing is that the entire patch is consumed for testing, thus precluding any re-testing. Moreover, there is no proficiency program to support sweat testing and there is still very little knowledge on the pharmacokinetics and drug disposition into sweat as well as the possible susceptibility of the sweat patches to environmental contamination *(14)*.

The cutoff values for sweat testing in the proposed revisions of the SAMHSA guidelines are listed in Table 2.

### *2.4. Meconium*

Meconium is a specimen matrix used exclusively for documentation of in utero drug exposure. Meconium is the a green, viscous material in the newborn's stool during its first 2–3 days of life and it begins to form in the second trimester. Drugs and metabolites are deposited in the meconium either from excretion from the bile or from the fetus's swallowing of amniotic fluid, which contains the compounds eliminated by fetal urine. Thus, meconium, as a waste product of the fetus that accumulates, provides a window of detection of drug exposure that is much longer than that of urine, the latter being a few days from the most recent episode of drug use by the mother. Therefore, meconium testing provides a higher detection rate of fetal drug exposure *(15,16)*. Meconium is easily collected from a diaper and usually available in

quantities in excess of minimum amount required for analysis. This is in contrast to urine specimens, which are difficult to collect from newborns in quantities sufficient for screening and confirmation testing. Meconium, being a tarry material, is not easy to handle, particularly in the weighing process, and there are more sample handling steps, including extraction of drugs, before initial testing using standard immunoassays *(17)*.

## 3. URINE DRUG TESTING PROCESSES

Testing for drugs of abuse in urine generally is restricted to alcohol and several drugs that have high prevalence of abuse, not all of which are illicit drugs. The test menu varies with the intents of the testing programs and includes various combinations of the following drugs or metabolites:

- Alcohol (ethanol).
- Amphetamines* (amphetamine* and methamphetamine*, sympathomimetic amines).
- Barbiturates (amobarbital, butalbital, and secobarbital).
- Benzodiazepines (alprazolam, clonazepam, diazepam, nitrazepam, nordiazepam, oxazepam, and temazepam).
- Buprenorphine.
- Cannabinoids*.
- Cocaine metabolites* (cocaine, benzoylecgonine*).
- Fentanyl.
- Methylenedioxyamphetamine (MDA).
- Methylenedioxymethamphetamine (MDMA).
- Methadone.
- Opiates* (morphine*, codeine*, 6-acetylmorphine*, hydrocodone, and hydromorphone).
- Oxycodone.
- Phencyclidine*.
- Propoxyphene.

For example, the Federal Drug Testing Programs mandate the testing of those drugs which have an asterisk (the "NIDA five"), and a pain management clinic may be interested in monitoring its patients for the opioids (buprenorphine, opiates, oxycodone, methadone and propoxyphene). Typical testing protocol is based on an initial test (screening test) using immunoassays in a qualitative mode. Results are designated as positive or negative according to the cutoffs chosen, and, depending on the testing requirements, positive results are then subjected to confirmation testing (see Section 4).

The immunoassays are calibrated at the cutoff concentrations, and a specimen that yields a response equal to or greater than that of the cutoff calibrator is positive, and negative if the response is less. The cutoffs mandated by the Federal Drug Testing Programs for Federal Employees (SAMHSA) and those by the DOT are listed in Table 1. These cutoffs are not at the limits of detection of the assays. Each cutoff is chosen as a compromise: higher than the assay detection limit to distinguish reliably a positive response from analytical noise and yet low enough not to miss the detection of drug use. Therefore, a negative result should not be interpreted as being devoid of drug.

It should be pointed out that workplace drug testing programs that do not fall under the regulations of the Federal and DOT Guidelines can choose not to use the cutoffs

mandated for the federal programs. Although these are forensic drug testing cutoffs, many clinical laboratories have adopted them as well. Workplace drug testing cutoffs, however, may be too high for clinical testing; using lower cutoffs will greatly increase the clinical sensitivity of the test *(18,19)*.

### *3.1. Initial Test Immunoassays*

Initial immunoassays are either performed on an automated instrument in the laboratory, or by single-use point-of-care or POC devices (see Section 5). The most commonly used immunoassays are RIA, enzyme-multiplied immunoassay technique (EMIT; Dade Behring Diagnostics), fluorescence polarization immunoassay (FPIA, Abbott Diagnostics), cloned enzyme donor immunoassay (CEDIA, Microgenics Corp.), kinetic interaction of microparticles in solution (KIMS, Roche Diagnostics), and ELISA. These assays differ from each other in their immunospecificity, assay dynamic range, linearity and slope of the response curve around the cutoff, and susceptibility to the actions of adulterants.

The immunospecificity of an immunoassay determines its accuracy. An immunoassay with poor specificity can yield erroneous results, which can be either false positive [e.g., dextromethorphan triggering the phencyclidine (PCP) assay] or false negative (the opiate assay failing to detect oxycodone). In the following sections for individual drug groups, the issue of immunospecificity will be discussed in terms of (a) lack of specificity—detecting a compound other than the target analyte, yielding a false-positive result; (b) too restricted or inadequate specificity—inability to detect some important members of a drug group.

### *3.2. Amphetamines*

Immunoassays for amphetamine and methamphetamine can be divided into two general types: those designed to favor the detection of amphetamine and methamphetamine only and those that also have variable cross-reactivities with "designer amphetamines" such as MDA, MDMA, MDEA methylenedioxyethylamphetamine and with sympathomimetic amines such as ephedrine, pseudoephedrine, phenylpropanolamine, and phentermine. In workplace drug-of-abuse screening, those amphetamine immunoassays that have high specificity for amphetamine and methamphetamine are considered operationally advantageous because they reduce the number of initial positives caused by sympathomimetic amines; consequently, fewer samples have to go to costly confirmation testing. For clinical toxicology, however, amphetamines "class" assays directed toward a broad spectrum of sympathomimetic amines should be used so that patients exposed to sympathomimetic amines will not be missed *(20)*. Immunoassays designed specifically to detect the designer amphetamines are available *(21,22)*.

Pharmaceutical methamphetamine is *d*-methamphetamine; amphetamine, however, is available as *d*-amphetamine as well as a racemic mixture (e.g., Adderall®). Illicit methamphetamine products are either the *d*-isomer or racemic mixture, and *l*-methamphetamine, compared to the *d*-isomer, has much lower potency as a central nervous system stimulant and is of little interest to drug abusers and is available as

a nonprescription nasal inhalant. For detection of illicit amphetamine and methamphetamine use, immunoassays are designed to detect the *d*-isomers of methamphetamine and amphetamine, and assays are calibrated with *d*-isomer of either amphetamine or methamphetamine *(23)*. Definitive identification of the enantiomer is needed to distinguish between illicit use of *d*-methamphetamine and over-the-counter use of *l*-methamphetamine. Standard gas chromatography-mass spectrometry (GC-MS) confirmation methods cannot distinguish between these isomers. The isomers must be converted by optically active derivatizing reagent into diasteriomers, which then can be chromatographically separated prior to mass spectrometric analysis *(24)*.

### *3.3. Barbiturates*

Short to intermediate-acting (amobarbital, butalbital, pentobarbital, and secobarbital) barbiturates are most commonly abused, whereas phenobarbital, a long-acting barbiturate used as an antiepileptic, is rarely abused. Several immunoassays for barbiturates are available: EMIT, FPIA, CEDIA, KIMS, and RIA. Some of the assays can be run at 200 or 300 ng/ml cutoffs, with secobarbital as the calibrator. Most of these assays have sufficient cross-reactivity to detect butalbital and amobarbital also *(25)*. Immunospecificity for other barbiturates such as phenobarbital and pentobarbital varies with the assays.

### *3.4. Benzodiazepines*

The benzodiazepines are prescribed for their anxiolytic, sedative, and anticonvulsant activities. They are among the most prescribed drugs in the United States, but they are rarely the sole drug of abuse; abusers of benzodiazepines are usually poly-drug users.

Several commercial immunoassays for benzodiazepines are available: EMIT, KIMS, FPIA, CEDIA, and RIA. These assays have different specificities for benzodiazepines and their metabolites. The benzodiazepines are extensively metabolized; for some (e.g., lorazepam), very little of the parent drug is excreted unchanged and the major urinary metabolites are the glucuronide conjugates. Therefore, immunoassays that are designed to detect the parent benzodiazepine compounds, and not the glucuronides metabolites, can improve their detection rate greatly by a hydrolysis step using glucuronidase *(26)*. Hydrolysis will improve the detection rate of benzodiazepines such as oxazepam, temazepam, and lorazepam in particular.

A well-documented positive interference with several commonly used immunoassays for benzodiazepines (EMIT, FPIA, CEDIA, and Triage) is the nonsteroidal anti-inflammatory drug oxaprozin *(27)*.

### *3.5. Cannabinoids*

11-nor-delta 9-tetrahydrocannabinoid (THC) is the principle psychoactive cannabinoid in marijuana. Its major urinary metabolites are 11-nor-delta 9-tetrahydrocannabinoid carboxylic acid (THCA) and its glucuronide conjugate. Cannabinoid metabolites immunoassays (EMIT, FPIA, CEDIA, and KIMS) are designed for THCA detection and are calibrated with this compound; typical cutoffs used are 20, 25, 50, and 100 ng/ml of THCA. These assays do have substantial

cross-reactivity with other cannabinoids, and THCA concentrations estimated semi-quantitatively (as THCA equivalents) can be 2–5 times higher than the actual THCA concentration determined quantitatively by GC-MS. Hence, the most commonly adopted GC-MS cutoff for THCA is 15 ng/ml.

THC has a very large volume of distribution (large tissue store), and continued release of THC from tissue storage can lead to a window of detection of THCA in urine that can range from 2 to 5 days for infrequent users to over 70 days for chronic heavy users *(28)*. The excretion profile of THCA since the last exposure should reflect a downward trend. Variation in hydration status, however, means that the same amount of THCA excreted into urine can be higher in concentration in a dehydrated individual (with lower urine volume) than a fluid-loaded individual. This can lead to fluctuation in urine THCA concentrations, including "spikes" in the excretion profile, which could be interpreted as new use of marijuana. Because creatinine excretion into urine is at a fairly constant daily rate, it is possible to normalize the fluctuation of urine THCA concentration due to variation in hydration status by relating urine THCA concentration to creatinine concentration *(29)*. It has been proposed, based on a carefully controlled study, that an increase of 50% in the THCA : creatinine ratios of two urine specimens collected 24 h apart would indicate reuse of marijuana *(30)*.

### *3.6. Cocaine*

All the immunoassays for initial testing for cocaine exposure are assays for benzoylecgonine, the major metabolite in urine. These assays, including EMIT, FPIA, CEDIA, and KIMS, have very low reactivity with cocaine or the other major urinary metabolite, ecgonine methyl ester. Cocaine-specific assays are available (RIA and ELISA) and they are used mostly for forensic analysis and only rarely in clinical testing. Exposure to cocaine administered as a local anesthetic during otolarynological procedures (in 10–20% solutions) or in ophthalmological procedures (in 1–4% solutions) will result in a positive urine test for benzoylecgonine. But these are analytical true positive results because the source of benzoylecgonine is cocaine.

The current initial benzoylecgonine test cutoff used in both clinical and workplace drug testing is 300 ng/ml. The window of detection is 1–3 days, but can be greater than 10 days among patients who are chronic heavy users *(31)*. The sensitivity (detection rate) can be increased if the cutoff is reduced to 150 ng/ml or lower *(19,32)*. The proposed revisions of the Federal guidelines call for a decrease to 150 ng/ml (Table 2).

### *3.7. Lysergic Acid Diethylamide*

Lysergic acid diethylamide (LSD), a very potent hallucinogen taken in very small doses (typically $<1$ mg), is extensively metabolized. As a result, urinary concentration of LSD is very low, usually in the sub-nanogram per ml range. The cutoffs used in LSD immunoassays (EMIT, CEDIA, KIMS, ELISA, and RIA) are usually 0.5 ng/ml. The window of detection is 24 h or less *(33)*. The homogenous immunoassays, the enzyme immunoassay in particular (EMIT), are known to have poor immunospecificities, and false-positive results have been reported as a result of the interferences of tricyclic antidepressant and antipsychotic medications *(34,35)*. LSD is light sensitive and urine specimen should be protected from light during storage of specimen prior to analysis.

### 3.8. Methadone

Urine tests to detect methadone use are based on immunoassays that are designed to detect either methadone or its major urinary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) formed by cytochrome P450 3A4 system. Immunoassays for methadone have low reactivity with EEDP and vice versa. Polymorphism of CYP 3A4 can lead to substantial variation in methadone metabolism and elimination. Those patients who rapidly metabolize the methadone dose taken can have methadone concentrations in urine below the typical cutoff of 300 ng/ml. These urine specimens, however, do contain EDDP in high concentrations and yield positive results if an EDDP-specific immunoassay is used. It has been suggested that EDDP can be a marker for monitoring methadone compliance *(36)*. Huestis and co-workers *(37)* showed in their study that 2% of the specimens collected from patients who have taken methadone tested negative for methadone, whereas all specimens tested positive by the EDDP assay at a cutoff of 100 ng/ml. Theoretically, it is possible to have positive methadone and negative EEDP in a noncompliant patient who takes a dose of methadone just before urine collection, and there is insufficient time to metabolize the methadone to reach a detectable level of EDDP.

### 3.9. Opiates

The term opiates refer to naturally occurring alkaloids (morphine and codeine) obtained from the opium poppy, *Papaver somniferum,* as well as the semi-synthetic alkaloids (e.g., buprenorphine, dihydrocodeine, heroin, hydrocodone, hydromorphone, levophanol, oxycodone, and oxymorphone). The term opioids refers to a group of compounds that have pharmacological properties similar to morphine and affinity toward the opioid receptors. Opioids include not only the opiates, but also synthetic compounds that are structurally unrelated to morphine: fentanyl, meperidine, methadone, pentazocine, propoxyphene, and tramadol. Thus, all opiates are opioids but the reverse is not true. The opiate immunoassays are designed to detect morphine and codeine as the target analytes, and the cross-reactivity with other opiates varies with the assays. For example, most opiate assays have high cross-reactivity for hydrocodone and hydromorphone to detect their presence, much lower reactivity for oxycodone for reliable detection, and negligible reactivity for buprenorphine. With the recent popularity of oxycodone as a drug of abuse and the introduction of buprenorphine in the US market for treatment of heroin dependency, immunoassays specific for these two opiates are now available. Opiate assays have no reactivity with the opioids that are not opiates. Individual immunoassays developed to detect specific opioids are available for fentanyl, methadone (and EDDP), and propoxyphene. Clearly, consultation with manufacturer's package insert for assay immunospecificity is crucial to understanding the applicability and limitations of opiate assays for different drug testing programs.

It is important to understand the metabolism of the opiates for proper interpretation, particularly of the minor metabolites: approximately 10% of codeine is metabolized to morphine; hydromorphone and dihydrocodeine are minor metabolites of hydrocodone; oxymorphone is a metabolite of oxycodone; and hydrocodone and hydromorphone are minor metabolites found in the presence of very high codeine and morphine concentrations, respectively *(38,39)*. Otherwise, detection of a minor metabolite (e.g., hydro-

morphone) in addition to the prescribed medication (morphine) may be mistakenly interpreted as illicit drug use.

Poppy seeds may contain morphine and codeine. Therefore, consumption of poppy seed food may result in urinary presence of morphine and codeine in concentrations exceeding the 300 ng/ml cutoff. ElSohly and co-workers *(40)* have proposed guidelines to interpret urinary morphine and codeine results as to the source of the morphine. Alternatively, the initial test cutoff has been raised to 2000 ng/ml, a level sufficiently higher than those seen following ingestion of contaminated poppy seeds. An intermediary metabolite of heroin (diacetylmorphine) is 6-monoacetylmorphine (6-AM). Thus, the detection of 6-AM in the presence of morphine in urine points to heroin as the source of morphine. As 6-AM has a very narrow window of detection (6–12 h), its brief presence after heroin use can be missed. Recent reports have indicated that it is possible to have detectable 6-AM even if morphine concentration is below the 300 ng/ml threshold *(41)*. Therefore, a testing program using an opiate assay as the first test will miss these patients, hence, the proposal to use both opiates and 6-AM assays to screen for heroin abuse. The opiates cutoff used in workplace drug testing (2000 ng/ml) may be inappropriately high for clinical or postmortem toxicological testing for possible heroin use as many cases that had documented 6-AM had total opiates $<2000$ ng/ml *(42)*.

### *3.10. Phencyclidine*

PCP is one of the "NIDA five" drugs and is included in most workplace drug testing programs. For clinical drug testing, however, the prevalence of PCP abuse may be too low in some regions to justify routine testing for PCP. Immunoassays available for PCP include EMIT, FPIA, KIMS, and CEDIA. False-positive results have been reported due to interference by dextromethorphan *(43)*, diphendyramine *(44)*, and thioridazine *(45)*. In view of the low prevalence of PCP abuse and the poor specificity of the immunoassay, one should expect that the test has low positive predictive value and most presumptive positive results by immunoassay are likely to be false positives. Therefore, it is important to confirm a PCP positive immunoassay result by another analytical technique.

### *3.11. Propoxyphene*

Propoxyphene is a nonopiate opioid analgesic. It is extensively metabolized and the major urinary metabolite, norpropoxyphene, may accumulate to concentration levels higher than those of the parent compound. Most of the available immunoassays (EMIT, FPIA, KIMS, and CEDIA) do not have significant cross-reactivity with the norpropoxyphene. Diphenhydramine has been reported to cause a positive interference with the EMIT assay *(46)*

## 4. CONFIRMATION TESTS

As immunoassays lack absolute specificity for their target analytes, an initial immunoassay positive result should be considered as a presumptive positive only. Definitive identification of the drug must be based on confirmation testing. The standard of practice in analytical toxicology defines a confirmation test as a technique that

employs a different analytical principle and has better specificity and equivalent or better sensitivity than the initial test. Therefore, re-testing by a second immunoassay is not confirmation, because the second test is not based on a different analytical principle. Nor is an high performance liquid chromatography (HPLC) result confirmed by immunoassay acceptable because the second test is less specific than the first. While confirmation using a method based on MS is mandatory in forensic testing, other analytical techniques such as thin layer chromatography, GC, or HPLC are acceptable for clinical confirmation as long as the confirmation assay has been vigorously validated for its precision and accuracy.

Confirmation testing may not be needed in clinical toxicology in certain settings *(20)*. Physicians interpreting the initial test results have other information on the patient—history, physical examination, and other laboratory tests. Therefore, clinical drug test results are interpreted within the clinical context of the patient. In clinical settings where administrative or punitive actions maybe taken (e.g., removing a patient from drug treatment program because of repeated positive drug tests), confirmation testing may be indicated. Laboratories performing clinical testing that may have high potential for being involved in a medical legal challenge should consider instituting confirmation testing. An example would be drug testing of newborns for suspected in utero drug exposure because results, in some localities, are reported to local health authorities.

### *4.1. Mass Spectrometry*

In forensic testing, GC-MS has been the "gold standard" for confirmation technique. The advances in LC-MS instrumentation design have resulted in many laboratories adopting this newer MS technology. This section gives only a brief overview of MS. Interested readers are encouraged to refer two excellent book chapters on MS *(47,48)*.

A mass spectrometer consists of the following basic components: inlet, ion source, vacuum system, mass analyzer, and computer (Fig. 1): The "inlet" is a device through which samples are introduced into the mass spectrometer. The most common inlets are those interfacing GC or LC with the mass spectrometer. Because the molecules must be in the gas phase and the mass analyzer operates under vacuum, the bulk of the mobile phase—carrier gas in GC and liquid solvent in LC—delivering the molecules to the mass spectrometer must be removed, hence, the challenge of designing the interface, particularly for LC-MS, because the mobile phase is liquid. The "ion source" is where molecules in the gas phase are ionized (the different modes of ionization are discussed in sections 4.2 and 4.3). The ions formed are delivered to the "mass analyzer" (the

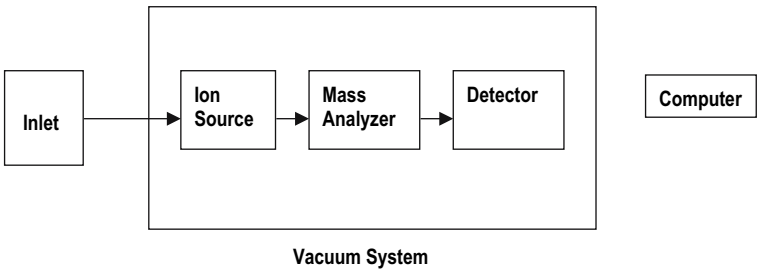


**Fig. 1.** Basic components of a mass spectrometer

different mass analyzer types are discussed in Section 4.4.) under vacuum where the ions of specific mass are selected and directed to the detector, usually an electron multiplier device.

### *4.2. Gas Chromatography-Mass Spectrometry*

GC-MS is still the mainstay confirmation technique in drugs of abuse testing, although in recent years there has been a greater use of LC and tandem MS. The successful interface of a GC to the mass spectrometer offers the advantage of combining chromatographic separation and spectral analysis for identification of analytes. For confirmation of drugs of abuse, electron ionization is the most common form of ionization. The molecules in the ion source, which is under vacuum, are exposed to a beam of electrons, resulting in the loss of an electron from a molecule to form a positively charged molecular ion ($M^+$). The molecular ion is usually fragmented due to the high energy of the electrons (70 eV). The fragmentation pattern (the mass spectrum) is characteristic of a molecule and can be used for identification of the compound. This can be accomplished by comparing the unknown spectrum to a library of mass spectra of known reference standards. A search algorithm helps to identify the unknown and provides an indication of quality of match of the mass spectra ("full spectral matches" or "full scan mode"). As the typical confirmation procedures in drugs of abuse testing are for a limited number of drugs or metabolites, the structural characteristics of which are well known, identification can be based on retention time data combined with the monitoring of several selected characteristic ions (usually 3) in place of full spectral matching. This mode of identification, called selected ion monitoring (SIM), is based on comparing the ratios of these ions ("ion ratios") with those obtained from a known reference standard. Because only a few mass fragments, not the whole spectrum of fragments, are analyzed, the data acquisition time for each of the selected ions is longer, allowing collection of more data points and improving signal-to-noise ratio. Therefore, SIM mode analysis is a more sensitive technique than full scan mode. For quantitative analysis in the SIM mode, the internal standard method is most often used. Because a mass spectrometer is capable of separating ions by mass, the best internal standards are the stable-isotope analogs of the analyte of interest. If the isotope is deuterium, then each molecule or fragment containing a deuterium atom instead of hydrogen will have one additional mass unit which can easily be discerned by the mass spectrometer.

### *4.3. Liquid Chromatography-Mass Spectrometry*

In recent years, successful interfacing of a LC to a mass spectrometer has led to a gradual movement to performing confirmation of drugs of abuse using LC-MS. This is particularly true for confirmation testing on specimens of alternate matrices (hair, sweat, and oral fluid). One advantage of liquid chromatographic over gas chromatographic separation is that as the mobile phase is aqueous, sample extraction procedures are less extensive. Unlike GC, LC does not require vaporization of the compounds at high temperature for chromatographic separation. LC can analyze the more polar and nonvolatile compounds without resorting to chemical derivatization to make these compounds suitable for analysis by GC. As LC can be performed at much lower

temperature, compounds that are unstable at the high operating temperature of GC can be analyzed by LC.

A technical challenge for the design of a LC-MS instrument is the large volume of liquid (mobile phase) that must be removed before the analytes can enter the mass spectrometer, which is under vacuum, for ionization and detection. In recent years, techniques have been developed for the ionization of analyte molecules to take place outside of the mass analyzer at atmospheric pressure (i.e., atmospheric pressure ionization). The ability to have the ionization process occurring at atmospheric pressure outside of the mass analyzer is a major advantage of LC-MS over GC-MS. Two atmospheric ionization techniques in common use for drugs of abuse testing are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

ESI involves the passage of the LC effluent though a small capillary nozzle to which a voltage has been applied. This results in a spray of small solvent droplets inside the ionization chamber and the transfer of energy (charge) to the droplets. As the droplets evaporate (assisted by gas flowing through the chamber), they decrease in size until they break apart, and a charge is transferred to the molecules inside. The charged molecules are then delivered to the mass analyzer for analysis.

APCI uses a fine spray of LC effluent that is vaporized in a high temperature tube. At the exit of the tube is applied a high voltage, resulting in the formation of ions between the solvent molecules and the sheath gas. These ions, in turn, react with analyte molecules to form ions, which are drawn into the mass analyzer.

### *4.4. Mass Analyzer*

The quadrupole mass spectrometer is the most common mass detector in use for drugs of abuse testing. The quadrupole analyzer is a set of four rods. At one radio frequency (rf) and DC voltage on the two sets of diagonally opposed rods, only ions of a single m/z value are "selected" and travel through the analyzer to the detector; all other ions with different m/z values are lost on collision to the rods. By scanning the rf and DC voltages, ions of increasing m/z values can be selected to travel to the detector, thus generating a mass spectrum.

Another mass analyzer, the ion trap, is a special version of the quadrupole. Ions are "trapped" within a three-dimensional space formed by the four "rods" rather than allowed to pass through as in a standard quadrupole. By changing the applied rf voltage, ions of increasing m/z values are ejected from the "trap" and directed to the detector.

### *4.5. Tandem Mass Spectrometry*

Tandem MS, or mass spectrometry/mass spectrometry (MS/MS), is the linking of several quadrupoles together. The most common configuration is the triple quads where three quadrupole analyzers are linked in series (Fig. 2). The first quadrupole acts as the mass filter allowing only ions having specific m/z ("precursor" or "parent" ions) to pass to the second quadrupole, called the "collision cell" where further fragmentation into "product" (or "daughter") ions takes place. The third quadrupole will further select one or more of the product ions to pass through to the detector.

Tandem MS offers tremendous advantages in selectivity of compounds. The added selectivity due to additional characterization of the product ions reduces the dependence on the chromatographic method to provide a single pure compound to the mass

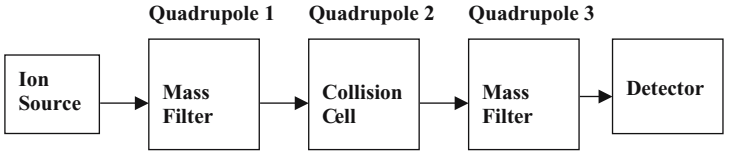


**Fig. 2.** Schematic of a triple quadrupole tandem mass spectrometer. Quadrupoles 1 and 3 are mass filters, quadrupole 2 is where further fragmentation of product ions selected by quadrupole 1 takes place.

spectrometer. Thus, tandem MS decreases the need for an elaborate sample preparation step and complicated chromatography protocol. When coupled with the added selectivity of an HPLC chromatographic separation, interferences in a well-designed MS/MS assay can be very low.

## 5. ONSITE TESTING

Onsite site is also referred to as point-of-care testing or point-of-collection testing (POCT). There has been much recent interest in the utility of rapid, easy-to-use urine drug testing devices in a variety of settings: emergency departments, drug treatment clinics, workplace drug testing, criminal justice, and enforcement of traffic laws. The major advantage of POCT is the reduction in turnaround time; shipping of specimens to centralized laboratories for testing is time-consuming and costly. Performing analysis at the site of collection and having results available without delay greatly increase the impact of test results. POCT testing provides (a) in the ED, rapid implementation of therapeutic intervention; (b) in drug treatment clinics, particularly if done in the presence of patients who tested positive, a powerful tool in deterring denial of drug use; (c) in the workplace, permits rapid hiring decision or deployment of workers in fitness for duty programs *(49)*.

POCT devices require no sophisticated instrumentation or a permanent laboratory. Urine testing devices in single- and multi-analyte configurations come in different formats, such as a dipstick, a test strip enclosed in a cassette, and a cup where the specimen is collected into a cup containing a test strip *(50)*. These devices are generally easy to use and rapid, with results usually available within a few minutes. Numerous devices for saliva testing are available and a few have been evaluated *(10)*.

The most common of the devices utilize immunochromatography (also described as lateral flow immunoassay) where a drug in urine migrates along a porous membrane and inhibits a drug–antibody capture on the membrane. The designs of many of the commercially available test kits are based on variations of this basic concept. In one version, for example, drug molecules in a urine specimen and drug conjugates, which are immobilized on the test zone, compete for binding to specific antibody conjugates, which are mobilized as the applied test specimen migrates on the device. If the drug in the urine is present above threshold (cutoff) concentration, it will bind all the antibody conjugates leaving no antibody conjugates to bind with the immobilized drug conjugates to form a color line. Thus, the end point for a specimen containing drug at concentration at or above below threshold is absence of a color line; that for a negative

specimen is the presence of a color line—a negative-indicating reaction. Most of the devices also have control lines to serve as internal quality control.

The accuracy of testing by these devices, however, is dependent on the experience and training of the operators *(51,52)*.

## 6. URINE COLLECTION

The quality of testing, be it for clinical or forensic purposes, can only be as good as the quality of the specimen. Hence, individuals who want to escape detection of their drug use would attempt to tamper with the testing process to produce negative test results. Various attempts to accomplish this include substitution with negative urine, dilution of the urine so that drug concentrations fall below the cutoffs, and addition of various chemicals to either inactivate the assays or chemically modify the analytes so that they become "invisible" to the initial and confirmation tests. Urine specimens can be easily tampered with and the collection protocol must be designed to deter and detect attempts at tampering. An observed collection is the most effective way to guard against attempts at tampering, but it is invasive and is objectionable to both the donor and the collector. Simple procedures, such as visual inspection for unusual appearance and taking specimen temperature, are used in all collection protocols to assess the validity of a urine specimen, and these can be supplemented with urine creatinine and specific gravity measurements. SAMHSA and DOT have published urine specimen collection guidance documents *(53,54)*, and SAMHSA has promulgated a set of definitions for specimen validity and also laboratory tests to assess validity *(55)*. Although these are specific requirements for the Federal Drug Testing Programs, many of the procedures can be adapted for use in nonregulated workplace or clinical testing programs (also see Chapter 17).

## 7. CONCLUSION

Drug testing is a useful laboratory tool for diagnosis and treatment of drug abuse and an effective deterrent to drug use in the workplace. To realize fully the usefulness of this tool requires thorough understanding of the various technical components of drug testing in terms of the standard of practice, the relative merits of different specimen matrices, the limitation of immunoassays used for initial testing, the need for confirmation, and the role of MS in confirmation testing.

## REFERENCES

1. Caplan Y, Goldberger B. Alternate specimens for workplace drug testing. J Anal Toxicol 2001; 25:396–399.
2. Cone E. Alternate samples: oral fluid (saliva), sweat, hair, and meconium. The Clinical Toxicology Laboratory, Contemporary Practice of Poisoning Evaluation. Washington, DC: AACC Press, 2001.
3. Kintz P. Drug Testing in Hair. Boca Raton, FL: CRC Press, 1996.
4. Strano-Rossi S, Bermejo-Barrera A, Chiarotti M. Segmental hair analysis for cocaine and heroin abuse determination. Forensic Sci Int 1995; 70:211–216.
5. Rollins DE, Wilkins DG, Kreuger GG, Augsburger MP, Mizuno A, O'Neal C, et al. The effect of hair color on the incorporation of codeine into human hair. J Anal Toxicol 2003; 27:545–551.
6. Koren G. Measurement of drugs in neonatal hair; a window to fetal exposure. Forensic Sci Int 1995; 70:771–782.

7. Samyn N, Verstraete A, Van Haeren C, Kintz P. Analysis of drugs of abuse in saliva. Forensic Sci Rev 1999; 11:1–19.
8. National Highway Traffic Safety Administration. Conforming products list of screening devices to measure alcohol in body fluids. Fed Regist 2005;70:72502–72503.
9. Crouch D, Walsh JM, Flegel R, Cangianelli L, Baudys J, Atkins R. An evaluation of selected oral fluid point-of-collection drug-testing devices. J Anal Toxicol 2005;29:254–257.
10. Walsh JM, Flegel R, Crouch D, Cangianelli L, Baudys J. An evaluation of rapid point-of-collection oral fluid drug-testing devices. J Anal Toxicol 2003; 27:429–439.
11. Preston LP, Huestis MA, Wong CJ, Umbricht A, Goldberger B. Monitoring cocaine use in substance-abuse-treatment patients by sweat and urine testing. J Anal Toxicol 1999; 23:313–322.
12. Levisky JA, Bowerman DL, Jemkins W, Johnson DG, Levisky JS, Karch SB. Comparison of urine to sweat patch results in court ordered testing. Forensic Sci Int 2001; 122:65–68.
13. Uemura N, Nath RP, Harkey MR, Henderson GL, Mendelson J, Jones RT. Cocaine levels in sweat collection patches vary by location of patch placement and decline over time. J Anal Toxicol 2004;28:253–259.
14. Kidwell DA, Smith FP. Susceptibility of PharmChek drugs of abuse patch to environmental contamination. Forensic Sci Int 2001; 116:89–106.
15. Ostrea EM, Jr, Brady MJ, Parks PM, Asensio DC, Naluz A. Drug screening of meconium in infants of drug-dependent mothers: an alternative to urine testing. J Pediatr 1989; 115:474–477.
16. Ryan RM, Wagne CL, Schultz JM, Varley J, DiPreta J, Sherer DM, et al. Meconium analysis for improved identification of infants exposed to cocaine in utero. J Pediatr 1994; 125:435–440.
17. ElSohly MA, Standford DF, Murphy TP, Lester BM, Wright LL, Smeriglio VL, et al. Immunoassay and GC-MS procedures for the analysis of drugs of abuse in meconium. J Anal Toxicol 1999; 23:436–445.
18. Wingert W. Lowering cutoffs for initial and confirmation testing for cocaine and marijuana: large scale study of effects on the rates of drug-positive results. Clin Chem 1997; 43:100–103.
19. Hicks JM, Morales A, Soldin SJ. Drugs of abuse in a pediatric outpatient population. Clin Chem 1990; 36:1256–1257.
20. Wu AHB, McKay C, Broussard LA, Hoffman RS, Kwong TC, Moyer TP, et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: recommendations for the use of laboratory tests to support poisoned patients who present to the Emergency Department. Clin Chem 2003; 49:357–379.
21. Hsu J, Liu C, Hsu CP, Tsay W-L, Li J-H, Lin D-L, Liu RH. Performance characteristics of selected immunoassays for preliminary test of 3,4-methylenedioxyamphetamine, methamphetamine, and related drugs in urine specimens. J Anal Toxicol 2003; 27:471–478.
22. Stout PR, Klette KL, Wiegand R. Comparison and evaluation of DRI methamphetamine, DRI Ecstasy, Abuscreen Online amphetamine, and a modified Abuscreen Online amphetamine screening immunoassays for the detection of amphetamine (AMP), methamphetamine (MTH), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) in human urine. J Anal Toxicol 2003; 27:265–269.
23. Verstraete AG, Heyden FV. Comparison of the sensitivity and specificity of six immunoassays for the detection of amphetamines in urine. J Anal Toxicol 2005; 29:359–364.
24. Cody JT. Important issues in testing of methamphetamine enantiomer ratios in urine by gas chromatography-mass spectrometry. In: Liu RH, Goldberger B, eds. Handbook of Workplace Drug Testing. Washington, DC: AACC Press, 1995:239–288.
25. Magnani B. Concentrations of compounds that produce positive results. In: Shaw L, Kwong T, eds. The Clinical Toxicology Laboratory, Contemporary Practice of Poisoning Evaluation. Washington, DC: AACC Press, 2001:482–497.
26. Borrey D, Meyer E, Duchateau L, Lambert W, Van Peteghem C, De Leenheer A. Enzymatic hydrolysis improves the sensitivity of Emit screening for urinary benzodiazepines. Clin Chem 2002; 48:2047–2049.
27. Nishikawa T, Kamijo Y, Ohtani H, Fraser AD. Oxaprozin interference with urinary benzodiazepine immunoassays and noninterference with receptor assay. J Anal Toxicol 1999; 23:125–126.

28. Ellis GM, Mann MA, Judson BA, Schramm NT, Tashchian A. Excretion patterns of cannabinoid metabolites after last use in a group of chronic users. Clin Pharmacol Ther 1985; 38:572–578.
29. Lafolie P, Beck O, Hjemdahl P, Borg S. Using relation between urinary cannabinoid and creatinine excretions to improve monitoring of abuser adherence to abstinence. Clin Chem 1994; 40:170–171.
30. Huestis MA, Cone E. Differentiating new marijuana use from residual drug excretion in occasional marijuana users. J Anal Toxicol 1998; 22:445–454.
31. Weiss RG, Gawin FH. Protracted elimination of cocaine metabolites in long-term high dose cocaine abusers. Am J Med 1988; 85:879–880.
32. Cone E, Sampson-Cone AH, Darwin WD, Huestis MA, Parks PM. Urine testing for cocaine abuse: metabolic and excretion patterns following different routes of administration and methods for detection of false-negative results. J Anal Toxicol 2003; 27:386–401.
33. Reuschel SA, Percey SE, Liu S, Eades DM, Foltz RL. Quantitative determination of LSD and a major metabolite, 2-oxo-3-hydroxy-LSD, in human urine by solid-phase extraction and gas chromatography-tandem mass spectrometry. J Anal Toxicol 1999; 3:306–312.
34. Ritter D, Cortese CM, Edwards LC, Barr JL. Interference with testing for lysergic acid diethylamide. Clin Chem 1997; 43:635–637.
35. Cody JT, Valtier S. Immunoassay analysis of lysergic acid diethylamide. J Anal Toxicol 1977; 21:459–464.
36. George S, Braithwaite RA. Application and validation of a urinary methadone metabolite (EDDP) immunoassay to monitor methadone compliance. Ann Clin Biochem 2000; 37:350–354.
37. Preston LP, Epstein DH, Davoudzadeh D, Huestis MA. Methadone and metabolite urine concentrations in patients maintained on methadone. J Anal Toxicol 2003; 27:332–341.
38. Oyler JM, Cone E, Joseph RE, Jr, Huestis MA. Identification of hydrocodone in human urine following controlled codeine administration. J Anal Toxicol 2000; 24:530–535.
39. Cone E, Heit HA, Caplan Y, Gourly D. Evidence of morphine metabolism to hydromorphone in pain patients chronically treated with morphine. J Anal Toxicol 2006; 30:1–5.
40. ElSohly HN, ElSohly MA, Standford DF. Poppy seed ingestion of opiate urinalysis: a closer look. J Anal Toxicol 1990; 14:308–310.
41. Beck O, Bowerman DL. Paradoxical results in urine drug testing for 6-acetylmorphine and total opiates: implications for best analytical strategy. J Anal Toxicol 2006; 30:73–79.
42. Moore KA, Addision J, Levine B, Smialek JE. Applicability of opiate cutoffs to opiate intoxication cases. J Anal Toxicol 2000; 25:657–658.
43. Boeckx RL. False positive EMIT DAU PCP assay as a result of an overdose of dextromethorphan. Clin Chem 1987; 33:974–975.
44. Levine B, Smith ML. Effects of diphenhydramine on immunoassay of phencyclidine in urine. Clin Chem 1990; 36:1258.
45. Long C, Crifasi J, Maginn D. Interference of thioridazine (Mellaril) in identification of phencyclidine. Clin Chem 1996; 42:1885–1886.
46. Schneider S, Wenning R. Interference of diphenhydramine with the EMIT II immunoassay for propoxyphene. J Anal Toxicol 1999; 23:637–638.
47. Annesley T, Rockwood AL, Sherman NE. Mass spectrometry. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. St Louis, MO: Elsevier Saunders, 2006:165–190.
48. Cody JT. Mass spectrometry. In: Levine B, ed. Principle of Forensic Toxicology. Washington, DC: AACC, 2006.
49. Jenkins AJ, Goldberger B. Onsite Drug Testing. Totowa, NJ: Humana Press, 2002.
50. Crouch D, Hersch RK, Cook RF, Frank JF, Walsh JM. A field evaluation of five on-site drug-testing devices. J Anal Toxicol 2002; 26:493–499.
51. George S, Braithwaite RA. Use of on-site testing for drugs of abuse. Clin Chem 2002; 48:1639–1646.
52. Kranzler HR, Stone J, McLaughlin L. Evaluation of a point-of-care testing product for drugs of abuse; testing site is a key variable. Drug Alcohol Depend 1995; 40:55–62.
53. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration. Urine specimen collection handbook for federal agency workplace drug testing programs.

http://dwp.samhsa.gov/DrugTesting/Level_1_Pages/HHS%20Urine%20Specimen%20Collection%20Handbook%20(Effective%20November%201,%202004).aspx (Accessed 10-12-2006).

54. Department of Transportation. Urine Specimen Collection Guidelines. http://www.dot.gov/ost/dapc/testingpubs/200612_urine_guidelines.pdf(Access 8-2-2007)
55. Department of Health and Human Services SAMHSA. Mandatory Guidelines for Federal Workplace Drug Testing Programs. Fed Regist 2004; 69:19644.

# 17 Urinary Adulterants and Drugs of Abuse Testing

*Amitava Dasgupta, PhD*

***Contents***



**Summary**

Persons abusing drugs attempt to adulterate urine specimens in order to escape detection. Household chemicals such as bleach, table salt, laundry detergent, toilet bowl cleaner, vinegar, lemon juice and Visine eye drops are used for adulterating urine specimens. Most of these adulterants except Visine eye drops can be detected by routine specimen integrity tests (creatinine, pH, temperature and specific gravity). However, certain adulterants such as Klear, Whizzies, Urine Luck and Stealth cannot be detected by using routine specimen integrity testing. These adulterants can successfully mask drug testing if the concentrations of certain abused drugs are moderate. Several spot tests have been described in the literature to detect the presence of such adulterants in urine, and recently, urine dipsticks are commercially available (AdultaCheck 4, AdultaCheck 6, Intect 7 and MASK Ultrascreen) for detecting the presence of such adulterants along with creatinine, pH and specific gravity.

**Key Words:** Adulterants; drugs of abuse; nitrite; pyridinium chlorochromate.

## 1. INTRODUCTION

Drug abuse is a critical problem not only in the United States but also throughout the world. Commonly abused drugs are cocaine, cannabinoids, amphetamine, phencyclidine and benzodiazepines. For many years, diazepam was the most prescribed drugs in the United States. Moreover, designer drugs such as 3,4-methylenedioxy

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amphetamine and 3, 4-methylenedioxy methamphetamine ("ecstasy") are commonly used in rave parties along with rohypnol (flunitrazepam) and gamma-hydroxy butyric acid.

On September 15, 1986, President Reagan issued Executive Order No 12564 directing federal agencies to achieve a drug-free work environment. Then the Department of Health and Human Services (DHHS, Formerly NIDA) developed guidelines and protocols for drugs of abuse testing. The mandatory guidelines for Federal Workplace Drug Testing Program were first published in the Federal Register on April 11, 198 (53 FR 11970) and have since been revised in the Federal Register on June 9, 1994 (59 FR 29908) and also on September 30, 1997 (62 FR 51118). Another notice was issued on April 13, 2004 (Federal Register, Vol. 69, No. 71). The overall testing process under mandatory testing consists of proper collection of specimen, initiation of chain of custody and finally analysis of specimen [screening and gas chromatography/mass spectrometry (GC/MS) confirmation if needed] by a Substance Abuse and Mental Health Services Administration (SAMHSA)-certified laboratory. The screening by immunoassay should be performed using an FDA-approved method. The confirmation should be performed by a second technique, preferably by GC/MS. Federal guidelines for cut-off levels of five abused drugs in screening and confirmation phase of drug testing program are summarized in Table 1.

It is estimated that approximately 20 million employees are screened each year in the United States for illicit drugs. Marijuana is the most frequently abused drug in the United States. Drug testing programs in the United States can be classified as mandatory or non-mandatory. In the first category (e.g., the Department of Transportation), a regulated employer is required by federal regulation to test the employees. In the second category, employers choose to test for reasons other than the federal requirements.

**Table 1**
**Federal Guideline for Cut-off Levels for Screening and Confirmation of Five Abused Drugs in Urine**

| it Drug | *Screening level (ng/mL)* | *Confirmation level (ng/mL)* |
|---|---|---|
| Marijuana metabolites (Delta -9-tetrahydrocannabinol-9-carboxylic acid) | 50 | 15 |
| Cocaine metabolite (Benzoylecgonine) | 300 | 150 |
| Opiate metabolites | 2000 | |
| Morphine | | 2000 |
| Codeine | | 2000 |
| 6-Acetyl morphine[a] | | 10 |
| Phencyclidine | 25 | 25 |
| Amphetamines | 1000 | |
| Amphetamine | | 500 |
| Methamphetamine[b] | | 500 |

[a] Tested only when morphine concentration is $\geq$2000 ng/mL by the initial screening tests.
[b] Specimen must also contain amphetamine at a concentration $\geq$200 ng/mL.

Private employers who are not mandated to test under federal authority have instituted employee drug testing in order to create a drug-free work place. These programs also formalized the role of a specialist physician termed as Medical Review Officer (MRO). The MRO is an integral part of a drug testing program that can determine the cause of positive results in drug testing (interference, other prescription drugs and so on) and counsel the employee. An MRO shall be a licensed physician (MD or DO) and should have thorough knowledge and clinical experience in controlled substance abuse disorders, detailed knowledge of alternative medical explanations for laboratory positive drug test results, knowledge about issues relating to adulterated or substituted urine specimens and knowledge about possible medical causes for specimens that may be reported as having an invalid result. A certifying scientist should be an individual with at least a Bachelor's degree in Chemical or Biological Sciences or Medical Technology or equivalent who reviews all pertinent data and quality control results. The individual shall have training in the theory and practice of all methods used in the laboratory as well as a thorough knowledge of Chain of Custody procedure (a process that is used to track the handling and storage of specimen submitted for drugs of abuse testing). It is required that a laboratory should submit a drug testing result to the MRO within 5 working days of receiving the specimen and result must be certified by the certifying scientist.

Federal guidelines defined adulterated specimen as a urine specimen containing a substance that is not a normal constituent or containing an endogenous substance at a concentration that is not a normal physiological concentration. In the military where the urine collection process is supervised, the chances of receiving adulterated specimens are reduced, but in pre-employment screening where direct supervision of specimen collection is not practiced, a person may attempt to escape detection of drugs of abuse by adulterating specimens to avoid unwanted consequences of failing a drug test. Several precautions are taken by the personnel of the collection site to avoid such adulteration of submitted specimens such as asking the donor to remove outer garments (coat or jacket) that may contain concealed adulterating substances. The collector should ensure that all personal belongings such as a purse or a briefcase stay with the collector.

When a donor is unable to provide a urine specimen, the donor may have intentionally urinated prior to arriving at the collection site, has a physical disability making it impossible to provide a specimen or has a "shy bladder." The term "shy bladder" usually refers to an individual who is unable to provide a specimen either upon demand or when someone is nearby during the attempted urination.

If a donor tells the collector, upon arrival at the collection site, that he or she cannot provide a specimen, the collector must unwrap or open a collection container and request the donor to try to provide a specimen. If that failed, the donor is given a reasonable amount of fluid to drink distributed reasonably through a period of up to 3 h, or until the donor has provided a new sufficient amount of urine, whichever occurs first. If the donor refuses to drink fluids as directed or refuses to attempt to provide a urine specimen, the collection procedure is discontinued and deemed a "refusal to test."

## 2. FORENSIC DRUG TESTING

Forensic drug testing can be more complex than regular hospital-based drug testing programs because in order to investigate the cause of death, specimens can be tested for the presence of a wide variety of drugs including commonly abused drugs. The toxicology section of the American Academy of Forensic Sciences has issued the following policy statement "confirmation of results is essential in forensic toxicology. Positive results of toxicological screening tests, regardless of the method used, and positive toxicological analysis results obtained by immunoassay methods should be adequately confirmed before the results are used for forensic purposes, or clearly be designated as unconfirmed results. Analysis methods used for attempted confirmation of presumptive results must be appropriately sensitive and specific or unequivocally selective for analyte in question, and must be based upon different chemical or physical principles than the initial method." GC/MS is widely used in a forensic laboratory for confirmation of a variety of drugs. In addition, high performance liquid chromatography coupled with mass spectrometry is also a very useful technique in a forensic toxicology laboratory. Both polar and non-polar drugs can be analyzed using HPLC/MS.

## 3. COMMERCIALLY AVAILABLE PRODUCTS TO ADULTERATE URINE SPECIMENS

Common household chemicals such as laundry bleach, table salt, toilet bowl cleaner, hand soap and vinegar have been for many years used as adulterants of urine specimens in an attempt to avoid a positive drug test. There is also a popular belief that drinking Golden Seal tea helps to escape detection of an abused drug. More recently, a variety of products have become commercially available which can be ordered through Internet sites (http://www.bdtzone.com, http://pass-drug-test.com and so on) and toll-free numbers. Home test kits are also available commercially to test for certain drugs. Synthetic urine is available from these Internet sites as a sure method to beat a drug test in settings where collection of a urine specimen is not supervised. The Quick Fix Synthetic Urine is a bottle of premixed urine with all the characteristics of natural urine (correct pH, specific gravity and creatinine). The product can be heated in a microwave oven for up to 10 s in order to achieve a temperature between 90 and 100°F. It can also be taped to a heater pad in order to maintain the normal temperature of urine for up to 6 h in a pocket.

Commercially available products to beat drug tests can be classified under two broad categories. The first category includes specific fluids or tablets, along with substantial water intake to flush out drugs and metabolites. Many of these products can produce dilute urine and the concentrations of drugs or metabolites can be significantly reduced. Common products are Absolute Detox XXL drink ($31.00), Absolute Carbo Drinks ($28.50), Ready Clean Drug Detox Drink ($29.50), Fast Flush Capsules ($29.50) and Ready Clean Gel Capsules ($28.50). All products are available from the Internet site (http:///www.testself.com). The second category of products available is in vitro urinary adulterants, which should be added to urine after collection in order to pass a drug test. Stealth (contains peroxidase and peroxide), Klear (nitrite), Clean ADD-IT-ive (glutaraldehyde) and Urine Luck [pyridinium chlorochromate (PCC)] are urinary adulterants available through the Internet. Iodine is a strong oxidizing agent and may

potentially destroy abused drugs, especially tetrahydrocannabinoid (THC), if present in urine *(1)*. A recent article indicates that papain with intrinsic ester hydrolysis ability can significantly reduce the concentration of 11-norcarboxy delta-9-tetrahydrocannibinol (THC-COOH), a metabolite of marijuana, if added to the urine specimen in vitro. This product is relatively inexpensive and commercially available from Sigma-Aldrich Chemical Company. Papain, however, did not significantly decrease concentrations of the other drugs analyzed [screening by both enzyme multiplied immunoassay technique (EMIT) and fluorescence polarization immunoassay] except nordiazepam *(2)*.

### *3.1. Diluted Urine*

A negative result for the presence of abused drugs in a urine specimen does not mean that no drug is present. It is possible that the amount of drug is below the cut-off values for detection in the laboratory assays. Diluting urine is a simple way to make an otherwise positive drug tests negative if the original concentrations of drugs in the urine are slightly above the cut-off values. Federal guidelines recommend placing a toilet bluing agent in the toilet tank if possible so that the reservoir of water in the toilet bowl always remains blue. There should be no other source of water in the enclosure where urination takes place.

Consumption of a large amount of fluid prior to drug testing is a way to avoid a positive test *(3)*. A creatinine concentration below 20 mg/dL or a specific gravity below 1.003 should be considered as an indication of diluted urine. Creatinine analysis in urine is a very effective method to detect diluted urine. Needleman and Porvaznik *(4)* considered a creatinine value of less than 10 mg/dL as suggestive of replacement of a urine specimen largely by water. Beck et al. *(5)* reported that 11% of all urine specimens submitted to their laboratory for drugs of abuse testing was diluted. The SAMHSA program does not currently allow analysis of dilute urine specimens at lower screening and confirmation cut-off values. However, in Canada, the Correctional Services of Canada (CSC), for diluted urine specimens, incorporates lower screening and confirmation cut-off for drug/metabolites (amphetamine: screening cut-off, 100 ng/mL; confirmation cut-off, 100 ng/mL; benzoylecgonine (BE): screening and confirmation, cut-off 15 ng/mL; opiates: screening and confirmation cut-off, 120 ng/mL; phencyclidine: screening and confirmation cut-off, 5 ng/mL and cannabinoids: screening cut-off, 20 ng/mL; confirmation cut-off 3 ng/mL). Fraser and Zamecnik reported that 7912 urine specimens between 2000 and 2002 analyzed collected by the CSC were dilute, and out of that 26% screened positive using SAMHSA cut-off values. When lower values for cut-off and confirmation were adopted, 1100 specimens tested positive for one or more illicit drugs. The positive rate of diluted specimens was 18.2% in the CSC Institutes and 22.3% in parolee specimens. The drug most often confirmed positive in a diluted specimen was marijuana. Codeine and/or morphine were also commonly confirmed in these urine specimens and ranked second after marijuana in prevalence *(6)*. Soldin *(7)* reported earlier that there was more than a 100% increase in cocaine-positive specimens when the cut-off was lowered to 80 ng/mL from 300 ng/mL in a pediatric population because neonates are not capable of concentrating urine to the same extent as adults. Luzzi et al. investigated the analytic performance criteria of three immunoassay systems [EMIT, Beckman EIA and Abbott fluorescence polarization assay (FPIA)] for detecting abused drugs below established cut-off values.

The authors concluded that drugs can be screened at concentrations much lower than that of established SAMHSA cut-off values. For example, the authors proposed a THC-COOH cut-off value of 35 ng/mL using EMIT and 14 ng/mL for the Beckman EIA and the Abbott FPIA, where SAMHSA guidelines stated a cut-off value of 50 ng/mL. The proposed cut-off values were based on impression studies where coefficient of varience (CV) was less than 20%. Such lowering of cut-off values increased the number of positive specimens in the screening tests to 15.6%. A 7.8% increase was also observed in the confirmation stage of drugs of abuse testing *(8)*.

New SAMHSA regulations (49 Code of Federal Regulation Part 40) indicate that a specific gravity lower than 1.0010 (new refractometers are capable of detecting such low concentration to four decimal places accurately) or above 1.020 and creatinine concentration less than 5 mg/dL are inconsistent with normal human urine. Edgell et al. performed a controlled hydration study with 56 volunteers to investigate whether it is possible to produce such diluted urine. Subjects were given 2370 mL of fluid, and urine specimens were collected at the end of each hour for a 6-h test period. No urine specimen satisfied the paired substitution criteria (specific gravity $\leq$ 1.001 or above 1.020 and creatinine $\leq$5.0 mg/dL) for diluted urine (although 55% subjects produce at least one dilute urine specimen during the first 3 h of hydration with creatinine $<$20 mg/dL and specific gravity $<$1.003). This supports the criteria set by SAMHSA for classifying a specimen as substituted *(9)*. Barbanel et al. studied specific gravity and/or creatinine concentrations in 803,130 random urine specimens submitted to the laboratory. Out of these, 13,467 specimens had both creatinine and specific gravity measurements and none of them met the lower limit of specific gravity (1.001) and creatinine (5 mg/dL). The patients who met one of the two criteria (creatinine $<$5.0 mg/dL or specific gravity $<$1.001) were neonatal or so severely ill unlike anyone in the work force undergoing testing for abused drugs. Eleven patients met the criteria of substituted urine (creatinine $<$5 mg/dL, specific gravity $>$1.020), but all of them were seriously or terminally ill *(10)*. Cook et al. *(11)* demonstrated that an osmolality substation cut-off of $<$50 mOsm/kg can be indicative of substituted urine.

### *3.2. Flushing, Detoxification Agents, Diuretics and Herbal Tea to Escape Detection*

Flushing and detoxification are frequently advertised as effective means of passing drug tests. Cone et al. evaluated the effect of excess fluid ingestion on false-negative marijuana and cocaine urine test results. The authors studied the ability of Naturally Clean Herbal tea, Golden Seal root and hydrochlorothiazide to cause false-negative results. Volunteers drank 1 gallon of water (divided in four doses over a 4-h period) or herbal tea or hydrochlorothiazide 22 h after smoking marijuana cigarettes or intranasal administration of cocaine. The creatinine levels dropped below the cut-off 2 h after intake of excessive fluid. Marijuana and cocaine metabolite levels (as measured by both EMIT and TDx) reduced significantly, and results frequently switched from positive to negative in subjects after consuming 2 quarts of fluid. Even excess water was effective in diluting a urine specimen to cause false-negative results. Consumption of herbal tea produced dilute urine faster compared to subjects who drank water alone *(12)*. Consumption of Golden Seal tea produces dark urine and can be identified by visual inspection *(13)*. A more sophisticated approach to identify marker compounds of

Golden Seal tea in a specimen suspected of contamination requires application of high performance liquid chromatography to identify such compounds *(14)*. Diuretics are used in sports for two purposes: first to flush out previously taken banned substances by forced diuresis and second to achieve quick weight loss to qualify for a group with a lower weight class. Ingestion of salicylate-containing drugs and sodium bicarbonate by individuals can also occur in order to avoid positive results in drug testing *(2)*. The Medical Commission of International Olympic Committee bans diuretics. There is no commercially available immunoassay for detecting diuretics such as hydrochlorothiazide in urine. Therefore, a sophisticated technique such as liquid chromatography combined with tandem mass spectrometry is necessary to confirm the presence of diuretics in doping analysis *(15)*.

### *3.3. Common Household Chemicals as Urinary Adulterants*

People try to beat drug testing by adding adulterants into urine specimens. Several adulterants can cause false-negative results in drug testing by immunoassays. Common adulterants for masking drug testing are as follows and detection of these adulterants by specimen integrity tests is given in Table 2.

1. Table salt.
2. Household vinegar.
3. Liquid laundry bleach.
4. Concentrated lemon juice.
5. Golden Seal tea (produces dark urine).
6. Visine eye drops.

Although FPIA is less subjected to interference from adulterants compared to the EMIT, some interference has also been reported with FPIA. Sodium chloride caused negative interference with all drugs tested by EMIT and caused a slight decrease in measured concentrations of benzodiazepines by FPIA. Sodium bicarbonate caused false positive with an EMIT opiate assay and with a PCP assay by FPIA. Hydrogen peroxide caused false-positive benzodiazepine results by FPIA *(16)*. Uebel and Wium studied the effect of

**Table 2**
**Common Household Adulterants and Specimen Integrity Tests**

| | *Specimen integrity tests*[a] | | | |
|---|---|---|---|---|
| *Household chemicals* | *pH* | *Creatinine* | *Temperature* | *Specific Gravity* |
| Sodium chloride | | | | X |
| Vinegar | X | | | |
| Laundry bleach | X | | | |
| Liquid soap | X | | | Cloudy |
| Drano | X | | | |
| Golden Seal | | | | Dark Urine |
| Visine eye drops | | | | |

X-denotes measurable change.
[a]Abnormal test indicative of the adulterant.

household chemicals sodium hypochlorite, Dettol (chloroxylenol), glutaraldehyde, Pearl hand soap, ethanol, isoproponal and peroxide on cannabis and methaqualone tests using EMIT assays. Most of the agents tested interfered with the tests, and the greatest effect was observed with glutaraldehyde and Pearl hand soap for methaqualone (false negative). Dettol and Pearl hand soap also caused false-negative results in cannabis tests. Addition of isoproponal, ethanol and peroxide invalidated methaqualone tests *(17)*.

Schwarzhoff and Cody studied the effect of 16 different adulterating agents: ammonia-based cleaner, L-ascorbic acid, Visine eye drops, Drano, Golden Seal root, lemon juice, lime solvent, Clorox, liquid hand soap, methanol, sodium chloride, tribasic potassium phosphate, toilet bowl cleaner (Vanish, Drackett Products), white vinegar, ionic detergent (Multi-Terge) and whole blood anticoagulated with EDTA on FPIA analysis of urine for abused drugs. The authors tested these adulterating agents at 10% by volume concentration of urine with the exception of Golden Seal because of the insolubility. For Golden Seal tea, one capsule was suspended in 60 mL urine. Out of six drugs tested (cocaine metabolites, amphetamines, opiates, phencyclidine, cannabinoid and barbiturates), the cannabinoid test was most susceptible to adulteration. Approximately half of the agents (ascorbic acid, vinegar, bleach, lime solvent, Visine eye drops and Golden Seal) tested caused false negatives. Both cannabinoid and opiate assays were susceptible to bleach, and actual degradation of THC was confirmed by GC/MS analysis. The PCP and BE (the metabolite of cocaine) analysis were affected by alkaline agents *(18)*. Baiker et al. reported that hypochlorite (a common ingredient of household bleach) adulteration of urine caused a decreased concentration of THC as measured by GC/MS. A false-negative result was also observed with the FPIA screen as well as the Roche Abuscreen *(19)*. Another report described adulteration of urine specimens with denture cleaning tablets *(20)*.

The ability of Visine eye drops to cause false-negative drug testing in the screening phase of the analysis is troublesome because the presence of components of Visine eye drops in adulterated urine cannot be detected by routine specimen integrity testing or any routine urine analysis. Pearson et al. studied in detail the effect of Visine eye drops on drugs of abuse testing as well as the mechanism by which components of Visine eye drops produce false-negative drug testing results. Visine eye drops are effective in causing false-negative result in the analysis of the THC metabolite 11-nor-9-carboxy-delta 9-tetrahydrocannabinol. The GC/MS analysis showed that there was no modification in the structure of THC metabolite by the components of Visine eye drops. At low concentrations of Visine eye drops, the false-negative cannabinoid result was due to the benzalkonium chloride ingredient of Visine. Visine decreased the THC assay results in both EMIT-d.a.u. assays and Abuscreen (Abbott Laboratories, Abbott Park, IL) although Visine had no effect on glucose 6-phosphate dehydrogenase–drug conjugate used in the EMIT assay. Results of ultrafiltration studies with Visine eye drops suggest that the THC metabolite partitions between the aqueous solvent and the hydrophobic interior of benzalkonium chloride micelles, thus reducing the availability of THC metabolite in antibody-based assay *(21)*. Visine eye drops and Ben Gay ointment can also cause false-negative drug testing with sweat testing *(22)*. Components of Visine eye drops in urine may be detected by using high performance liquid chromatography combined with UV detection at 262 nm, a method originally developed for analysis of ophthalmic formulations *(23)*.

### 3.4. Specimen Integrity Tests

Both the collection site and the laboratory have a number of mechanisms to detect potentially invalid specimens. The temperature of the urine, for instance, should be within 90.5–98.9°F. The specific gravity should be between 1.005 and 1.030 and the pH should be between 4.0 and 10.0. The creatinine concentration should be 20–400 mg/dL. A specimen is considered as diluted if the creatinine is <20 mg/dL and the specific gravity is <1.003. It is advised that the laboratory should perform creatinine and pH analysis of all specimens submitted for drugs of abuse testing. Additional tests are also recommended to detect the presence of other adulterants. Determination of specific gravity is mandatory for any specimen with a creatinine concentration of <20 mg/dL. Substituted urine specimens have creatinine concentrations <5 mg/dL and a specific gravity 1.001 or over 1.020. The urine is adulterated if pH is <3 or >11 *(2)*. Adulteration with sodium chloride at a concentration necessary to produce a false-negative result always produces a specific gravity over 1.035. Use of household chemicals such as bleach, acid, soap and detergent as well as adulteration with vinegar alter the pH of urine to a value outside the physiological range and can be easily detected by specimen integrity tests. Specimens adulterated with liquid soap are usually cloudy. The presence of Visine eye drops in adulterated urine cannot be detected by routine specimen integrity testing. Moreover, newer urine adulterants such as Urine Luck, UrinAid, Klear and Whizzies can also cause false-negative results in drug tests. The presence of these compounds in urine may escape detection by routine specimen integrity tests.

### 3.5. Adulteration Product "Urine Luck"

Wu et al. reported that the active ingredient of "Urine Luck" is 200 mmol/L of PCC. The authors reported a decrease in the response rate for all EMIT II drug screens and for the Abuscreen (Abbott Laboratories) morphine and THC assays. In contrast, Abuscreen amphetamine assay produced a higher response and no effect was observed on the results of BE and PCP. This adulteration of urine did not alter GC/MS confirmation of methamphetamine, BE and PCP. However, apparent concentrations of opiates and THC as determined by GC/MS were reduced *(24)*. Paul et al. also studied the effect of "Urine Luck" on testing for drugs of abuse. When THC-COOH-containing urine specimens were treated with 2 mmol/L of PCC, 58–100% of the THC-COOH was lost. The loss increased with decreasing pH and increasing time of incubation (0–3 days). There was no effect on the concentration of free codeine or free morphine if the pH of the urine was in the range of 5–7, but at lower pH, significant loss of free morphine was observed. Amphetamine, methamphetamine, BE and PCP remained unaffected by PCC at urine pH 3–7 *(25)*.

### 3.6. Spot Tests for Detecting Urine Luck (Pyridinium Chlorochromate) in Urine

#### 3.6.1. Spot Test 1

Wu et al. also described the protocol for detection of PCC in urine using spot tests. The indicator solution contains 10 g/L of 1,5-diphenylcarbazide (DPC) in methanol. The indicator detects the presence of chromium ion and is colorless when prepared.

Two drops of indicator solution is added to 1.0 mL of urine. If a reddish-purple color develops, the test is positive *(24)*. Paul et al. also used DPC for detection of PCC in urine. When this reagent is added, a red-violet color appears immediately if PCC is present. The chromium–DPC complex shows a characteristic absorption peak at 544 nm and a shoulder peak at 575 nm. The ratio of absorption can be used to detect the presence of PCC as chromium in urine, and concentration of chromium can be estimated by measuring absorption at 544 nm, with a linear association between concentrations of 0.5 and 20 μg/mL *(25)*.

#### 3.6.2. Spot Test 2

Stock solution: 1% potassium iodide in distilled water.

1. In a test tube a few drops (~200 μl) of stock potassium iodide solution is added (~6–7 drops from a transfer pipette).
2. Add about 100 μl of urine specimen (~3–4 drops) suspected of PCC adulteration.
3. Add two drops of 2 N hydrochloric acid. Immediate release of iodine from the colorless potassium iodide solution is seen if PCC is present in the urine. Shaking of this solution with n-butanol results in the transfer of iodine in the organic phase. If no nitrite is present, the potassium iodide solution remains colorless. There is no interference from high glucose or ketone bodies if present in the urine.

#### 3.6.3. Spot Test 3

Addition of four to five drops of 3% hydrogen peroxide in approximately 200 μL of urine adulterated with PCC (~6–7 drops from a transfer pipette) caused rapid formation of a dark brown color and a dark brown precipitate appeared on standing. In contrast, unadulterated urine turned colorless after addition of hydrogen peroxide *(26)*.

#### 3.6.4. Other Tests for Detecting Chromate

Freslew et al. *(27)* described a capillary ion electrophoresis technique for detecting chromate ion, as well as nitrite ion, in urine specimens suspected of adulteration. The DPC colorimetric test for chromate, which can be easily automated, can serve as a screening test. Capillary electrophoretic analysis can be used to confirm the presence of chromate in adulterated specimen, if necessary. A good correlation was observed between chromate concentrations in urine using the colorimetric test and the capillary electrophoretic analysis *(28)*. Paul described six spectroscopic methods for detection of oxidants including chromate. The presence of oxidants (as adulterants in urine) was established by initial oxidation of ferrous to ferric ion and then detecting ferric ion by chromogenic oxidation or complex formation. The author used N,N-dimethylamino-1,4-phenylenediamine, 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) or 2-amino-para-cresol for chromogenic oxidation. The reagents for the chromogenic complex formation were xylenol orange, 8-hydroxy-7-iodo-5-quinolinesulfonic acid and 4, 5-dihydroxy-1, 3-benzene-di sulfonic acid *(29)*.

### *3.7. Adulteration of Urine with Nitrite-Containing Agents*

The product "Klear" comes in two microtubes containing 500 mg of white crystalline material. This product readily dissolves in urine without affecting color or temperature.

Klear may cause a false-negative GC/MS confirmation for marijuana. ElSohly et al. first reported this product as potassium nitrite and provided evidence that nitrite leads to decomposition of ions of 9-THC and its internal standard. The authors reported that using a bisulfite step at the beginning of sample preparation could eliminate such interference *(30)*. Tsai et al. further investigated the effect of nitrite on immunoassay screening of other drugs. These drugs include cocaine metabolites, morphine, THC metabolites (THC-COOH), amphetamine and phencyclidine. Nitrite at a concentration of 1.0 M had no effect on the Abuscreen assay. At a higher nitrite concentration, the amphetamine assay becomes more sensitive and the THC metabolite assay becomes less sensitive. The GC/MS analyses of BE, morphine, amphetamine and phencyclidine were not affected while recovery of the THC metabolite was significantly reduced. Again, this interference could be eliminated by bisulfite treatment *(31)*. Both duration of nitrite exposure and the urine matrix affect the THC-COOH assay. In an in vitro study, 40 clinical urine specimens confirmed positive for THC-COOH were supplemented with 1.15 or 0.30 M nitrite. The results indicated that the pH of the urine and the original drug concentrations play major roles in dictating the effectiveness of nitrite in causing false-negative THC metabolite tests. With acidic pH significant decreases in the immunoassay screening, results can be observed in all urine specimens within 4 h of adulteration with nitrite regardless of original concentrations of THC-COOH (range of concentrations 33–488 ng/mL as determined by GC/MS). All specimens were negative for THC-COOH after 1 day. In contrast, the immunoassay results of urine specimens with basic or neutral pH were less affected by nitrite exposure. Approximately two-thirds of the samples with pH values greater than 7.0 were immunoassay positive even 3 days after supplementing with nitrite *(32)*.

Nitrite in urine may arise in vivo and is found in urine in low concentration. Patients receiving medications such as nitroglycerine, isosorbide dinitrate, nitroprusside and ranitidine may have increased nitrite levels in their blood. However, concentrations of nitrite were below 36 μg/mL in specimens cultured positive for microorganisms, and nitrite concentrations were below 6 μg/mL in patients receiving medications that are metabolized to nitrite. On the other hand, nitrite concentrations were 1910–12, 200 μg/mL in urine specimens adulterated with nitrite *(33)*. Whizzies is another urine adulterant available from the Internet. This adulterant also contains potassium nitrite.

## *3.8. Spot Tests for Nitrite*

### 3.8.1. Spot Test 1

Stock solution: 2% potassium permanganate in distilled water and 2 N hydrochloric acid.

1. In a test tube add about 200 μL of stock potassium permanganate solution (~6–7 drops from a transfer pipette).
2. Add about 100 μl of urine specimen (~3–4 drops) suspected of nitrite adulteration.
3. Add two drops of 2 N hydrochloric acid.

The pink permanganate solution turns colorless with effervescence immediately after addition of hydrochloric acid if nitrite is present. This is due to reduction of heptavalent manganese ion of potassium permanganate by nitrite. The presence of very

high glucose in urine (glucose > 1000 mg/dL) and ketone bodies may cause false positive. However, it takes approximately 2–3 min for the solution to turn colorless. On the other hand, if nitrite is present, the solution turns colorless immediately.

#### 3.8.2. Spot Test 2

Stock solution: 1% potassium iodide in distilled water.

1. In a test tube add approximately 200 μL of stock potassium iodide solution (~6–7 drops from a transfer pipette).
2. Add about 100 μL of urine specimen (~3–4 drops) suspected of nitrite adulteration.
3. Add two drops of 2 N hydrochloric acid. Immediate release of iodine from the colorless potassium iodide solution is observed if nitrite is present in the urine. Shaking of this solution with n-butanol results in the transfer of iodine in the organic phase. If no nitrite is present, the potassium iodide solution remains colorless. There is no interference from high glucose or ketone bodies if present in the urine *(26)*.

#### 3.8.3. Other Tests to Detect Nitrite

Nitrite can also be detected by diazotizing sulfanilamide and coupling the product with N-(1-napthyl) ethylenediamine. The presence of nitrite in urine can also be confirmed by analysis using high performance liquid chromatography using an IonPac AS 14 analytical column with an anion self-generating suppressor and conductivity detector. Using a single-point calibration, the assay was linear up to a nitrite concentration of 12,000 μg/mL. The detection limit was 30 μg/mL *(34)*. Kinkennon et al. described a capillary electrophoresis method for detection of nitrite in urine specimens suspected of adulteration. The method involved separation of nitrite by capillary electrophoresis and direct UV detection at 214 nm. Separation was achieved by using a bare fused silica capillary column and 25 mM phosphate buffer at pH 7.5. The method was linear for a nitrite concentration of 80–1500 μg/mL, with a limit of detection of 20 μg/mL. However, $CrO_4^{2-}$ and $S_2O_8^{2-}$ as well as high concentrations of $Cl^-$ interfered with the chromatography *(35)*.

### *3.9. Stealth as a Urinary Adulterant*

Stealth is an adulterant advertised as an effective way to escape detection in a urine drug test. Stealth consists of two vials, one containing a powder (peroxidase) and another vial containing a liquid (hydrogen peroxide). Both products should be added to the urine specimen. Stealth is capable of producing false-negative results using Roche ONLINE and Microgenic's CEDIA immunoassay methods when marijuana metabolites, LSD and opiates (morphine) were present in the urine at 125–150% of cut-off values. Adulteration of an authentic positive sample provided by a marijuana user caused that sample to screen negative using these immunoassay reagents *(36)*. Low concentration of morphine (2500 ng/mL) could be effectively masked by Stealth, but higher concentrations (6000 ng/mL) tested positive by immunoassay (Roche ONLINE and Microgenic's CEDIA immunoassay). GC/MS confirmation step can be affected if Stealth is present in the urine. Cody et al. *(37)* reported that GC/MS analysis of Stealth-adulterated urine using standard procedures proved unsuccessful in several cases, and in 4 of 12 cases, neither the drug nor the internal standard was recovered.

Valtier and Cody described a rapid color test to detect the presence of Stealth in urine. Addition of 10 µL of urine to 50 µL of tetramethylbenzidine working solution followed by addition of 500 µL of 0.1 M phosphate buffer solution caused a dramatic color change of the specimen to dark brown. Peroxidase activity could also be monitored by using a spectrophotometer. Routine specimen integrity check using pH, creatinine, specific gravity and temperature did not detect the presence of Stealth in urine *(38)*. Our experiences show that if a few drops of a urine specimen adulterated with Stealth is added to potassium dichromate followed by a few drops of 2 N hydrochloric acid, a deep blue color develops immediately which usually fades with time.

### *3.10. Glutaraldehyde as an Adulterant to Urine*

Glutaraldehyde has also been used as an adulterant to mask urine drug tests *(39)*. This product is available under the trade name of "UrinAid." The manufacturer, Byrd Laboratories (Topanga, CA), sells this product for $20–30 per kit. Each kit contains 4–5 mL glutaraldehyde solution, which is added to 50–60 mL of urine. Glutaraldehyde solutions are available in hospitals and clinics as a cleaning or sterilizing agent. A 10% solution of glutaraldehyde is available from pharmacies as over-the-counter medication for treatment of warts. Glutaraldehyde at a concentration of 0.75% volume can lead to false-negative screening results for a cannabinoid test using the EMIT II drugs of abuse screen. Amphetamine, methadone, benzodiazepine, opiate and cocaine metabolite tests can be affected at glutaraldehyde concentration between 1 and 2% using EMIT immunoassays. At a concentration of 2% by volume, the assay of cocaine metabolite is significantly affected (apparent loss of 90% sensitivity). A loss of 80% sensitivity was also observed with the benzodiazepine assay.

Wu et al. *(40)* reported that glutaraldehyde also interfered with the CEDIA immunoassay for screening of abused drugs. Goldberger and Caplan *(41)* reported that glutaraldehyde caused false-negative results with EMIT but also caused false-positive phencyclidine results with the FPIA (Abbott Laboratories) and Kinetic Interaction of Microparticles in a Solution Immunoassay (KIMS, Roche Diagnostics).

Although the presence of glutaraldehyde as an adulterant in urine can be detected by GC/MS, Wu et al. described a simple fluorometric method. When 0.5 mL of urine was heated with 1 mL of 7.7 mmol/L potassium dihydrogen phosphate (pH 3.0) saturated with diethylthiobarbituric acid for 1 h at 96–98°C in a heating block, a yellow green fluorophore developed if glutaraldehyde was present. Shaking the specimen with n-butanol resulted in the transfer of this adduct to the organic layer which can be viewed under long wavelength UV light. Glutaraldehyde in urine can also be estimated using a fluorimeter *(42)*.

## 4. MECHANISM OF ACTION OF ADULTERANTS

Adulterants such as bleach cause a false-positive result in THC radioimmunoassay but false-negative results with both FPIA and EMIT assay. These erroneous results are due to direct effect of bleach on the reagents in the immunoassays *(13,43)*. Adulterants that are strong oxidizing agents such as Klear (potassium nitrite), Urine Luck (PCC) and Stealth (peroxidase and hydrogen peroxide) cause false-negative results in the

immunoassays used for screening drugs by directly destroying THC metabolites (THC-COOH). In the GC/MS confirmation stage (GC/MS), these adulterants interfere with confirmation of THC-COOH because of destruction of THC-COOH and internal standard as well as interference during the extraction phase. To overcome this problem, the use of reducing agents such as sodium hydrosulfite or sulfamic acid prior to extraction has been recommended *(30)*. However, such steps can allow detection of remaining THC-COOH but cannot recover the lost concentration of the marijuana metabolite. Most oxidizing agents used as adulterants are more effective if the pH of urine is acidic. To prevent destruction of drugs by oxidizing agents, addition of carbonate as a buffering agent prior to or after urine void has been recommended *(44)*. Other oxidizing agents such as potassium permanganate, hydrogen peroxide/ferrous ammonium sulfate, periodic acid, potassium persulfate, and sodium oxychloride can also destroy THC-COOH within 48 h. The effect of oxidizing agents on THC-COOH primarily depends on the reduction potential ($E^0$), pH, temperature, time of reaction and urine constituents. Horseradish peroxidase with hydrogen peroxide, combination of hydrogen peroxide with Japanese radish, and black mustard seed and red radish are all effective in destroying THC-COOH. Interestingly, hydrogen peroxide alone had no effect in destroying any drug *(45)*.

Adulterants can also interfere with the extraction process. Stealth is known to interfere with extraction of codeine and morphine for GC/MS confirmation *(37)*. Our experience indicates that PCC is effective for decreasing the semi-quantitative response rate for THC and opiates using Abuscreen (FPIA). The incubation time played an important role in decreasing the response rate. Nitrite is very effective in reducing the response rate of THC, but PCP assay was also affected.

## 5. FEDERAL GUIDELINE OF ADDITIONAL TESTING TO DETECT ADULTERANTS

SAMHSA guidelines require additional tests for urine specimens with abnormal physical characteristics or ones that show characteristics of an adulterated specimen during initial screening or confirmatory tests (non-recovery of internal standard, unusual response and so on). A pH less than 3 or more than 11 and nitrite concentrations greater than 500 μg/mL indicate the presence of adulterants. A nitrite colorimetric test or a general oxidant colorimetric test can be performed to identify nitrite. These criteria are summarized in Table 3. Similarly, the presence of chromium can be confirmed by a chromium colorimetric test or a general test for the presence of oxidant. A confirmatory test can be performed using multi-wavelength spectrophotometry, ion chromatography, atomic absorption spectro-photometry, capillary electrophoresis or inductively couples plasma mass spectrometry. The presence of halogen (chloride, fluoride or bleach) should be confirmed by a halogen colorimetric test or a general test for the presence of oxidants. Confirmatory tests may employ multi-wavelength spectrophotometry, ion chromatography, atomic absorption spectrophotometer, capillary electrophoresis or inductively couples plasma mass spectrometry. The presence of glutaraldehyde should be detected by a general aldehyde test or the characteristic immunoassay response in one or more drug immunoassay tests for initial screening. The presence of PCC should be confirmed by using a general test for the presence of oxidant and a GC/MS confirmatory test. The presence of a surfactant should be verified by using a surfactant

**Table 3**
**Identification of Adulterated and Substituted Specimens Following SAMHSA Guidelines**

| *Parameter* | *Diluted specimen* | *Adulterated specimen* | *Substituted urine* |
|---|---|---|---|
| Creatinine | <20 mg/dL | | >5 mg/dL |
| pH | | <3 or >11 | |
| Specific gravity | <1.003 | | <1.0010 or >1.020 |
| Nitrite | | ≥500 μg/mL | |
| Chromium | | ≥50 μg/mL | |
| PCC | | ≥50 μg/mL chromium (VI) equivalent or 200 μg/mL nitrite equivalent | |
| Glutaraldehyde | | Present | |
| Halogen (Chloride, fluoride, bleach) | | Halogen colorimetric test/ ≥200 μg/mL nitrite equivalent/ ≥50 μg/mL chromium equivalent | |
| Surfactant | | Colorimetric test with >100 μg/mL dodecylbenzene sulfonate equivalent | |

PCC, Pyridinium chlorochromate; SAMHSA, Substance Abuse and Mental Health Services Administration.

colorimetric test with ≥100 μg/mL dodecylbenzene sulfonate equivalent cut-off. Jones et al. described a modified methylene blue procedure for detection and quantitation of surfactants in urine. Based on the analysis of negative samples, an anionic surfactant level of 100 μg/mL or greater could be considered adulterated but most likely such specimens will have levels greater than 800 μg/mL *(46)*.

### *5.1. On-Site Adulteration Detection Devices (Dipsticks) for Urine Specimens*

Standard urinalysis test strips such as Multistix from Bayer Diagnostics and Combur-Test from Roche Diagnostics are sometimes used to detect the presence of adulterants in urine. However, among various pads in the test strip, only pads for detection of nitrite, pH and specific gravity have some value. The specific gravity test does not differentiate between specific gravity of 1.000 and 1.005, and therefore, it is very difficult to apply it to identify substituted or diluted urine. The nitrite pad also detects a clinically significant range. Our experience indicates that a much deeper color develops with Multistix if nitrite is present in high concentrations as expected in an adulterated specimen. Moreover, some non-specific color change may be observed with the glucose test pad. More recently, on-site adulterant detection devices have become commercially available. These dipstick devices offer an advantage over spot tests because an adulteration check can also be performed at the collection site. Peace and Tarani evaluated the performance of three on-site devices, Intect 7 (Branan Medical Corporation), MASK Ultrascreen (Kacey Inc) and AdultaCheck 4 (Sciteck Diagnostics). Intect 7 can simultaneously test for creatinine, nitrite, glutaraldehyde, pH, specific gravity, PCC

and bleach. Ultrascreen tests for creatinine, nitrite, pH, specific gravity and oxidants. AdultaCheck 4 tests creatinine, nitrite, glutaraldehyde and pH. The authors adulterated urine specimens with Stealth, Urine Luck, Instant Clean ADD-IT-ive and Klear at their optimum usage concentration and concluded that Intect 7 was most sensitive and correctly identified adulterants. AdultaCheck 4 did not detect Stealth, Urine Luck or Instant Clean ADD-it-ive. Ultra Screen detected a broader range of adulterants than AdultaCheck 4. However in practice, it only detected these adulterants at levels well above their optimum usage, making it less effective than Intect 7 *(47)*. King *(48)* reported that AdultaCheck 4 is an excellent way to detect contamination in urine specimen.

### *5.2. Adulta Check 6 and Intect 7 Test Stripes for Detection of Urine Adulteration*

AdultaCheck 6 test strips are recently available which can be used to detect creatinine, oxidants, nitrite, glutaraldehyde, pH and chromate. In our experience, both AdultaCheck 6 and Intect 7 effectively identified the presence of low and high concentrations of PCC as well as nitrite in urine. Moreover, Intect 7 can also detect small amount of bleach (10 μL of bleach/mL of urine).

AdultaCheck 6 and Intect 7 test strips were effective for detecting the presence of glutaraldehyde in urine. When glutaraldehyde was present at a concentration of less than 0.4% by volume, neither AdultaCheck 6 nor Intect 7 showed expected color change. However, at glutaraldehyde concentration above 2% by volume, both urine test strips showed the desired color change in the pad designed to detect the presence of glutaraldehyde.

AdultaCheck 6 has a test pad for determining the creatinine concentration in urine. The possible readings are 0, 10, 20, 50, 100 and 400 mg/dL. Intect 7 test pad shows reading of 0, 10, 20, 50 and 100 mg/dL depending on color change of the test pad. Precise concentration of creatinine cannot be determined. Similarly, neither test strip can determine precise pH of a urine specimen but only can show the range. However, both AdultaCheck 6 and Intect 7 test strips successfully differentiated between abnormal creatinine and pH from normal creatinine and pH in urine as determined by precise measurement of creatinine using the Synchron LX 20 analyzer and pH using a pH meter *(49)*.

## 6. ADULTERATION OF HAIR AND SALIVA SPECIMEN FOR DRUG TESTING

Hair and saliva are considered as alternative specimens to urine for drugs of abuse testing. Drugs can be trapped in the segment of hair as it develops in a hair follicle and the hair segment emerges from the follicle and becomes keratinized and it carries the drug already trapped in the hair. This trapping of drugs permits analysis a few months later of the actual abuse and has a much longer window of detection than urine or saliva testing. However, several factors may influence amount of drug trapped in the hair such as hair color. Saliva is also an alternative specimen to urine for testing of abused drugs. The main advantage of saliva and hair testing is that the donor has a little chance to adulterate the specimen. Already in use in the transportation and

insurance industries, there is an increasing interest in saliva testing in the workplace, roadside test for driving under influence of drugs and criminal justice. It also appears that given the advances of technology and reproducibility of test results, oral fluid test results will be able to survive legal challenges *(50)*. Several products are available for sale through the Internet that claims by washing hair with these shampoos can aid a person to pass a drug test. These issues are discussed in detail in Chapter 18.

## 7. CONCLUSIONS

Adulterants impose a new challenge in the testing for abused drugs. Routine specimen integrity testing involving pH, creatinine, specific gravity and temperature is not adequate to detect the presence of more recently introduced adulterants such as Urine Luck, Klear and Stealth. These agents can cause false negative in the immunoassay screening steps and may also affect the GC/MS confirmation step if the abused drugs are present in modest concentrations (100–150% of cut-off concentrations). Therefore, true presence of a drug can be missed if these agents are used for adulteration. Fortunately, spot tests have been introduced and several dipstick tests (AdultaCheck 4, AdultaCheck 6 and Intect 7) are available for validation of specimen integrity. Studies are also needed to investigate effectiveness of hair shampoo to cause false negatives in a hair drug test and mouthwash products to invalidate saliva testing for abused drugs.

## REFERENCES

1. Paul B, Jacobs A. Spectrophotometric detection of iodide and chromic (III) in urine after oxidation to iodine and chromate. J Anal Toxicol 2005; 29: 658–663.
2. Burrows DL, Nicolaides A, Rice PJ, Duforc M, et al. Papain: a novel urine adulterant. J Anal Toxicol 2005; 29: 275–295.
3. George S, Braithwaite RA. An investigation into the extent of possible dilution of specimens received for urinary drugs of abuse screening. Addiction 1995; 90: 967–970.
4. Needleman SD, Porvaznik M. Creatinine analysis in single collection urine specimens. J Forensic Sci 1992; 37: 1125–1133.
5. Beck O, Bohlin M, Bragd F, Bragd J, Greitz O. Adulteration of urine drug testing-an exaggerated cause of concern [article in Swedish]. Lakartidningen 2000; 97: 703–706.
6. Fraser AD, Zamecnik J. Impact of lowering the screening and confirmation cutoff values for urine drug testing based on dilution indicators. Ther Drug Monit 2003; 25: 723–727.
7. Soldin SJ, Morales AJ, D'Angelo LJ, Bogema SC, Hicks JC. The importance of lowering the cut-off concentrations of urine screening and confirmatory tests for benzoylecgonine/cocaine [Abstract] Clin Chem 1991; 37: 993.
8. Luzzi VI, Saunders AN, Koenig JW, Turk J, et al. Analytical performance of immunoassays for drugs of abuse below established cutoff values. Clin Chem 2004; 50: 717–722.
9. Edgell K, Caplan YH, Glass LR, Cook JD. The defined HHS/DOT substituted urine criteria validated through controlled hydration study. J Anal Toxicol 2002; 26: 419–423.
10. Barbanel CS, Winkelman JW, Fischer GA, King AJ. Confirmation of the department of transportation criteria for a substituted urine specimen. J Occup Environ Med 2002; 44: 407–416.
11. Cook JD, Hannon MW, Vo T, Caplan YH. Evaluation of freezing point depression for classifying random urine specimens defined as substituted under HHS/DOT criteria. J Anal Toxicol 2002; 26: 424–429.
12. Cone EJ, Lange R, Darwin WD. In vivo adulteration: excess fluid ingestion cause false negative marijuana and cocaine urine test results. J Anal Toxicol 1998; 22: 460–473.

13. Mikkelsen SL, Ash KO. Adulterants causing false negative in illicit drug testing. Clin Chem 1988; 34: 2333–2336.
14. McNamara CE, Perry NB, Follett JM, Parmenter GA, Douglas JA. A new glucosyl feruloyl quinic acid as a potential marker for roots and rhizomes of goldenseal, Hydrastis canadenis. J Nat Prod 2004; 67: 1818–1822.
15. Deventer K, Delbeke FT, Roels K, Van Ecnoo P. Screening for 18 diuretics and probenecid in doping analysis by liquid chromatography-tandem mass spectrometry. Biomed Chromatogr 2002; 16: 529–535.
16. Warner A. Interference of household chemicals in immunoassay methods for drugs of abuse. Clin Chem 1989; 35: 648–651.
17. Uebel RA, Wium CA. Toxicological screening for drugs of abuse in samples adulterated with household chemicals. S Afr Med J 2002; 92: 547–549.
18. Schwarzhoff, R, Cody, JT. The effects of adulterating agents on FPIA analysis of urine for drugs of abuse. J Anal Toxicol 1993; 17: 14–17.
19. Baiker C, Serrano L, Lindner B. Hypochlorite adulteration of urine causing decreased concentration of delta-9-THC-COOH by GC/MS. J Anal Toxicol 1994; 18: 101–103.
20. Stolk LM, Scheijen JL. Urine adulteration with denture cleaning ts [Letter]. J Anal Toxicol 1997; 21: 403.
21. Pearson SD, Ash KO, Urry FM. Mechanism of false negative urine cannabinoid immunoassay screens by Visine eye drops. Clin Chem 1989; 35: 636–638.
22. Fogerson R, Schoendorfer D, Fay J, Spiehler V. Qualitative detection of opiates in sweat by EIAS and GC–MS. J Anal Toxicol 1997; 21: 451–458.
23. Rojsitthisak P, Wichitnithad W, Pipitharome O, Sanphanya K, Thanawattanawanich P. Simple HPLC determination of benzalkonium chloride in ophthalmic formulations containing antazoline and tetrahydrozoline. PDA J Pharm Sci Technol 2005; 59: 323–327.
24. Wu A, Bristol B, Sexton K, Cassella-McLane G, et al. Adulteration of urine by Urine Luck. Clin Chem 1999; 45: 1051–1057.
25. Paul BD, Martin KK, Maguilo J, Smith ML. Effects of pyridinium chlorochromate adulterant (urine luck) on testing of drugs of abuse and a method for quantitative detection of chromium (VI) in urine. J Anal Toxicol 2000; 24: 233–237.
26. Dasgupta A, Wahed A, Wells A. Rapid spot tests for detecting the presence of adulterants in urine specimens submitted for drug testing. Am J Clin Pathol 2002; 117: 325–329.
27. Freslew KE, Hagardorn AN, Roberts TA. Capillary ion electrophoresis of endogenous anions and anionic adulterants in human urine. J Forensic Sci 2001; 46: 615–626.
28. Ferslew KE, Nicolaides AN, Robert TA. Determination of chromate adulteration of human urine by automated colorimetric and capillary ion electrophoretic analyses. J Anal Toxicol 2003; 27: 36–39.
29. Paul BD. Six spectrometric methods for detection of oxidants in urine: implication in differentiation of normal and adulterated urine. J Anal Toxicol 2004; 28: 599–608.
30. ElSohly MA, Feng S, Kopycki WJ, Murphy TP, et al. A procedure to overcome interferences caused by adulterant "Klear" in the GC-MS analysis of 11-nor-Δ9-THC-9-COOH. J Anal Toxicol 1997; 20: 240–242.
31. Tsai SC, ElSohly MA, Dubrovsky T, Twarowska B, et al. Determination of five abused drugs in nitrite-adulterated urine by immunoassay and gas chromatography-mass spectrometry. J Anal Toxicol 1998; 22: 474–480.
32. Tsai LS, ElSohly MA, Tsai SF, Murphy TO, et al. Investigation of nitrite adulteration on the immunoassay and GC-MS analysis of cannabinoids in urine specimens J Anal Toxicol 2000; 24: 708–714.
33. Urry F, Komaromy-Hiller G, Staley B, Crockett D, et al. Nitrite adulteration of workplace drug testing specimens: sources and associated concentrations of nitrite and distinction between natural sources and adulteration. J Anal Toxicol 1998; 22: 89–95.
34. Singh J, Elberlind JA, Hemphill DG, Holmstrom J. The measurement of nitrite in adulterated urine samples by high performance ion chromatography. J Anal Toxicol 1999; 23: 137–140.
35. Kinkennon AE, Black DL, Robert TA, Stout PR. Analysis of nitrite in adulterated urine samples by capillary electrophoresis. J Forensic Sci 2004; 49: 1094–1100.

36. Cody JT, Valtier S. Effects of stealth adulteration on immunoassay testing for drugs of abuse. J Anal Toxicol 2001; 25: 466–470.
37. Cody JT, Valtier S, Kuhlman J. Analysis of morphine and codeine in samples adulterated with Stealth. J Anal Toxicol 2001; 25: 572–575.
38. Valtier S, Cody JT. A procedure for the detection of Stealth adulterant in urine samples. Clin Lab Sci 15 2002; 111-115.
39. George S, Braithwaite RA. The effect of glutaraldehyde adulteration of urine specimens on Syva EMIT II drugs of abuse assay. J Anal Toxicol 1996; 20: 195–196.
40. Wu AH, Forte E, Casella G, Sun K, et al. CEDIA for screening drugs of abuse in urine and the effect of adulterants. J Forensci Sci 1995; 40: 614–618.
41. Goldberger BA, Caplan YH. Effect of glutaraldehyde (UrinAid) on detection of abused drugs in urine by immunoassay [Letter]. Clin Chem 1994; 40: 1605–1606.
42. Wu A, Schmalz J, Bennett W. Identification of Urin-Aid adulterated urine specimens by fluorometric analysis [Letter]. Clin Chem 1994; 40: 845–846.
43. Cody JT, Schwarzhoff RH. Impact of adulterants on RIA analysis of urine for drugs of abuse. J Anal Toxicol 1989; 13: 277–284.
44. Lewis SA, Lewis LA, Tuinman A. Potassium nitrite reaction to 11-nor-delta 9-tetrahydrocannabinol-9-carboxylic acid in urine in relation to the drug screening analysis. J Forensic Sci 1999; 44: 951–955.
45. Paul BD, Jacobs A. Effects of oxidizing adulterants on detection of 11-nor-$\Delta^9$-THC-9-carboxylic acid in urine. J Anal Toxicol 2002; 26: 460–463.
46. Jones JT, Esposito FM. An assay evaluation of the methylene blue method for the detection of anionic surfactants in urine. J Anal Toxicol 2000; 24: 323–327.
47. Peace MR, Tarnai LD. Performance evaluation of three on-site adulteration detection devices for urine specimens. J Anal Toxicol 2002; 26: 464–470.
48. King EJ. Performance of AdultaCheck 4 test stripes for the detection of adulteration at the point of collection of urine specimens used for drugs of abuse testing. J Anal Toxicol 1999; 23: 72.
49. Dasgupta A, Chughtai O, Hannah C, Davis B, Wells A. Comparison of spot tests with AdultaCheck 6 and Intect 7 urine test strips for detecting the presence of adulterants in urine specimens. Clin Chem Acta 2004; 348: 19–25.
50. Kadehjian L. Legal issues in oral fluid testing [Review]. Forensic Sci Int 2005; 150: 151–160.

# 18 Hair, Oral Fluid, Sweat, and Meconium Testing for Drugs of Abuse

*Advantages and Pitfalls*

*Uttam Garg, PhD*

***Contents***



**Summary**

Urine is the most widely used specimen for the detection of and analysis of abused drugs. However, commonly abused drugs can only be detected 1–3 days after abuse using urine specimens. Urine collection, for forensic purposes, requires a special collection facility. Despite strict rules for specimen collection, due to privacy issues in urine collection, sample adulteration is not uncommon. The other matrices provide solutions to some of these problems and have additional advantages for drug testing programs. Hair analysis provides non-invasive and supervised sample collection with the largest window of drug detection period (>90 days). Oral fluid analysis provides a convenient way of sample collection under direct supervision and is useful in assessing very recent drug use. The sweat drug analysis provides continuous monitoring of drug use for several weeks. Meconium is a good specimen for detection of intrauterine drug exposure. Despite many advantages, these matrices pose special challenges, particularly analytical and interpretation of results.

**Key Words:** Hair; oral fluid; sweat; meconium; drugs of abuse.

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## 1. INTRODUCTION

Currently, urine and blood are the most commonly used specimen types for drugs of abuse testing. In these specimen types, commonly abused drugs and/or their metabolites can generally be detected for a few hours to a few days. One of the reasons for common use of these samples is high concentration of drugs in these specimens. In recent years, with the advent of sensitive methods, the interest in alternate samples such as hair, oral fluid, sweat and meconium has grown. These alternative specimens provide unique and sometimes additional information on drugs of abuse. The advantages of these specimens include ease of sample collection, less intrusion during sample collection, decreased potential of sample adulteration and sample stability. Despite many advantages, these alternative specimens also pose a number of challenges for the analytical laboratory including interpretation of data. Relative drug detection times for various specimens are shown in Fig. 1. Advantages and disadvantages of these specimens are listed in Table 1.

## 2. HAIR

Hair is a useful specimen when assessment of repeated or chronic drug abuse is desired. Due to high affinity of heavy metals for keratin, hairs have been used for evaluation of chronic exposure of heavy metals such as arsenic, cadmium and mercury since the 1960s. At that time, analysis of drugs in hair was not possible due to unavailability of sensitive methods. However, using radioactive drugs, it was established that the drugs move from blood to hair. With the availability of sensitive methods, a number of drugs have been detected in hair. In 1979, Baumgartner et al. *(1)*,

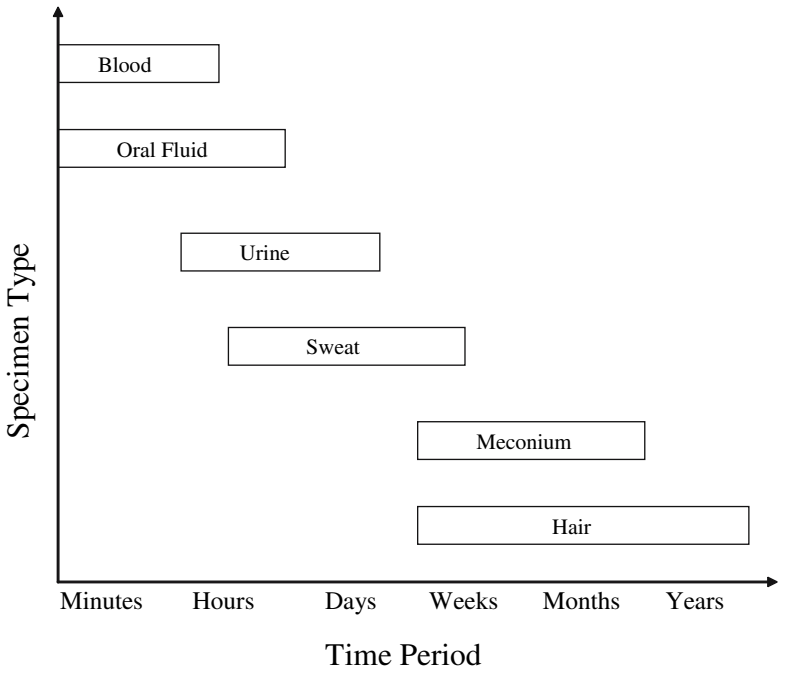


**Fig. 1.** Approximate drug detection periods for various specimen types.

**Table 1**
**Advantages and Disadvantages of Different Specimen Types for Drugs of Abuse Testing**

| *Specimen* | *Advantages* | *Disadvantages* |
|---|---|---|
| Blood | Indicates recent use<br>Better correlation with impairment as compared to other specimen types<br>Difficult to adulterate | Difficult sample collection<br>Requires laboratory analysis; Point of care tests not available |
| Hair | Longer detection window (weeks to years)<br>Relatively non-invasive<br>Difficult to adulterate<br>Very stable specimen, for years<br>Possibility of repeat collection<br>Can be used in mummified or exhumed bodies | Inability to detect recent use<br>More expensive<br>Sample may not be available<br>Hair color bias issues<br>Requires laboratory analysis; Point of care tests not available |
| Oral fluid | Indicates recent use<br>Relatively non-invasive<br>Difficult to adulterate<br>Point of care tests available | Shorter window of detection<br>Possibility of external/passive contamination<br>Inability to produce sample by some individuals |
| Sweat | Longer detection window (3–14 days)<br>Relatively non-invasive<br>Patches tamper resistant | Passive/environmental contamination<br>Expensive<br>Questionable accuracy<br>Requires laboratory analysis; Point of care tests not available |
| Urine | Well-established and studied specimen type<br>Well-established standards among laboratories<br>Less expensive<br>Larger menu<br>Point of care tests available | Needs special facility for sample collection<br>High adulteration potential<br>Shorter detection window<br>Collection may be considered invasive |

using radioimmunoassay (RIA), reported the detection of morphine in hair. The authors also found that presence of morphine along the hair shaft correlated with the time of drug use. Hair consists of hair follicle and hair shaft. In keratin matrix of hair, drugs are incorporated permanently. In contrast, the presence of an abused drug can be detected in serum or urine from a few hours to a few days. Circulating drugs or their metabolites are deposited in the hair follicles first and eventually get entrapped in the core of the hair shaft as the hair grows out of the hair follicle. The drugs can also incorporate in the hair from sweat and sebum and from external environment.

Specimen collection for hair analysis at the present time is not well standardized. Hair is best collected from the area at the back of the head called vertex posterior. This area has less variability in hair growth and number of hairs in the growth phase and is also less influenced by age and sex *(2)*. Hair in this area grows at a relatively constant rate of approximately 0.5–1.0 cm/month, although there are variations among sex, age and race. Sample size varies considerably among laboratories, some requiring

**Table 2**
**SAMHSA-Proposed Initial Cut-off Concentrations for Hair Samples**

| *Analyte* | *Concentration (pg/mg)* |
|---|---|
| Marijuana metabolites | 1 |
| Cocaine metabolites | 500 |
| Opiate metabolites[a] | 200 |
| Phencyclidine | 300 |
| Amphetamines[b] | 500 |
| MDMA | 500 |

MDMA, methylenedioxymethamphetamine; SAMHSA, Substance Abuse Mental and Health Services Administration.

[a] Laboratories are permitted to initial test all specimens for 6-acetylmorphine using a 200 pg/mg cut-off.

[b] Methamphetamine is the target analyte.

only a single hair and others requiring up to a 200 mg sample. The distance from the hair root reflects the time elapsed since drug use.

Substance Abuse Mental and Health Services Administration (SAMHSA) proposes use of approximately 1.5-inch-long hairs which represent a time period of approximately 90 days. Hair samples are useful for pre-employment, random, return to duty or follow-up testing. Hair is not a suitable specimen for reasonable suspicion or cause and post accident as it takes 7–10 days for drugs and drug metabolites to appear in hair. SAMHSA-proposed screening and confirmatory cutoff for hair drug testing are summarized in Tables 2 and 3 *(3)*.

There are a large number of procedures described in the literature for drug hair analysis. The common steps in these procedures include external decontamination of hair, extraction of drug(s) and analysis of drug(s) of interest. Before extraction of drugs, it is important that the hair samples are thoroughly washed to remove any external contamination. Though it depends on the drug of interest, hair is generally washed with organic solvents such as acetone, methanol, methylene chloride and isopropanol. Aqueous solutions such as water and phosphate buffer are not very effective. The sample is then dried and cut into 1–3 mm long pieces or grounded by mechanical beating using metal or glass beads. This is followed by drug extraction and analysis. Common methods of drug hair analyses include highly sensitive immunoassays (RIA, enzyme immunoassays and fluorescent immunoassays), gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-mass spectrometry (HPLC-MS). Tandem mass spectrometry (MS/MS) using gas or liquid chromatography (LC) has emerged in recent years. This analytical tool is particularly useful for determination of concentrations of abused drugs in hair specimens due to high sensitivity and specificity of this technique compared to conventional GC-MS or HPLC-MS.

### *2.1. Detection of Specific Drugs in Hair*

In the following paragraphs analysis of specific class of abused drugs in hair specimens will be discussed.

**Table 3**
**SAMHSA-Proposed Confirmatory Cut-off Concentrations for Hair Samples**

| *Analyte* | *Concentration (pg/mg)* |
|---|---|
| Marijuana metabolite[a] | 0.05 |
| Cocaine | |
| Cocaine [b] | 500 |
| Cocaine metabolites [b] | 50 |
| Opiates | |
| Morphine | 200 |
| Codeine | 200 |
| 6-Acetylmorphine [c] | 200 |
| Phencyclidine | 300 |
| Amphetamines | |
| Amphetamines | 300 |
| Methamphetamine[d] | 300 |
| MDMA | 300 |
| MDA | 300 |
| MDEA | 300 |

MDA, methylenedioxyamphetamine; MDEA 3,4-methylenedioxyethylamptamine; MDMA, methylenedioxymethamphetamine; SAMHSA, Substance Abuse Mental and Health Services Administration.

[a] Delta-9-tetrahydrocannabinol-9-carboxlic acid.

[b] Cocaine concentration is greater than or equal to confirmatory cut-off, and benzoylecgonine /cocaine ratio is $\geq$ 0.05 or cocaethylene $\geq$ 50 pg/mg or norcocaine $\geq$ 50 pg/mg.

[c] Specimen must also contain morphine at a concentration $\geq$ 200 pg/mg.

[d] Specimen must also contain amphetamine at a concentration $\geq$ 50 pg/mg.

### *2.2. Amphetamines*

Early experiments on guinea pigs suggested that amphetamines incorporate into hair and may be considered as a sensitive and long-term marker of amphetamine consumption in humans *(4)*. Over the years, large number of publications has appeared in the literature describing the methods for detection of amphetamine and other sympathomimetic amines in hair. These methods include immunoassays, GC, GC-MS and HPLC *(5–10)*.

Rothe et al. *(11)* described GC-MS protocol with selected ion monitoring for simultaneous analysis of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) for hair samples obtained from subjects with a self-reported history of amphetamine or ecstasy use. The samples were digested with sodium hydroxide, followed by solid-phase extraction using a C-18 column and derivatization with pentafluoropropionic anhydride. Though amphetamine was detected in 17 of 20

samples, methamphetamine was not detected in any sample. Also, despite enormous interindividual variations, there was a correlation between increasing ecstasy abuse and concentration of MDA, MDMA and MDEA in the proximate 3-cm segments. Using a similar method, Cooper et al. *(12)* analyzed hair samples for amphetamine, methamphetamine, MDA, MDMA and MDEA from 100 subjects recruited from the "dance scene" in and around Glasgow, Scotland. These subjects self-reported drug use. The hair samples were analyzed in two 6-cm segments or in full, ranging from 1.5 to 12 cm depending on the length of the hair. Of the 139 segments analyzed, 77 (52.5%) were positive for at least one of the five amphetamines. Despite more than 50% concordance between the drugs consumed and the drugs detected in hair, there was no correlation between the reported number of "ecstasy" tablets consumed and the drug levels detected in hair.

Koide et al. *(13)* described head-space solid-phase microextraction (SPME) and gas chromatography with nitrogen-phosphorus detection method for detection of amphetamine and methamphetamine. The authors used 1 mg of hair. The methods for amphetamine and methamphetamine were linear in the ranges of 0.4–15 and 4–160 ng/mg hair samples with limit of detection of 0.1 and 0.4 ng/mg of hair, respectively.

Like urine, the guidelines require the presence of amphetamine on methamphetamine-positive samples *(3)*.

### 2.3. Cannabinoids

$\Delta^9$-Tetrahydrocannabinol (THC) is a major active constituent of cannabis (e.g., marijuana and hashish). The two major metabolites of THC are 11-hydroxytetrahydrocannabinol (active) and 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) (inactive). When testing for cannabinoids in hair, several analytical considerations should be considered. In hair, THC is present in much higher concentrations than THC-COOH. In contrast, THC-COOH is the major cannabinoid present in urine and blood, which is frequently used to demonstrate cannabinoid consumption. Therefore, due to very low concentration, detection of THC-COOH in hair is analytically challenging. However, detection of THC-COOH in hair is considered a better proof of drug use as it is not present in the smoke or dust and is an in vivo metabolite of THC. Kintz et al. *(14)* used GC-MS-negative chemical ionization (NCI) for detection of THC-COOH in hair. In their method, samples (100 mg) were decontaminated with methylene chloride and pulverized in 1 mL of 1 N sodium hydroxide for 30 min at 95°C in the presence of 10 ng THC-COOH-$d_3$. After acidification of the sample, the drug was extracted with n-hexane/ethyl acetate and derivatized with pentafluoropropionic anhydride/pentafluoropropanol. The concentration of THC-COOH found in their study ranged from 0.02 to 0.39 ng/mg. Wilkins et al. *(15)* used high-volume injection coupled with NCI-MS for quantitation of THC-COOH in hair. In this method, hair specimens were washed, incubated in sodium hydroxide and subjected to solid-phase extraction before analyses. Several other methods for analysis of cannabinoids including THC, cannabidiol (CBD), cannabinol (CBN) and THC-COOH have been described. These methods include GC-MS with electron impact ionization, GC-MS

with MS operated in NCI mode as well as capillary electrophoresis with electrochemical detection *(16–21)*.

### *2.4. Cocaine*

Cocaine and its metabolites have been detected in hair using various techniques, the most common being immunoassay and GC-MS. Immunoassays were the earlier techniques used for the detection of cocaine in hair. Baumgartner et al. *(22)* used RIA for analysis of cocaine metabolite benzoylecgonine in the hair of patients undergoing drug rehabilitation and admitting cocaine use in the last 6 months. Due to high sensitivity, rapid analysis time and low cost, immunoassays are still frequently employed for screening cocaine in hair. Confirmation is generally performed by using GC-MS. Before analysis, hair samples are washed and digested to release cocaine and its metabolites from hair matrix. Wash solutions, which are frequently used, include phosphate buffer, sodium dodecyl sulphate and methanol. When using lower cutoff, it is important that robust wash method be used to avoid any false-positive result from external contamination. Schaffer et al. *(23)* compared isopropanol/phosphate buffer and methanol wash procedures for removal of externally applied cocaine. Isopropanol/phosphate buffer was more effective than methanol wash. With methanol wash procedure, 8 of 14 samples exceeded a cutoff of 5 ng/10 mg hair as compared to none with isopropanol/phosphate buffer wash *(23)*.

Correlation has been found between the concentration of cocaine in hair and amount of cocaine consumed. Graham et al. *(24)* reported benzoylecgonine concentration of 0.64–29.1 ng/mg in heavy users and 0.032–1.21 ng/mg in occasional users. Moreover, there is also a correlation between time elapsed after use of cocaine and cocaine concentration in hair. Seven subjects who used cocaine for 2–12 months were tested before and after 2 months of abstinence. Benzoylecgonine concentrations, estimated by RIA, were 0.6–6.4 and 0.3–0.5 ng/mg hair, respectively. Hair samples from infants born to cocaine-addict mothers had benzoylecgonine concentration between 0.2 and 2.75 ng/mg *(24)*. No drug was detected after 10 weeks. This phenomenon is due to loss of fetal hair. A study on maternal urine and neonatal hair testing for cocaine showed hair analysis for gestational cocaine exposure had a sensitivity of 88%, specificity of 100% and positive and negative predictive values of 100% and 85%, respectively *(25)*. A 10-month follow-up of a female cocaine user, by urine and hair cocaine testing, showed disappearance of cocaine in the hair sections closet to the root, in the first 3 months after the last consumption of cocaine *(26)*.

Due to question of external contamination, it is commonly desired that the cocaine metabolites be also measured. Detection of cocaine metabolites increases the possibility of detection of an active drug user and reduces the possibility of false-positive results due to external contamination. SPME method for simultaneous determination of cocaine and cocaethylene (transesterification product of cocaine and ethanol) has been described *(27)*. A highly sensitive GC-MS/MS method using ion trap in positive chemical ionization for simultaneous determination of cocaine, anhydroecgonine methyl ester, ecgonine methyl ester and cocaethylene metabolites has been reported. The limit of detection for these compounds was 0.005, 0.050, 0.025, 0.005 ng/mg hair, respectively *(28)*.

### 2.5. Opiates

Over the years, a number of reports on detection of opiates in hair have appeared in the literature. Using RIA, in 1979, Baumgartner et al. *(1)* reported the presence of heroin and morphine in hair samples collected from heroin addicts. All hair samples were positive as compared to only 30% urine samples being positives in these known heroin addicts. The authors also found a correlation between drug concentration and duration of drug use. However, in another study on 20 subjects taking part in a heroin maintenance program and receiving drug doses of 30–800 mg/day with total doses ranging from 14,100 to 71,540 mg, no significant correlation was found between heroin dose and total opiate concentration *(29)*. The method used was GC-MS with detection limit of 0.03, 0.05 and 0.04 ng/mg hair for morphine, 6-acetylmorphine and heroin, respectively. Jones et al. *(30)* described a solid-phase extraction GC-MS method for simultaneous determination of several opiates including codeine, morphine, hydrocodone, hydromorphone, 6-acetylmorphine and oxycodone in hair and oral fluid. A highly sensitive and specific GC-MS-MS method for determination of opiates, cocaine, amphetamine and anabolic steroids has been described *(31)*. Lachenmeier et al. *(32)* used oral fluid microplate enzyme immunoassays for hair opiate testing and found them to have good correlation with GC-MS. Cooper et al. *(33)* evaluated Cozart microplate ELISA method for opiate detection in hair. The authors extracted the drugs at 60°C from hair using methanol. The methanolic extract was dried and derivatized with N,O-bis (trimethylsilyl) trifluoro-acetamide and analyzed with GC-MS. Using a cutoff of 200 pg/mg hair, Cozart Enzyme Immunoassay (EIA) had sensitivity and specificity of 98 and 93%, respectively.

### 2.6. Phencyclidine

The presence of phencyclidine (PCP) and its metabolites have been reported in human and animal hair. Baumgartner et al. *(34)* used RIA for the detection of PCP in hair from subjects admitting the use of PCP. Using analysis of hair specimens, all seven subjects tested were positive for PCP. Only 1 of 7 subjects tested positive with urine analysis. PCP concentrations were 0.3–5.2 and 0.3–2.8 ng/mg in unwashed and washed hair samples, respectively. There was also a correlation between duration of PCP use and concentration of PCP found in hair. In another study using psychiatric patients, hair analysis detected PCP in 11 of 47 patients. Blood and urine was negative on all these 47 patients *(35)*. Studies also demonstrate the stability and long-term detection of PCP in hair samples. Swartz et al. *(36)* showed better positive predictive value of hair analysis compared to urine analysis for PCP.

### 2.7. Issues of Special Interest in Hair Drug Testing

Several factors affect concentrations of abused drugs in hair including color of hair, external contamination and environmental factors.

#### 2.7.1. Hair Color

Concerns about the role of hair color and preferential incorporation of drugs into different hair color have been raised. Melanin, a polyanionic polymer of eumelanin and pheomelanin, determines the hair color. Eumelanin concentration is highest in black

color hair and pheomelanin concentration is highest in red color hair, whereas melanin is absent in white color hair. It is postulated that differences in drug concentrations in different color hair are due to differences in concentrations of these pigments. Human and animal studies have been reported in the literature investigating the effect of hair color on drug incorporation in hair.

Borges et al. *(37)* used Long–Evans rats to study the affect of hair color on amphetamine incorporation. Long–Evans rat provides an ideal model for the study of drug incorporation into pigmented versus non-pigmented hair as it produces both pigmented and non-pigmented hair. In that particular study, black hair showed significantly higher concentration of amphetamine as compared to white hair, $6.44 \pm 1.31$ and $2.04 \pm 0.58$ ng /mg hair, respectively. Kronstrand et al. *(38)* analyzed methamphetamine and amphetamine in pigmented and non-pigmented hair from patients on long-term selegiline medication; selegiline metabolizes to methamphetamine and amphetamine. Concentrations of amphetamine and methamphetamine were significantly higher in pigmented hair as compared to non-pigmented hair. Effect of hair color and amount of cocaine incorporation has also been studied in vitro by incubation of hair with cocaine and benzoylecgonine. Relative incorporation for benzoylecgonine was black > brown > blond hair *(39)*. In another study on self-reported cocaine users, 37 of 38 hair samples were positive for cocaine. The authors reported significantly more cocaine in black hair as compared to brown or blond hair *(40)*. A study by Goldberger et al. *(41)* on 20 subjects found higher concentrations of cocaine in Africoid group as compared to Caucasoid group. The question of PCP incorporation in pigmented versus non-pigmented hair has been explored using animal models. Using Long–Evans rat as experimental model, Slawson et al *(42)* reported PCP levels of $14.33 \pm 1.43$ and $0.47 \pm 0.04$ ng/mg in pigmented and non-pigmented hair, respectively. The authors used intraperitoneal PCP dose of 12 mg/kg.

Rollins et al. *(43)* studied the effect of hair color on the incorporation of codeine. Human volunteers were given 30 mg codeine three times a day for 5 days. Codeine and melanin concentrations were measured for several weeks. The mean ± SE hair codeine concentrations 5 weeks after dosing were $1429 \pm 249$, $208 \pm 17$, $99 \pm 10$ and $69 \pm 11$ in black, brown, blond and red hair, respectively. These differences in codeine concentrations could be attributed to different melanin concentrations in different hair types. Normalization of the codeine concentration with the melanin concentration reduced the hair color differences. From the data, the authors confer that assuming if this dosing protocol is used, the proposed federal guideline cutoff of 200 pg/mg of codeine would result in 100% of subjects with black hair and 50% of subjects with brown hair being reported as positive, while subjects with blond or red hair would be reported as negative.

However, there are a number of studies that found no significant difference in the amounts of drugs between different colors of hair, race or ethnicity. Schaffer et al. *(44)* investigated the effects of cocaine concentrations and hair porosity on contamination and decontamination. Hairs of different color (blond, auburn, brown and black) were incubated with different concentrations of cocaine. Uptake and concentration of cocaine correlated with the time of incubation, but no correlation was found between hair color and cocaine concentration. When hair was permed, there was an increase in cocaine uptake. The authors concluded that porosity, not hair color, determined the penetration

of cocaine in hair. In another study using 1852 people that classified themselves as "black" or "white" showed no evidence of a group adversely affected by hair testing, compared to urine testing, for cocaine and marijuana *(45)*. Kelly et al. *(46)* examined 2000 randomly selected hair samples, 500 negative and 500 positive for each of three drugs—cannabinoids, cocaine, and amphetamine, and evaluated ethnic/racial factors and hair color in relation to positive hair and urine samples. The authors found that the observed outcome patterns were largely consistent with differences in drug preferences among the various ethnic/racial groups and there was no bias based on hair color alone. Likewise, there was no discernible pattern associated with race or ethnicity that would lend support to a "race effect" in drug analysis.

Despite these controversies, the SAMHSA proposes hair drug testing and emphasizes that irrespective of hair color there is no question about the fact that the hair drug testing can identify drug users and deter those who intend to use drugs.

### 2.7.2. Environmental or External Contamination

Concerns have been raised regarding environmental contamination of hair. For example, a person can claim that the positive hair test was due to the individual being present in a room where others were using marijuana or cocaine or use of hair shampoo that contained marijuana. Although effective methods are available for removing external contamination of drugs from hair, some studies suggest that despite extensive washings, under certain conditions, complete removal of external contaminants is not possible *(47)*. Moreover, different wash procedures lead to significant differences in the measured concentrations of drugs in hair indicating that certain wash procedures, in addition to removing external contamination, may also remove incorporated drugs from hair *(48,49)*. In one study, four washing procedures for removing cocaine and its metabolites benzoylecgonine, norcocaine, ecgonine methyl ester and cocaethylene produced significantly different results in concentration of these analytes *(48)*.

Cannabis plants are used in many products such as shampoo, oils, noodles, crackers and beverages. These products often contain $<1\%$ THC in order to eliminate psychoactive effects, but some may contain as high as 3% THC such as hair shampoos *(50)*. One study on Cannabio shampoo revealed concentrations of THC, CBD and CBN as 412, 4079 and 380 ng/mL, respectively *(50)*. Because these shampoos contain significant amount of cannabinoids, questions have been raised about the possibility of false-positive laboratory results by the use of these shampoos. To explore this possibility, in one study, three subjects washed their hair with Cannabio shampoo once daily for 2 weeks. After this time period, the hair samples were analyzed for THC, CBD and CBN and were found negative for the presence of these compounds. The limits of detection for THC, CBD and CBN were 0.05, 0.02, and 0.01 ng/mg, respectively. To study the effect of longer exposure time to Cannabio shampoo, hair specimens (200 mg) were incubated with 10 mL water/Cannabio shampoo (20:1, v/v) for 30 min, 2 and 5 h. After an incubation period of 30 min, the analysis of hair by GC-MS did not reveal any presence of THC, CBD and CBN. When samples were incubated for 2 and 5 h, the specimens tested positive for CBD and CBN. However, THC was not detected in any specimens at any incubation time period. The study concluded that unrealistic use of Cannabio shampoo can cause drug-free hair to test positive for CBD and CBN but not for the primary psychoactive drug THC *(50)*.

Studies also suggest that external contamination of marijuana can be effectively ruled out by detecting marijuana metabolite. Uhl and Sachs *(51)* reported a case in which hair samples from a couple living together in an apartment tested positive for THC and CBN. The male subject admitted smoking marijuana whereas the female subject denied any consumption. Analysis of the hair samples for THC-COOH showed a high level (>6.6 pg/mg) in the sample of the male and negative results [limit of quantitation (LOQ) 0.1 pg/mg] in the sample of the female. Therefore, it appears that detection of THC-COOH in hair sample is an effective strategy to demonstrate active use versus passive exposure as well as to rule out external contamination. SAMHSA guidelines propose guidelines for hair analysis of THC-COOH *(3)*.

Detection of PCP without detection of PCP metabolites may be due to external contamination. The detection of PCP metabolites provides better evidence of active use rather than due to external contamination. Nakahara et al. *(52)* reported simultaneous detection of PCP and its two major metabolites 1-(1-phenylcyclohexyl)-4-hydroxypiperidine and trans-1-(1-phenyl-4-hydroxycyclohexyl)-4'-hydroxypiperidine (t-PCPdiol) by GC-MS. As t-PCPdiol is a major metabolite, the authors recommend using this metabolite as an indicator of active PCP use. In SAMHSA guidelines, analysis of PCP is recommended without any mention of PCP metabolites. This is probably due to limited understanding on PCP metabolites in hair samples and infrequent availability of methods for measurement of PCP metabolites.

Detection of cocaine metabolites is useful in distinguishing active use of cocaine from external contamination. Koren et al. *(53)* showed that pyrolysis of crack results in accumulation of cocaine in hair, but not its metabolite benzoylecgonine, and in cocaine users, both cocaine and benzoylecgonine are detectable in hair. However, Romano et al. *(49)*, despite extensive washings, detected small quantities of benzoylecgonine (generally <0.5 ng/mg) from externally contaminated hair.

Despite these controversies, it appears that use of appropriate wash procedures and appropriate cutoff values for drugs or their metabolites can effectively distinguish external contamination from active drug use.

### *2.8. Products Claiming to Beat Hair Drug Test*

There are numerous sites on the Internet that sell products and claim to beat or pass the hair drug test. Searching on Google using the phrase "pass hair drug test" showed 6,620,000 hits in 0.22 s. Although most of these sites claim that their product works 100%, and some with money back guarantee, there is very little scientific evidence whether these products actually work.

Rohrich et al. *(54)* investigated the effect of shampoo Ultra Clean (Zydot Unlimited, Tulsa, Ok) on eliminating THC, cocaine, amphetamine, MDA, MDMA, MDEA, heroin, 6-monoacetylmorphine (6-MAM), morphine, codeine, dihydrocodeine and methadone from hair samples. The authors used postmortem hair samples from subjects with known history of drug abuse. Their findings suggested that single use of Ultra Clean did not sufficiently remove these drugs to cause negative results.

## 3. ORAL FLUID

The terms oral fluid and saliva have been used interchangeably in the literature. However, oral fluid is a preferred term as it is the fluid that is collected and analyzed. It contains saliva (secretion of salivary glands), mucosal transudate and crevicular fluid.

Oral fluid is becoming a popular specimen for drug of abuse testing. The use of oral fluid in impaired subjects is of particular interest because it is easy to collect on-site as compared to blood and urine and provides a better indication of recent drug use. In forensic situations, it can be collected under close supervision to avoid adulteration and substitution. Concentrations of drugs in oral fluid correlate better with blood concentrations as compared to urine.

Collection of oral fluid is not very well standardized, and there are various techniques for its collection. In direct non-stimulated technique, there is more froth than actual liquid resulting in viscous and small sample size and causing problems in sample analysis. For this reason, many investigators suggest salivation stimulation by sour candy or citric acid crystals. Manual stimulation can be achieved by chewing on inert material such as Teflon. Substances such as Parafilm should be avoided as they may absorb lipophilic drugs. Various devices including Salivette, Omni-Sal, Intercept, Accusorb, Saliva Sampler and SalivaSac are commercially available for oral fluid collection. Samples collected using collection devices generally provide cleaner specimens as compared to direct spitting in which the sample contains cell debris, food particles and strings of mucous.

Once the oral fluid is collected, screening and confirmation are generally performed in the laboratory. Screening generally involves immunoassays, and confirmation is performed by GC-MS or LC-MS. In recent years, point of care devices have become available. These devices include OralLab, RapiScan, Drugwipe and SalivaScreen. Walsh et al. *(55)* compared these on-site devices and concluded that these devices perform well for the detection of methamphetamine and opiates, but poor for the detection of cannabinoids. The ability to accurately and reliably detect cocaine and amphetamine was dependent on the individual device *(55)*. SAMHSA-proposed screening and confirmation cutoff values in oral fluid for various drugs of abuse are shown in Tables 4 and 5 *(3)*.

Using a large number of oral fluid specimens ($n$ = 77,218), one study investigated the rate of positivity for amphetamines, cannabinoids, cocaine, opiates and PCP in non-regulated workplace drug testing programs *(56)*. The oral fluid samples were collected using Intercept Oral Collection device (OraSure Technologies, Bethlehem, PA). The device consists of an absorbent cotton fiber pad on which sample is collected by placing the pad between the lower gum and cheek for 2–5 min. The collected sample was placed in preservative solution for transportation to the laboratory. The samples were screened by EIA using cutoffs of 3, 15, 30, 3 and 120 ng/mL for THC (parent drug and metabolite), cocaine metabolites, opiate metabolites, PCP and amphetamines, respectively. The positive samples were confirmed by GC-MS-MS using cutoffs of 1.5, 6, 30, 30, 3, 1.5, 120 and 120 ng/mL for THC (parent drug), benzoylecgonine, morphine, codeine, 6-acetylmorphine, PCP, amphetamine and methamphetamine, respectively. Of 77,218 samples tested, 3908 (5.06%) confirmed positive. The frequency of positivity was THC (3.22%) > cocaine (1.12%) > amphetamines (0.47%) > opiates (0.23%) > PCP (0.03). In this study, the overall prevalence rate for drug detection was comparable to the urine drug prevalence rates in the general workforce ($n > 5,200,000$, positive rate of 4.46%). Oral fluid positivity rates, for amphetamine and cocaine, were 60% higher as compared to urine suggesting that these drugs are more efficiently accumulated in oral fluid as compared to urine. Another remarkable finding in this study was

**Table 4**
**SAMHSA-Proposed Initial Cut-off Concentrations for Oral Fluid Samples**

| *Analyte* | *Concentration (ng/mL)* |
|---|---|
| THC parent drug and metabolite | 4 |
| Cocaine metabolites | 20 |
| Opiate Metabolites[a] | 40 |
| Phencyclidine | 10 |
| Amphetamines[b] | 50 |
| MDMA | 50 |

MDMA, methylenedioxymethamphetamine; SAMHSA, Substance Abuse Mental and Health Services Administration; THC, $\Delta^9$-tetrahydrocannabinol

[a] Labs are permitted to initially test all specimens for 6-acetylmorphine using a 4 ng/mL cut-off.

[b] Methamphetamine is the target analyte.

**Table 5**
**SAMHSA-Proposed Confirmatory Cut-off Concentrations for Oral Fluid Samples**

| *Analyte* | *Concentration (ng/mL)* |
|---|---|
| THC parent drug | 2 |
| Cocaine[a] | 8 |
| Opiates | |
| Morphine | 40 |
| Codeine | 40 |
| 6-Acetylmorphine | 4 |
| Phencyclidine | 10 |
| Amphetamines: | |
| Amphetamine | 50 |
| Methamphetamine[b] | 50 |
| MDMA | 50 |
| MDA | 50 |
| MDEA | 50 |

MDA, methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, methylenedioxymethamphetamine; SHMHSA, Substance Abuse Mental and Health Services Administration; THC, $\Delta^9$-tetratydeocannabiol.

[a] Cocaine or benzoylecgonine.

[b] Specimen must also contain amphetamine at a concentration great than or equal to the limit of detection.

the presence of 6-acetylmorphine in 66.7% morphine-positive samples. However, in another study using 114 adult arrestees, for THC, saliva had sensitivity of only 5% when urinalysis was used as the reference standard. Cocaine and heroin had sensitivity of 100 and 88% and specificity of 99% and 100%, respectively *(57)*.

### *3.1. Detection of Specific Drugs in Oral Fluid*

Several methods have been reported in the literature for analysis of various drugs of abuse in oral fluid.

### *3.2. Amphetamines*

Amphetamine, methamphetamine, MDA and MDMA have been detected in oral fluid. The analytical methods include immunoassays *(58)*, GC-MS *(59)*, LC with fluorescent detector *(60)* and LC-MS *(61,62)*. Gunnar et al. *(63)* described a solid-phase extraction, long-column fast gas chromatography/electron impact mass spectrometry method for simultaneous determination of 30 drugs of abuse including amphetamines, opiates, methadone, cocaine, alprazolam, midazolam, fentanyl and zolpidem. Amphetamine-type stimulant drugs were acylated with heptafluorobutyric anhydride; benzodiazepines and THC were silylated with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide; and benzoylecgonine, codeine, ethylmorphine, 6-MAM, morphine, pholcodine, buprenorphine and norbuprenorphine were derivatized with N-methyl-N-(trimethylsilyl)trifluoroacetamide. The concentration of amphetamine in oral fluid is generally comparable to whole blood, and d-amphetamine is eliminated faster than l-amphetamine.

In another study using human volunteers who received four doses of 10 and/or 20 mg S-(+)-methamphetamine within 7 days, oral fluid methamphetamine concentrations were higher than plasma methamphetamine concentrations. Average area under the curve (AUC), for the 24-h time period for oral fluid, was on average 3.8 times higher than the plasma AUC. Disposition of methamphetamine in oral fluid was dose related, though there was high intra- and inter-individual variability *(64)*

SAMHSA-proposed guidelines provide cutoff values for amphetamine, methamphetamine, MDMA, MDA and MDEA. Like urine, these guidelines require the presence of amphetamine on methamphetamine-positive samples *(3)*.

### *3.3. Cannabinoids*

THC is a major component of cannabinoid in oral fluid, whereas THC-COOH is present in very low concentration. The presence of THC in oral fluid is from smoking, and ingestion as very little transport occurs from blood to oral fluid. Detection of cannabinoids in oral fluid is a better indicator of recent use than detection of the metabolite in urine. Due to low concentration of THC in oral fluid and limited amount of sample, sensitive methods for detection are required. A number of methods for detection of cannabinoids in oral fluid have been described. Immunoassays and GC-MS are the most commonly used methods for detection of cannabinoids in oral fluid. Maseda et al. *(65)* described a method for determination of THC as pentafluoropropyl derivatives using electron capture detector (GC/ECD). The method was linear over the range of 5–200 ng/mL with a detection limit of 1 ng/mL. It was demonstrated

that for at least 4 h after smoking the level of THC was sufficient for detection. A GC-MS method for detection of multiple drugs including cannabinoids CBD, THC, CBN and methadone, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate, cocaine, cocaethylene, amphetamine, methamphetamine, MDMA, MDEA and N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine has been described *(66)*.

One study evaluated the Triage, a rapid urine-screening test from Biosite Diagnostics, for the detection of drugs of abuse in oral fluid. In the study, urine and oral fluid samples from 21 drug abusers were collected and validated by GC-MS. Though methadone, opiates and barbiturates were detected in some samples, THC was not detected in any sample, indicating the limited use of this device in detection of drugs of abuse in oral fluid samples *(67)*.

### *3.4. Cocaine*

Because cocaine is weakly basic and oral fluid is generally more acidic than plasma, the concentration of ionized cocaine in oral fluid is generally higher than plasma and is detectable for longer time periods as compared to plasma. When cocaine is consumed by smoking or through intranasal routes, oral fluid/plasma ratio is higher for several hours. There are a number of reports on excretion of cocaine and its metabolites in oral fluid. Cocaine and its metabolites (anhydroecgonine, benzoylecgonine and ecgonine methyl ester) appear in oral fluid after intravenous injection, inhalation and intranasal administration of the drug. At higher concentrations, cocaine concentrations exceed benzoylecgonine and ecgonine methyl ester concentrations. However, at a level less than 100 ng/mL, the concentrations of cocaine, benzoylecgonine and ecgonine methyl ester in oral fluid are comparable. SAMHSA-proposed cutoffs are 20 ng/mL for screening (cocaine metabolites) and 8 ng/mL for confirmation (cocaine or benzoylecgonine). The methods of analyses of cocaine and its metabolites include immunoassays *(68)*, GC-MS *(69–71)* and LC-MS *(72)*.

### *3.5. Opiates*

A number of studies have shown the presence of opiates in oral fluid *(67,73–75)*. The major opiates studied were codeine, morphine, hydrocodone, hydromorphone, 6-acetylmorphine and oxycodone. Jones et al. *(30)* described a GC-MS method for simultaneous measurement of these opiates in hair and oral fluid. When performing opiate assay, keto-opiates (hydrocodone, hydromorphone and oxycodone) pose special problems as they form multiple derivatives that interfere in analysis of other opiates and are difficult to quantitate. Oximization with hydroxylamine or methoxamine is used to overcome this problem *(76)*. Singh et al. *(77)* used sodium bisulfite followed by solid-phase extraction to eliminate interference by keto-opiates in the analysis of opiates. Methadone which is extensively used in maintenance therapy of heroin addicts can also be detected in oral fluid.

### *3.6. Phencyclidine*

PCP has been detected in oral fluid, and good correlation has been found between oral fluid and serum concentrations. In one study using 100 patients from an emergency department suspected of using PCP, 74 oral fluid specimens and 75 serum specimens

were found positive for PCP. The method used was RIA and there was no correlation between PCP concentrations and severity of intoxication *(78)*. Though there is a good correlation between oral fluid and plasma PCP concentrations, intra- and inter-individual variability are high, and ratio of oral fluid to plasma concentration varies with pH. Therefore, oral fluid PCP concentrations cannot accurately predict plasma concentrations. The frequently used methods for analysis of PCP in oral fluid include immunoassays, GC-MS and LC-MS.

### *3.7. Issues of Special Interest in Oral Fluid Testing*

1. Although oral fluid can be collected under direct supervision and opportunity for sample adulteration is low, measurement of human IgG is recommended for sample validity.
2. To avoid adulteration, supervision of donor for 10–20 min is recommended prior to sampling. The donor should not put anything in his/her mouth during this period.
3. To ensure that positive THC result, using point of care testing device, on an oral fluid specimen is from active exposure, SAMHSA proposes collection of a urine sample along with oral fluid specimen for testing THC metabolite.
4. There are a number of products available on the Internet that guarantee to beat oral fluid drug of abuse tests. There is no scientific evidence that these products work. Using an on-site oral fluid drug screen, Oratect, Wong et al. *(79)* investigated the effects of various adulterants and foodstuffs. Common foods, beverages, food ingredients, cosmetics and hygienic products were demonstrated not to cause false-positive results when tested 30 min after their consumption. In addition, two commercial adulterant products "Clear Choice Fizzy Flush" and "Kleen Mouthwash" were tested and had no effect in destroying the drug compounds or changing the pH of the oral fluid. Their effect was through washing oral cavity and not different from a common mouthwash. In another study, a series of potential adulterants of oral fluid were evaluated and shown not to interfere in Cozart microplate EIA for opiates *(80)*.
5. Salivary pH ranges from 6.2 to 7.4. Stimulation of oral fluid flow raises pH due to secretion of bicarbonate in oral fluid. This can affect concentration of some drugs in oral fluid. Studies have shown that concentration of cocaine and its metabolites is higher in non-stimulated oral fluid as compared to mechanical stimulation. To avoid these effects, some investigators recommend spitting into a cup, but some donors may find spitting offensive especially when observed.
6. Parent drug is generally present in higher concentrations in oral fluid as compared to urine.
7. Some donors may experience dry mouth and may not be able to provide oral fluid sample.
8. Generally, there is a good correlation between oral fluid and free (active) drug in plasma.
9. Due to a short detection window, if more than 24 h prior notice is given for sample collection, oral fluid is not a specimen of choice.
10. There is significant inter-individual variation for sample volume of oral fluid, and there is no easy on-site method to check sample volume. In a study using 83 normal individuals, the volume ranged from 0.05 to 0.8 mL *(68)*.

## 4. SWEAT

With the developments of reliable sweat collection devices and sensitive analytical methods, interest in sweat drug testing has increased over the years. The advantages of sweat analysis for drugs of abuse include continuous drug monitoring for a longer period of time (from a few days to a few weeks) and less invasive and easy process of sample collection. Passive diffusion is thought to be the major mechanism of drugs transport in sweat. Sweat may be collected as liquid perspiration on sweat wipes or with a sweat patch. Sweat patches are commercially available and may be worn for extended period of time. They are waterproof, tamper resistant, comfortable to wear and are generally acceptable by patients. In contrast to urine testing to monitor compliance that requires three sample collections per week, sweat patch worn for 7–14 days provides continuous monitoring for the whole time period.

Sweat testing has been shown to be useful in drug treatment and work place drug testing programs *(81,82)*. SAMHSA-proposed screening and confirmation cutoff concentrations in sweat for various drugs of abuse are shown in Tables 6 and 7 *(3)*. As cutoff values are significantly lower than urine, sensitive methods are needed for sweat testing. Similar methods that are used for urine, hair and oral fluid testing such as immunoassays, GC-MS and LC-MS are frequently used for sweat testing.

Before the application of the sweat patch, the skin should be thoroughly cleaned with an organic solvent such as isopropyl alcohol to remove any dirt, skin oils or skin care products such as lotions and creams. Once the patch is in place, it is difficult to contaminate, as the outer polyurethane layer is impermeable to molecules larger than water. Contaminants applied to the outer layer do not reach the collection pad *(83)*.

### *4.1. Detection of Specific Drugs in Sweat*

Different methods are available for analysis of various abused drugs in sweat specimens.

**Table 6**
**SAMHSA-Proposed Initial Cut-off Concentrations for Sweat Patch Samples**

| *Analyte* | *Concentration (ng/patch)* |
|---|---|
| Marijuana metabolites | 4 |
| Cocaine metabolites | 25 |
| Opiate metabolites[a] | 25 |
| Phencyclidine | 20 |
| Amphetamines[b] | 25 |
| MDMA | 25 |

MDMA, methylenedioxymethamphetamine; SAMHSA, Substance Abuse Mental and Health Services Administration.

[a] Labs are permitted to initial test all specimens for 6-acetylmorphine at 25 ng/patch.

[b] Methamphetamine is the target analyte.

**Table 7**
**SAMHSA-Proposed Confirmatory Cut-off Concentrations for Sweat Patch Samples**

| *Analyte* | *Concentration (ng/patch)* |
|---|---|
| THC parent drug | 1 |
| Cocaine[a] | 25 |
| Opiates[b] | 25 |
| Phencyclidine | 20 |
| Amphetamine | |
| Methamphetamine[c] | 25 |
| MDMA | 25 |
| MDA | 25 |
| MDEA | 25 |

MDA, melnylenedioxyamphetamine; MEDA, 3,4-methylenedioxyetylamphetamine; MDMA, methylenedioxymethamphetamine; SMHHSA, Substance Abuse Mental and Health Services Administraction; TCH, $\Delta^9$-tetrahydrocannabinol.

[a] Cocaine or benzoylecgonine.

[b] Morphine, codeine or 6-acetylmorphine.

[c] Specimen must also contain amphetamine at a concentration greater than or equal to the limit of detection.

### *4.2. Amphetamines*

Presence of amphetamines in sweat has been reported in several studies. Fay et al. *(84)* collected sweat, using a FDA-approved device PharmChek (PharmChem Inc., Haltom City, TX) sweat patch, from volunteers dosed with 10, 20 and 25 mg methamphetamine. The drugs were eluted from the collection pad of the patch and tested using EIA and GC-MS. Sweat primarily contained parent methamphetamine. In another study using 180 drivers who failed the field sobriety tests, sweat samples collected from the forehead with a fleece moistened with isopropanol showed positive predictive value of 90% for amphetamines by GC-MS *(85)*. Samyn et al. *(86)* administered 75 mg MDMA to 12 volunteers who were known recreational MDMA users and then collected sweat for 5 h. The average concentration of MDMA was 25 ng/wipe. Like urine, the guidelines require the presence of amphetamine on methamphetamine-positive samples *(3)*.

### *4.3. Cannabinoids*

Using PharmChek, the presence of THC in sweat has been reported *(87)*. In another study, cosmetic pads were used for sweat collection from foreheads and tested for THC. Out of 198 injured drivers tested, 22 subjects were positive for THC-COOH in urine, 14 and 16 were positive for THC in oral fluid (1–103 ng/Salivette) and forehead wipe (4–152 ng/pad), respectively. 11-Hydroxy-THC and THC-COOH were not detected in oral fluid and sweat *(88)*. Sweat testing has overall good positive predictive values. In one study, the positive predictive value of sweat wipe analysis with GC-MS was over 90% for cocaine and amphetamines and 80% for cannabinoids *(85)*.

### *4.4. Cocaine*

In contrast to urine, sweat primarily contains parent cocaine. Kacinko et al. *(82)* performed a detailed study on 15 subjects with previous history of cocaine abuse. The subjects resided for 12 weeks in a closed research unit. The first 3 weeks were used as a washout period to excrete previously self-administered drug. In the 4th week, the participants received three subcutaneous injections of cocaine hydrochloride (75 mg/70 kg). Three weeks later, the participants received three additional doses of cocaine hydrochloride (150 mg/70 kg). These doses were comparable to the usual doses administered by cocaine drug users. PharmChek sweat patches ($n$ = 1390) were collected throughout a 3-week washout period, reflecting previously self-administered drugs, and during and after controlled dosing. The method of analysis was GC-MS. At a cut-off concentration of 2.5 ng/patch, 24% of patches were positive, whereas at a cut-off of 25 ng/patch (SAMHSA-proposed cut-off), only 7% of patches were positive for cocaine.

Cocaine metabolites have also been studied in sweat. Cocaine metabolite ecgonine methyl ester is generally present in higher concentration in sweat compared to benzoylecgonine *(82,89)*. Cocaine and ecgonine methyl ester also appear in the sweat earlier (within 2 h) as compared to benzoylecgonine (>4 h). In this study by Kacinko et al. *(82)* 5% of patches were positive for benzoylecgonine at the LOQ, whereas only 0.5% positive at SAMHSA-proposed cutoff of 25 ng/patch. Ecgonine methyl ester was detected in more patches than benzoylecgonine for a total of 9% positive patches at the LOQ and only 0.8% of patches at a concentration cut-off of 25 ng/patch. From this study, it appears that the SAMHSA cut-off values are too high and can miss significant number of cocaine users by sweat cocaine testing. The study also analyzed other cocaine metabolites such as cocaethylene, norcocaine, norcocaethylene, m- and p-hydroxycocaine, and m- and p-hydroxybenzoylecgonine.

### *4.5. Opiates*

Fogerson et al. *(83)* used PharmChek patches for sweat collection and EIA and GC-MS for opiate analysis on human subjects injected with known doses of codeine and heroin. The investigators found that sweat primarily contains parent opiates heroin and codeine and lipophilic metabolite 6-MAM. The EIA in which morphine was used as a calibrating analyte showed cross-reactivity with codeine of 588%, with hydrocodone of 143%, with diacetylmorphine of 28% and with 6-MAM of 30%. Using 215 patches from 95 subjects and receiver operator characteristics curves, the optimal cut-off was established at 10 ng/mL morphine equivalents. At this cutoff concentration, the assay had a diagnostic sensitivity of 86.9% and a diagnostic specificity of 92.8% as compared to GC-MS. The positive predictive value at a prevalence of 50% was 86%.

### *4.6. Phencyclidine*

Studies on sweat PCP testing are limited. Using radioactive PCP, Perez-Reyes et al. *(90)* found that PCP is excreted in sweat. After intravenous administration, PCP was detectable in sweat for 54 h. PCP was found to be highly concentrated in sweat as compared to blood during heavy exercise.

**Table 8**
**Effects of Various Adulterants on Sweat Patch Content of Heroin, Morphine or Codeine**

| *Adulterant* | *Heroin EIA % Recovery* | *Heroin GC-MS % Recovery* | *Morphine GC-MS % Recovery* | *Codeine GC-MS % Recovery* |
|---|---|---|---|---|
| Saline | 103 | 111 | 87 | 93 |
| Bleach | 111 | 100 | 80 | 92 |
| Hand soap no. 1 | 90 | 91 | 90 | 95 |
| Hand soap no. 2 | 105 | 111 | 84 | 98 |
| Liquid detergent | **171** | 100 | 88 | 82 |
| Tile cleaner | **200** | **57** | **56** | 93 |
| Drano | 123 | **57** | 85 | 86 |
| Vinegar | 104 | 105 | 90 | 95 |
| Sugar solution | 95 | 109 | 83 | 95 |
| Spot remover | 136 | **59** | 83 | 92 |
| Cranberry juice | 89 | 106 | 91 | 91 |
| Visine | 73 | 103 | 88 | 93 |
| Orange juice | 86 | 113 | 84 | 90 |
| Soda soft drink | 104 | 108 | 85 | 96 |
| Cough medication | **152** | 105 | 87 | 91 |
| Topical analgesic lotion | 81 | 103 | 90 | 92 |
| Mouthwash | 115 | 106 | 87 | 94 |
| Distilled water | 85 | 114 | 91 | 105 |

GC-MS, gas chromatography mass spectrometry
The patches were spiked with a 15 ng/mL of drug. Bolded values indicate potential for false-positive or false-negative results due to adulterant *(83)*.

### *4.7. Issues of Special Interest in Sweat Testing*

1. In one study, the effect of 18 adulterants on EIA and GC-MS for opiates was investigated *(83)*. The patches were spiked with adulterants and air-dried, then spiked with 15 ng/mL heroin, codeine or morphine and incubated at 37°C for 7 days. Most of the adulterants tested did not have any effect with the exception of a few (Table 8). Moreover, exposure of the skin to many of these adulterants causes visible inflammation and discomfort to the subject, decreasing the possibility of their use.
2. The average pH of sweat is 5.8. During exercise, the pH increases to an average of 6.4. The change in pH may affect the migration of drugs in sweat.

## 5. MECONIUM

Abuse of certain drugs among pregnant women remains a significant problem. In 2002, 3% of pregnant women aged between 15 and 44 used illicit drugs in the past months, 3% reported alcohol use and 17% reported smoking cigarettes in the past months *(91)*. It is well established that many drugs pass through placenta and cause fetal injury. Cocaine abuse during pregnancy is associated with an increased risk of prematurity, small for gestational age status, microcephaly, congenital anomalies including cardiac and genitourinary abnormalities, necrotizing enterocolitis, and central nervous

system stroke or hemorrhage *(92)*. Similarly, intrauterine exposure to amphetamine and methamphetamine is associated with low birth weight, premature delivery, increased maternal abruption, congenital brain hemorrhage and infarction. Moreover, the infants exposed to methamphetamine and/or cocaine show disorder in sleep patterns, tremors, poor feeding, hyperactive reflexes, abnormal cry and state of disorganization. In addition to an increased risk of prematurity and being small for gestational age, withdrawal symptoms are often seen in neonates exposed to opiates. In these babies, the symptoms include irritability, hypertonia, wakefulness, jitteriness, diarrhea, increased hiccups, yawning and sneezing, and excessive sucking and seizures. Withdrawal symptoms are also seen in infants exposed to cocaine and amphetamines *(92)*.

Methods used to find out prenatal exposure include interview, questionnaire and urine testing of mother and infant. Due to the consequences of admitting illicit drug abuse, maternal self-reporting drug use is not reliable, and underreporting of drug abuse by pregnant women has been reported in several studies *(93,94)*. To avoid underestimation of in utero exposure, the use of a combination of maternal history, newborn clinical symptoms and laboratory toxicological testing is the best approach *(92)*. In addition, a physician caring for a newborn may be required to investigate and report fetal drug exposure to child protective services. Due to legal implications, a positive maternal history of drug use may not be sufficient, and a definitive documentation of the presence of drug in the baby may be required. Urine underestimates drug exposure, as drugs are present in urine only for few days. Meconium has been shown to be a reliable source for detection of fetal drug exposure with higher sensitivity *(93–96)*. It is easy to collect and provides a wider gestational window for drug detection than fetal or maternal urine. Meconium starts forming between the 12th and 16th week of gestation and accumulates until after birth. It is a complex mixture of epithelial and squamous cells, amniotic fluid and many other products. The disposition of drugs in meconium is not well understood. It is proposed that the fetus excretes drugs into bile and amniotic fluid, and drugs accumulate in meconium either by direct deposition or through swallowing of amniotic fluid *(97)*. A number of methods have been described for detection of drugs of abuse in meconium *(94,98–104)*.

### *5.1. Detection of Specific Drugs in Meconium*

Although meconium is more easily collectable from a newborn as compared to urine, its analysis is considerably more difficult. Before analysis, it is important that the sample be homogenized, as meconium is a non-homogenous and is a gelatinous material. The methods of analysis include immunoassays for screening and GC-MS for confirmation. Although there is no FDA-approved immunoassay for drugs of abuse testing for meconium, the laboratories generally modify commercially available urine assays for drugs of abuse. The commonly used immunoassays are RIA, enzyme multiplied immunoassay technique (EMIT), kinetic interactions of microparticles in solution, cloned enzyme donor immunoassay and fluorescence polarization immunoassay.

Unlike urine or serum or plasma, drugs need to be extracted from meconium before analysis by immunoassays. Several methods have been described for extraction of drugs from meconium. Common methods include using 100–500 mg of meconium and extraction of drugs with 1–2 mL of organic solvents such as acetonitrile and methanol by vigorous agitation of the sample with a vortex mixer. The specimen is

then centrifuged at high speed ($\sim 10,000 \times g$), and supernatant is used for analysis using immunoassays *(97,105)*. Some laboratories evaporate the organic solvent extract and reconstitute the residue into a buffer prior to analysis *(106)*.

Moore et al. *(107)* compared several methods of drug detection from meconium. The authors compared extraction efficiency, false-positive and false-negative rates using hydrochloric acid, phosphate buffer/methanol and glacial acetic acid/ diphenylamine/acetone as extraction solvents. The study found that extraction performed with hydrochloric acid was poorest while glacial acetic acid/diphenylamine/acetone extraction protocol was the best. The confirmation method for this study was GC-MS, and the drugs studied were cocaine, THC-COOH, amphetamines, PCP and opiates.

ElSohly et al. *(100)* described a GC-MS method for confirmation of cannabinoids, cocaine, opiates, amphetamines and PCP in meconium. EMIT and TDx immunoassays were used as screening methods, and cut-off levels were 20 ng/g for THC-COOH and PCP, and 200 ng/g for benzoylecgonine, morphine and amphetamines. The GC-MS confirmation rate for the immunoassay-positive specimens was low, ranging from 0% for amphetamines to 75% for opiates. The lowest rate of confirmed positives was found with the cannabinoids, suggesting that THC metabolites other than free THC-COOH may be major contributors to the immunoassay response in meconium.

Pichini et al. *(108)* described an atmospheric pressure ionization-electrospray LC-MS method for determination of amphetamine, methamphetamine and methylenedioxy derivatives in meconium. The drugs were extracted in methanolic hydrochloric acid and cleaned by a solid-phase extraction. Chromatography involved C18 reversed-phase column and a mobile phase consisting of 10 mM ammonium bicarbonate (pH 9.0) and methanol. The method demonstrated excellent sensitivity with LOQ of 0.005 μg/g meconium for amphetamine, methamphetamine and 4-hydroxy-3-methoxymethamphetamine. The LOQ was 0.004 μg/g meconium for MDA, MDMA and MDEA.

### *5.2. Issues of Special Interest in Meconium Testing*

1. Unlike urine, cut-off values of abused drugs for meconium assays are not well standardized, and there are no suitable commercially available reference or control materials.
2. Infants with low birth weight (<1000 g) pass meconium at a median age of 3 days. Therefore, due to this delay, meconium collection may be missed *(109)*. A large study ($n$ = 3879) showed that meconium was collected only on 77.6% newborns *(93)*.
3. In a recent study, umbilical cord tissue was used to investigate its use for intrauterine drug exposure. Using GC-MS, the authors demonstrated that umbilical cord tissue performs as well as meconium in assessing fetal drug exposure to amphetamines, opiates, cocaine and cannabinoids *(104)*. Unlike meconium, umbilical cord tissue is available all the time and the testing can start just after delivery.
4. The metabolism of abused drugs as well as accumulation of drug metabolites in meconium may be different as compared to urine or blood. This may produce false-negative results by immunoassay and/or GC-MS. ElSohly et al. *(99)* showed that many meconium samples which tested positive by immunoassay tested negative by GC-MS when benzoylecgonine was used as a confirmatory analyte, and GC-MS confirmation rate could be substantially enhanced by inclusion of m- and p-hydroxybenzoylecgonine in the analysis along with benzoylecgonine. Similarly for

cannabinoids, in meconium, there seems to be another major metabolite of THC besides THC-COOH.

5. When lower cut-off values were used for urine, infant urine and meconium analyses yielded equivalent results for identifying newborns who have been exposed to cocaine in utero *(92,110)*.

## 6. OTHER MATRICES AND OTHER DRUGS

Several other matrices including amniotic fluid, vitreous fluid, breast milk, nails, semen, sebum, cerumen, breath, fat and placental tissue have been studied for various drugs. Similarly, number of other drugs not discussed above has been studied in these matrices *(30,63,111–113)*.

## 7. CONCLUSIONS

Although abused drugs are generally analyzed in urine and blood, other matrices such as hair, oral fluid, sweat and meconium offer certain advantages for special population of subjects. Each matrix provides unique perspective on drug detection. These matrices generally contain lower concentrations of drugs as compared to urine and thus pose special analytical challenges. Although some of the analytical challenges have been overcome by the newer technologies, still there is a need for improvement to overcome sensitivity and specificity issues. Also, there is need for better understanding of drug and metabolites disposition in these matrices to effectively overcome the issues of external contamination. Despite these issues, these matrices provide additional opportunities in drug detection, enforcement and deterrence programs.

## REFERENCES

1. Baumgartner AM, Jones PF, Baumgartner WA, Black CT. Radioimmunoassay of hair for determining opiate-abuse histories. J Nucl Med 1979;20:748–752.
2. Kintz P. Value of hair analysis in postmortem toxicology. Forensic Sci Int 2004;142:127–134.
3. Proposed revisions to mandatory guidelines for federal workplace drug testing programs. Substance abuse and mental health services administration. Federal Register vol. 69 no. 71, April 13, 2004, 19673–732.
4. Harrison WH, Gray RM, Solomon LM. Incorporation of D-amphetamine into pigmented guinea-pig hair. Br J Dermatol 1974;91:415–418.
5. Nakahara Y. Detection and diagnostic interpretation of amphetamines in hair. Forensic Sci Int 1995;70:135–153.
6. Vinner E, Vignau J, Thibault D, Codaccioni X, Brassart C, Humbert L, et al. Hair analysis of opiates in mothers and newborns for evaluating opiate exposure during pregnancy. Forensic Sci Int 2003;133:57–62.
7. Takayama N, Iio R, Tanaka S, Chinaka S, Hayakawa K. Analysis of methamphetamine and its metabolites in hair. Biomed Chromatogr 2003;17:74–82.
8. Sweeney SA, Kelly RC, Bourland JA, Johnson T, Brown WC, Lee H, et al. Amphetamines in hair by enzyme-linked immunosorbent assay. J Anal Toxicol 1998;22:418–424.
9. Quintela O, Bermejo AM, Tabernero MJ, Strano-Rossi S, Chiarotti M, Lucas AC. Evaluation of cocaine, amphetamines and cannabis use in university students through hair analysis: preliminary results. Forensic Sci Int 2000;107:273–279.
10. Miki A, Katagi M, Tsuchihashi H. Application of EMIT d.a.u. for the semiquantitative screening of methamphetamine incorporated in hair. J Anal Toxicol 2002;26:274–279.

11. Rothe M, Pragst F, Spiegel K, Harrach T, Fischer K, Kunkel J. Hair concentrations and self-reported abuse history of 20 amphetamine and ecstasy users. Forensic Sci Int 1997;89:111–128.
12. Cooper GA, Allen DL, Scott KS, Oliver JS, Ditton J, Smith ID. Hair analysis: self-reported use of "speed" and "ecstasy" compared with laboratory findings. J Forensic Sci 2000;45:400–406.
13. Koide I, Noguchi O, Okada K, Yokoyama A, Oda H, Yamamoto S, et al. Determination of amphetamine and methamphetamine in human hair by headspace solid-phase microextraction and gas chromatography with nitrogen-phosphorus detection. J Chromatogr B Biomed Sci Appl 1998;707:99–104.
14. Kintz P, Cirimele V, Mangin P. Testing human hair for cannabis. II. Identification of THC-COOH by GC-MS-NCI as a unique proof. J Forensic Sci 1995;40:619–622.
15. Wilkins D, Haughey H, Cone E, Huestis M, Foltz R, Rollins D. Quantitative analysis of THC, 11-OH-THC, and THCCOOH in human hair by negative ion chemical ionization mass spectrometry. J Anal Toxicol 1995;19:483–491.
16. Kim JY, Suh SI, In MK, Paeng KJ, Chung BC. Simultaneous determination of cannabidiol, cannabinol, and delta9-tetrahydrocannabinol in human hair by gas chromatography-mass spectrometry. Arch Pharm Res 2005;28:1086–1091.
17. Marsili R, Martello S, Felli M, Fiorina S, Chiarotti M. Hair testing for delta9-THC-COOH by gas chromatography/tandem mass spectrometry in negative chemical ionization mode. Rapid Commun Mass Spectrom 2005;19:1566–1568.
18. Baptista MJ, Monsanto PV, Pinho Marques EG, Bermejo A, Avila S, Castanheira AM, et al. Hair analysis for delta(9)-THC, delta(9)-THC-COOH, CBN and CBD, by GC/MS-EI. Comparison with GC/MS-NCI for delta(9)-THC-COOH. Forensic Sci Int 2002;128:66–78.
19. Backofen U, Matysik FM, Lunte CE. Determination of cannabinoids in hair using high-pH* non-aqueous electrolytes and electrochemical detection. Some aspects of sensitivity and selectivity. J Chromatogr A 2002;942:259–269.
20. Moore C, Guzaldo F, Donahue T. The determination of 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair using negative ion gas chromatography-mass spectrometry and high-volume injection. J Anal Toxicol 2001;25:555–558.
21. Sachs H, Dressler U. Detection of THCCOOH in hair by MSD-NCI after HPLC clean-up. Forensic Sci Int 2000;107:239–247.
22. Baumgartner WA, Black CT, Jones PF, Blahd WH. Radioimmunoassay of cocaine in hair: concise communication. J Nucl Med 1982;23:790–792.
23. Schaffer MI, Wang WL, Irving J. An evaluation of two wash procedures for the differentiation of external contamination versus ingestion in the analysis of human hair samples for cocaine. J Anal Toxicol 2002;26:485–488.
24. Graham K, Koren G, Klein J, Schneiderman J, Greenwald M. Determination of gestational cocaine exposure by hair analysis. JAMA 1989;262:3328–3330.
25. Katikaneni LD, Salle FR, Hulsey TC. Neonatal hair analysis for benzoylecgonine: a sensitive and semiquantitative biological marker for chronic gestational cocaine exposure. Biol Neonate 2002;81:29–37.
26. Felli M, Martello S, Marsili R, Chiarotti M. Disappearance of cocaine from human hair after abstinence. Forensic Sci Int 2005;154:96–98.
27. Bermejo AM, Lopez P, Alvarez I, Tabernero MJ, Fernandez P. Solid-phase microextraction for the determination of cocaine and cocaethylene in human hair by gas chromatography-mass spectrometry. Forensic Sci Int 2006;156:2–8.
28. Cognard E, Rudaz S, Bouchonnet S, Staub C. Analysis of cocaine and three of its metabolites in hair by gas chromatography-mass spectrometry using ion-trap detection for CI/MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2005;826:17–25.
29. Kintz P, Bundeli P, Brenneisen R, Ludes B. Dose-concentration relationships in hair from subjects in a controlled heroin-maintenance program. J Anal Toxicol 1998;22:231–236.
30. Jones J, Tomlinson K, Moore C. The simultaneous determination of codeine, morphine, hydrocodone, hydromorphone, 6-acetylmorphine, and oxycodone in hair and oral fluid. J Anal Toxicol 2002;26:171–175.

31. Gambelunghe C, Rossi R, Ferranti C, Bacci M. Hair analysis by GC/MS/MS to verify abuse of drugs. J Appl Toxicol 2005;25:205–211.
32. Lachenmeier K, Musshoff F, Madea B. Determination of opiates and cocaine in hair using automated enzyme immunoassay screening methodologies followed by gas chromatographic-mass spectrometric (GC-MS) confirmation. Forensic Sci Int 2006;159:189–199.
33. Cooper G, Wilson L, Reid C, Baldwin D, Hand C, Spiehler V. Validation of the Cozart microplate ELISA for detection of opiates in hair. J Anal Toxicol 2003;27:581–586.
34. Baumgartner AM, Jones PF, Black CT. Detection of phencyclidine in hair. J Forensic Sci 1981;26:576–581.
35. Sramek JJ, Baumgartner WA, Tallos JA, Ahrens TN, Heiser JF, Blahd WH. Hair analysis for detection of phencyclidine in newly admitted psychiatric patients. Am J Psychiatry 1985;142:950–953.
36. Swartz MS, Swanson JW, Hannon MJ. Detection of illicit substance use among persons with schizophrenia by radioimmunoassay of hair. Psychiatr Serv 2003;54:891–895.
37. Borges CR, Wilkins DG, Rollins DE. Amphetamine and N-acetylamphetamine incorporation into hair: an investigation of the potential role of drug basicity in hair color bias. J Anal Toxicol 2001;25:221–227.
38. Kronstrand R, Ahlner J, Dizdar N, Larson G. Quantitative analysis of desmethylselegiline, methamphetamine, and amphetamine in hair and plasma from Parkinson patients on long-term selegiline medication. J Anal Toxicol 2003;27:135–141.
39. Reid RW, O'Connor FL, Crayton JW. The in vitro differential binding of benzoylecgonine to pigmented human hair samples. J Toxicol Clin Toxicol 1994;32:405–410.
40. Ursitti F, Klein J, Sellers E, Koren G. Use of hair analysis for confirmation of self-reported cocaine use in users with negative urine tests. J Toxicol Clin Toxicol 2001;39:361–366.
41. Goldberger BA, Darraj AG, Caplan YH, Cone EJ. Detection of methadone, methadone metabolites, and other illicit drugs of abuse in hair of methadone-treatment subjects. J Anal Toxicol 1998;22:526–530.
42. Slawson MH, Wilkins DG, Foltz RL, Rollins DE. Quantitative determination of phencyclidine in pigmented and nonpigmented hair by ion-trap mass spectrometry. J Anal Toxicol 1996;20: 350–354.
43. Rollins DE, Wilkins DG, Krueger GG, Augsburger MP, Mizuno A, O'Neal C, et al. The effect of hair color on the incorporation of codeine into human hair. J Anal Toxicol 2003;27:545–551.
44. Schaffer M, Hill V, Cairns T. Hair analysis for cocaine: the requirement for effective wash procedures and effects of drug concentration and hair porosity in contamination and decontamination. J Anal Toxicol 2005;29:319–326.
45. Hoffman BH. Analysis of race effects on drug-test results. J Occup Environ Med 1999;41:612–624.
46. Kelly RC, Mieczkowski T, Sweeney SA, Bourland JA. Hair analysis for drugs of abuse. Hair color and race differentials or systematic differences in drug preferences? Forensic Sci Int 2000;107:63–86.
47. Blank DL, Kidwell DA. External contamination of hair by cocaine: an issue in forensic interpretation. Forensic Sci Int 1993;63:145–156; discussion 157–160.
48. Paulsen RB, Wilkins DG, Slawson MH, Shaw K, Rollins DE. Effect of four laboratory decontamination procedures on the quantitative determination of cocaine and metabolites in hair by HPLC-MS. J Anal Toxicol 2001;25:490–496.
49. Romano G, Barbera N, Lombardo I. Hair testing for drugs of abuse: evaluation of external cocaine contamination and risk of false positives. Forensic Sci Int 2001;123:119–129.
50. Cirimele V, Kintz P, Jamey C, Ludes B. Are cannabinoids detected in hair after washing with Cannabio shampoo? J Anal Toxicol 1999;23:349–351.
51. Uhl M, Sachs H. Cannabinoids in hair: strategy to prove marijuana/hashish consumption. Forensic Sci Int 2004;145:143–147.
52. Nakahara Y, Takahashi K, Sakamoto T, Tanaka A, Hill VA, Baumgartner WA. Hair analysis for drugs of abuse. XVII. Simultaneous detection of PCP, PCHP, and PCPdiol in human hair for confirmation of PCP use. J Anal Toxicol 1997;21:356–362.
53. Koren G, Klein J, Forman R, Graham K. Hair analysis of cocaine: differentiation between systemic exposure and external contamination. J Clin Pharmacol 1992;32:671–675.

54. Rohrich J, Zorntlein S, Potsch L, Skopp G, Becker J. Effect of the shampoo Ultra Clean on drug concentrations in human hair. Int J Legal Med 2000;113:102–106.
55. Walsh JM, Flegel R, Crouch DJ, Cangianelli L, Baudys J. An evaluation of rapid point-of-collection oral fluid drug-testing devices. J Anal Toxicol 2003;27:429–439.
56. Cone EJ, Presley L, Lehrer M, Seiter W, Smith M, Kardos KW, et al. Oral fluid testing for drugs of abuse: positive prevalence rates by intercept immunoassay screening and GC-MS-MS confirmation and suggested cutoff concentrations. J Anal Toxicol 2002;26:541–546.
57. Yacoubian GS, Jr., Wish ED, Perez DM. A comparison of saliva testing to urinalysis in an arrestee population. J Psychoactive Drugs 2001;33:289–294.
58. Laloup M, Tilman G, Maes V, De Boeck G, Wallemacq P, Ramaekers J, et al. Validation of an ELISA-based screening assay for the detection of amphetamine, MDMA and MDA in blood and oral fluid. Forensic Sci Int 2005;153:29–37.
59. Kankaanpaa A, Gunnar T, Ariniemi K, Lillsunde P, Mykkanen S, Seppala T. Single-step procedure for gas chromatography-mass spectrometry screening and quantitative determination of amphetamine-type stimulants and related drugs in blood, serum, oral fluid and urine samples. J Chromatogr B Analyt Technol Biomed Life Sci 2004;810:57–68.
60. Concheiro M, de Castro A, Quintela O, Lopez-Rivadulla M, Cruz A. Determination of MDMA, MDA, MDEA and MBDB in oral fluid using high performance liquid chromatography with native fluorescence detection. Forensic Sci Int 2005;150:221–226.
61. Mortier KA, Maudens KE, Lambert WE, Clauwaert KM, Van Bocxlaer JF, Deforce DL, et al. Simultaneous, quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2002;779:321–330.
62. Wood M, De Boeck G, Samyn N, Morris M, Cooper DP, Maes RA, et al. Development of a rapid and sensitive method for the quantitation of amphetamines in human plasma and oral fluid by LC-MS-MS. J Anal Toxicol 2003;27:78–87.
63. Gunnar T, Ariniemi K, Lillsunde P. Validated toxicological determination of 30 drugs of abuse as optimized derivatives in oral fluid by long column fast gas chromatography/electron impact mass spectrometry. J Mass Spectrom 2005;40:739–753.
64. Schepers RJ, Oyler JM, Joseph RE, Jr., Cone EJ, Moolchan ET, Huestis MA. Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. Clin Chem 2003;49:121–132.
65. Maseda C, Hama K, Fukui Y, Matsubara K, Takahashi S, Akane A. Detection of delta 9-THC in saliva by capillary GC/ECD after marihuana smoking. Forensic Sci Int 1986;32:259–266.
66. Fucci N, De Giovanni N, Chiarotti M. Simultaneous detection of some drugs of abuse in saliva samples by SPME technique. Forensic Sci Int 2003;134:40–45.
67. Lo Muzio L, Falaschini S, Rappelli G, Bambini F, Baldoni A, Procaccini M, et al. Saliva as a diagnostic matrix for drug abuse. Int J Immunopathol Pharmacol 2005;18:567–573.
68. Niedbala RS, Kardos K, Fries T, Cannon A, Davis A. Immunoassay for detection of cocaine/metabolites in oral fluids. J Anal Toxicol 2001;25:62–68.
69. Campora P, Bermejo AM, Tabernero MJ, Fernandez P. Quantitation of cocaine and its major metabolites in human saliva using gas chromatography-positive chemical ionization-mass spectrometry (GC-PCI-MS). J Anal Toxicol 2003;27:270–274.
70. Yonamine M, Tawil N, Moreau RL, Silva OA. Solid-phase micro-extraction-gas chromatography-mass spectrometry and headspace-gas chromatography of tetrahydrocannabinol, amphetamine, methamphetamine, cocaine and ethanol in saliva samples. J Chromatogr B Analyt Technol Biomed Life Sci 2003;789:73–78.
71. Cognard E, Bouchonnet S, Staub C. Validation of a gas chromatography-ion trap tandem mass spectrometry for simultaneous analysis of cocaine and its metabolites in saliva. J Pharm Biomed Anal 2006;41:925–934.
72. Clauwaert K, Decaestecker T, Mortier K, Lambert W, Deforce D, Van Peteghem C, et al. The determination of cocaine, benzoylecgonine, and cocaethylene in small-volume oral fluid samples by liquid chromatography-quadrupole-time-of-flight mass spectrometry. J Anal Toxicol 2004;28:655–659.

73. Piekoszewski W, Janowska E, Stanaszek R, Pach J, Winnik L, Karakiewicz B, et al. Determination of opiates in serum, saliva and hair addicted persons. Przegl Lek 2001;58:287–289.
74. Speckl IM, Hallbach J, Guder WG, Meyer LV, Zilker T. Opiate detection in saliva and urine – a prospective comparison by gas chromatography-mass spectrometry. J Toxicol Clin Toxicol 1999;37:441–445.
75. Kidwell DA, Holland JC, Athanaselis S. Testing for drugs of abuse in saliva and sweat. J Chromatogr B Biomed Sci Appl 1998;713:111–135.
76. Broussard LA, Presley LC, Tanous M, Queen C. Improved gas chromatography-mass spectrometry method for simultaneous identification and quantification of opiates in urine as propionyl and oxime derivatives. Clin Chem 2001;47:127–129.
77. Singh J, Burke RE, Mertens LE. Elimination of the interferences by keto-opiates in the GC-MS analysis of 6-monoacetylmorphine. J Anal Toxicol 2000;24:27–31.
78. McCarron MM, Walberg CB, Soares JR, Gross SJ, Baselt RC. Detection of phencyclidine usage by radioimmunoassay of saliva. J Anal Toxicol 1984;8:197–201.
79. Wong RC, Tran M, Tung JK. Oral fluid drug tests: effects of adulterants and foodstuffs. Forensic Sci Int 2005;150:175–180.
80. Cooper G, Wilson L, Reid C, Baldwin D, Hand C, Spiehler V. Validation of the Cozart microplate EIA for analysis of opiates in oral fluid. Forensic Sci Int 2005;154:240–246.
81. Huestis MA, Oyler JM, Cone EJ, Wstadik AT, Schoendorfer D, Joseph RE, Jr. Sweat testing for cocaine, codeine and metabolites by gas chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl 1999;733:247–264.
82. Kacinko SL, Barnes AJ, Schwilke EW, Cone EJ, Moolchan ET, Huestis MA. Disposition of cocaine and its metabolites in human sweat after controlled cocaine administration. Clin Chem 2005;51:2085–2094.
83. Fogerson R, Schoendorfer D, Fay J, Spiehler V. Qualitative detection of opiates in sweat by EIA and GC-MS. J Anal Toxicol 1997;21:451–458.
84. Fay J, Fogerson R, Schoendorfer D, Niedbala RS, Spiehler V. Detection of methamphetamine in sweat by EIA and GC-MS. J Anal Toxicol 1996;20:398–403.
85. Samyn N, De Boeck G, Verstraete AG. The use of oral fluid and sweat wipes for the detection of drugs of abuse in drivers. J Forensic Sci 2002;47:1380–1387.
86. Samyn N, De Boeck G, Wood M, Lamers CT, De Waard D, Brookhuis KA, et al. Plasma, oral fluid and sweat wipe ecstasy concentrations in controlled and real life conditions. Forensic Sci Int 2002;128:90–97.
87. Saito T, Wtsadik A, Scheidweiler KB, Fortner N, Takeichi S, Huestis MA. Validated gas chromatographic-negative ion chemical ionization mass spectrometric method for delta(9)-tetrahydrocannabinol in sweat patches. Clin Chem 2004;50:2083–2090.
88. Kintz P, Cirimele V, Ludes B. Detection of cannabis in oral fluid (saliva) and forehead wipes (sweat) from impaired drivers. J Anal Toxicol 2000;24:557–561.
89. Liberty HJ, Johnson BD, Fortner N. Detecting cocaine use through sweat testing: multilevel modeling of sweat patch length-of-wear data. J Anal Toxicol 2004;28:667–673.
90. Perez-Reyes M, Di Guiseppi S, Brine DR, Smith H, Cook CE. Urine pH and phencyclidine excretion. Clin Pharmacol Ther 1982;32:635–641.
91. http://www.drugabusestatistics.samhsa.gov/2k3/pregnancy/pregnancy.cfm (accessed June 13, 2006). US Department of Health and Human Services. Office of applied studies. Pregnancy and Substance use Report.
92. Kwong TC, Ryan RM. Detection of intrauterine illicit drug exposure by newborn drug testing. National Academy of Clinical Biochemistry. Clin Chem 1997;43:235–242.
93. Ostrea EM, Jr., Brady M, Gause S, Raymundo AL, Stevens M. Drug screening of newborns by meconium analysis: a large-scale, prospective, epidemiologic study. Pediatrics 1992;89: 107–113.
94. Ostrea EM, Jr., Knapp DK, Tannenbaum L, Ostrea AR, Romero A, Salari V, et al. Estimates of illicit drug use during pregnancy by maternal interview, hair analysis, and meconium analysis. J Pediatr 2001;138:344–348.

95. Wingert WE, Feldman MS, Kim MH, Noble L, Hand I, Yoon JJ. A comparison of meconium, maternal urine and neonatal urine for detection of maternal drug use during pregnancy. J Forensic Sci 1994;39:150–158.
96. Lewis DE, Moore CM, Leikin JB, Koller A. Meconium analysis for cocaine: a validation study and comparison with paired urine analysis. J Anal Toxicol 1995;19:148–150.
97. Ostrea EM, Jr., Brady MJ, Parks PM, Asensio DC, Naluz A. Drug screening of meconium in infants of drug-dependent mothers: an alternative to urine testing. J Pediatr 1989;115:474–477.
98. Moore C, Negrusz A, Lewis D. Determination of drugs of abuse in meconium. J Chromatogr B Biomed Sci Appl 1998;713:137–146.
99. ElSohly MA, Kopycki W, Feng S, Murphy TP. Identification and analysis of the major metabolites of cocaine in meconium. J Anal Toxicol 1999;23:446–451.
100. ElSohly MA, Stanford DF, Murphy TP, Lester BM, Wright LL, Smeriglio VL, et al. Immunoassay and GC-MS procedures for the analysis of drugs of abuse in meconium. J Anal Toxicol 1999;23:436–445.
101. Bennett AD. Perinatal substance abuse and the drug-exposed neonate. Adv Nurse Pract 1999;7:32–36; quiz 37–38.
102. Xia Y, Wang P, Bartlett MG, Solomon HM, Busch KL. An LC-MS-MS method for the comprehensive analysis of cocaine and cocaine metabolites in meconium. Anal Chem 2000;72:764–771.
103. Bar-Oz B, Klein J, Karaskov T, Koren G. Comparison of meconium and neonatal hair analysis for detection of gestational exposure to drugs of abuse. Arch Dis Child Fetal Neonatal Ed 2003;88:F98–F100.
104. Montgomery D, Plate C, Alder SC, Jones M, Jones J, Christensen RD. Testing for fetal exposure to illicit drugs using umbilical cord tissue vs meconium. J Perinatol 2006;26:11–14.
105. Bibb KW, Stewart DL, Walker JR, Cook VD, Wagener RE. Drug screening in newborns and mothers using meconium samples, paired urine samples, and interviews. J Perinatol 1995;15:199–202.
106. Ryan RM, Wagner CL, Schultz JM, Varley J, DiPreta J, Sherer DM, et al. Meconium analysis for improved identification of infants exposed to cocaine in utero. J Pediatr 1994;125:435–440.
107. Moore C, Lewis D, Leikin J. False-positive and false-negative rates in meconium drug testing. Clin Chem 1995;41:1614–1616.
108. Pichini S, Pacifici R, Pellegrini M, Marchei E, Lozano J, Murillo J, et al. Development and validation of a high-performance liquid chromatography-mass spectrometry assay for determination of amphetamine, methamphetamine, and methylenedioxy derivatives in meconium. Anal Chem 2004;76:2124–2132.
109. Verma A, Dhanireddy R. Time of first stool in extremely low birth weight (< or = 1000 grams) infants. J Pediatr 1993;122:626–629.
110. Callahan CM, Grant TM, Phipps P, Clark G, Novack AH, Streissguth AP, et al. Measurement of gestational cocaine exposure: sensitivity of infants' hair, meconium, and urine. J Pediatr 1992;120:763–768.
111. Huestis MA, Cone WJ. Alternative testing matrices. In: Karch SB, ed. Drug Abuse Handbook: CRC Press, Boca Raton, FL 1998:799–858.
112. Boumba VA, Ziavrou KS, Vougiouklakis T. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. Int J Toxicol 2006;25:143–163.
113. Kintz P, Villain M, Cirimele V. Hair analysis for drug detection. Ther Drug Monit 2006;28:442–446.

# 19 Abused and Designer Drugs and How They Escape Detection

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***Contents***



**Summary**

In most healthcare facilities, toxicology laboratories have moved from a more general toxicology screen to a more limited screen. This limited screen will usually consist of a serum ethanol test by an enzymatic assay and a urine immunoassay screen for 5–10 drug classes based on commercially available immunoassay kits. These may be supplemented by serum tests for salicylate, acetaminophen, benzodiazepines and tricyclic antidepressants by immunoassay or spot tests. Although immunoassays are quite useful for rapid screening of many drugs, there are some limitations associated with this limited screening approach. This chapter discusses these limitations with regard to drug-facilitated sexual assault drugs, hallucinogenic amines, miscellaneous hallucinogens and opiates. One limitation is the fact that not all drug classes or drugs within a class are detected by these tests. In addition, some of the drugs discussed have very limited detection windows or exhibit instability that may limit or prevent their detection. The end result of a "not detected" result may be due not only to the absence of analyte but also to the specimen being collected too late for analyte detection, a lack of sensitivity of the employed analytical methodology or the degradation of the analyte during storage.

**Key Words:** Designer drugs; hallucinogens.

## 1. INTRODUCTION

One difference between clinical and forensic toxicology laboratories is the importance of turnaround time in the completion of the results. In postmortem and human performance toxicology laboratories, a turnaround time of days or weeks may be

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acceptable. As a result, the approach to drug testing may be more comprehensive. A battery of tests, rather than a single type of test, can be utilized. On the other hand, a clinical toxicology laboratory needs to provide results back to the clinician more rapidly, usually within hours of specimen receipt. Moreover, with cost reduction becoming a significant factor in healthcare facilities, toxicology laboratories have moved from a more general toxicology screen to a more limited screen *(1)*. This limited screen will usually consist of a serum ethanol test by an enzymatic assay and a urine immunoassay screen for 5–10 drug classes based on commercially available immunoassay kits. These may be supplemented by serum tests for salicylate, acetaminophen, benzodiazepines and tricyclic antidepressants by immunoassay or spot tests. Because these tests are cheaper and less labor intensive than general screening techniques such as thin layer chromatography and gas chromatography, they have largely replaced these more classical techniques in the clinical toxicology laboratory.

Although immunoassays are quite useful for rapid screening of many drugs, there are some limitations associated with this limited screening approach. Obviously, there are drugs other than abused drugs that can necessitate a trip to the emergency department. Within a class of drugs, there will be differences in cross-reactivities between drugs. This chapter will discuss many of these limitations. Specifically, four areas will be covered: drug-facilitated sexual assault (DFSA) drugs, hallucinogenic amines, miscellaneous hallucinogens and opiates.

## 2. DRUG-FACILITATED SEXUAL ASSAULT DRUGS

Sexual assault refers to a broad array of sexual offenses that involve touching or penetration of an intimate part of another person's body without consent. A sexual assault is "drug-facilitated" when the victim is incapacitated or unconscious due to the effects of a drug and as a result is unable to give consent. The impairing drug may be taken knowingly by the individual or may be given surreptitiously to the victim by another *(2)*. The most commonly used drug in DFSA is ethanol; up to 75% of all DFSAs involve ethanol *(3)*. As stated earlier, ethanol testing is usually included in routine clinical testing; however, a number of drugs often implicated as DFSA agents are not detected by commonly performed immunoassay testing. Two such drugs are gamma-hydroxybutyrate (GHB) and flunitrazepam.

### *2.1. Gamma-Hydroxybutyrate*

GHB is a simple four-carbon molecule with a hydroxyl group on one end and a carboxylic acid group on the other end of the carbon chain. Before its use in DFSA, body builders who believed that it caused an increased release of human growth hormone abused GHB. Dieters have also abused it as an L-tryptophan replacement *(4)*. However, it is the central nervous system (CNS) effects of the drug that are utilized in DFSA. This CNS depression leads to reduced inhibitions, euphoria and sedation with possible loss of consciousness *(5)*. In March 2000, GHB was reclassified as a Schedule 1 controlled substance. However, there are several legally available substances that are readily converted to GHB in the body following oral administration. These substances include 1,4-butanediol and gamma-butyrolactone (GBL).

GHB is well absorbed orally, has a rapid onset of action and is rapidly cleared from the body. Its duration of action is 3–4 h. About 1% of a dose of GHB is recovered in the urine as unchanged drug *(6)*.

There are several reasons why GHB is difficult to detect in a clinical laboratory. There are no commercially available immunoassays to screen for GHB. It is too polar to be amenable to conventional thin layer chromatographic systems. There are color tests for the screening of suspected GHB powders *(7)*, but lack the sensitivity for use with biological specimens. It also has a small window of detectability in the urine, approximately 12 h after use *(6)*.

The analysis of GHB in biological specimens can be divided into two general types, with each type requiring a gas chromatographic or a gas chromatographic/mass spectrophotometry system. GHB may be converted to GBL by acidifying the specimen and heating it to high temperature. GBL has better chromatographic characteristics than GHB on a dimethylsilicone or methylphenylsilicone column. As a result, GBL can be extracted into an immiscible organic solvent, concentrated and chromatographed *(8)*. Alternatively, GHB can be extracted unchanged by solid-phase extraction, derivatized to a trimethylsilyl or heptafluorobutyric derivative and chromatographed *(9)*.

### *2.2. Flunitrazepam*

Flunitrazepam is a benzodiazepine that is approximately 10 times more potent than diazepam as a sedative/hypnotic drug *(10)*. It has never been approved for use therapeutically in the United States, but is available in a number of countries throughout the world. It started to appear in the United States as a DFSA drug in the 1990s because it produces heavy sedation and memory loss. When taken orally, the CNS depressant effects begin 15–20 min after ingestion and lasts from 6 to 12 h. It is extensively metabolized with 7-aminoflunitrazepam being the major urinary product *(11)*.

Most clinical laboratories include benzodiazepines in their immunoassay screening panel. The screen used is general for benzodiazepines. There are several problems with this. One problem is that there is a wide range of potencies of drugs within a class. For instance, diazepam and chlordiazepoxide are prescribed in dosages up to 25 mg whereas lorazepam and alprazolam are taken in sub-milligram dosages. Therefore, a positive result may indicate therapeutic use of some drugs within the class, but toxic use of other drugs. Moreover, a negative screening result may not mean that a benzodiazepine is not present. The common dose for flunitrazepam is $<2$ mg *(12)*, meaning that it will be present in low concentrations in the urine. The detection of flunitrazepam use is further complicated by the fact that the benzodiazepine immunoassays have different cross-reactivities to different drugs within the class. Table 1 gives a breakdown of the cross-reactivity of flunitrazepam and 7-aminoflunitrazepam in a variety of commercially available benzodiazepine immunoassays. Table 1 indicates variable cross-reactivity to flunitrazepam and 7-aminoflunitrazepam, from limited to good. Some manufacturers whose benzodiazepine immunoassays show lower cross-reactivity to flunitrazepam have developed more specific immunoassays for flunitrazepam and 7-aminoflunitrazepam. However, this requires laboratories to perform two immunoassays for benzodiazepines if they choose to use the particular technology. An additional factor is that benzodiazepines appear in the urine primarily as glucuronide conjugates. The antibodies in these assays are developed to recognize free drug or

Table 1
Flunitrazepam Cross-Reactivity in Commercially Available Benzodiazepine Immunoassays[a]

| *Assay* | *Flunitrazepam %reactivity* | *7-Amino FLU %reactivity* | *Calibrator* | *Cutoff concentration(s) tested* | *Intended specimen type(s)* |
|---|---|---|---|---|---|
| Abuscreen® ONLINE Benzodiazepines | 34% | Not available | Nordiazepam | 100 ng/mL | Urine |
| CEDIA® DAU Benzodiazepine | 109%/135% | 99% / Not available | Nitrazepam | 200 ng/mL w/β–Gluc<br>300 ng/mL | Urine |
| Emit® d.a.u.® Benzodiazepine | 200%/130% | Not available | Oxazepam | 200 ng/mL<br>300 ng/mL | Urine |
| Emit® II Plus Benzodiazepine | 145%/155% | 34% / 22% | Lormetazepam | 200 ng/mL<br>300 ng/mL | Urine |
| Immunalysis Benzodiazepines Direct ELISA | 31% | Not available | Oxazepam | 100 ng/mL | Whole blood, serum, plasma, urine |
| Immunalysis Flunitrazepam Direct ELISA | 83% | 100% | 7-Aminoflunitrazepam | 10 ng/mL | Whole blood, serum, plasma, urine |
| Neogen® Benzodiazepine Group ELISA | 3.2% | Not available | Temazepam | Not available | Multiple matrices |
| Neogen® Flunitrazepam Group ELISA | 100% | 212% | Flunitrazepam | Not available | Multiple matrices |
| OraSure Benzodiazepines ELISA | Not available | 6.4% | Nordiazepam | Not available | Multiple matrices |

[a] Data from manufacturer package inserts.

metabolite and not the conjugate. Performing a glucuronidase pretreatment prior to immunoassay analysis can enhance sensitivity in the immunoassays.

To determine the utility of the benzodiazepine immunoassays in identifying a single exposure to flunitrazepam, it is important to know the concentrations of 7-aminoflunitrazepam that are seen in urine after a single exposure. One study *(13)* measured 7-aminoflunitrazepam concentrations in urine for 72 h following a single 2 mg dose of flunitrazepam to four individuals. Peak urine concentrations of 7-aminoflunitrazepam occurred 12–18 h after ingestion. In another study involving 10 volunteers *(10)*, peak urine concentrations were observed 6 h after administration of a 2 mg oral dose in 9 subjects and 24 h after administration in the 10th subject. In both studies, a peak urine concentration up to about 0.5 mg/L was found; urine concentrations of 7-aminoflunitrazepam dropped below 0.1 mg/L by 72 h after ingestion. Combining this information with the cross-reactivity of a particular immunoassay to 7-aminoflunitrazepam, the clinical laboratory can assess its ability to detect flunitrazepam use.

There are a variety of methods available to confirm the presence of 7-aminoflunitrazepam in urine specimens. Either liquid-liquid extraction or solid-phase extraction *(14–16)* can be used to separate the metabolite from the urine. Cleaving the glucuronide can enhance sensitivity. This may be performed by adding a glucuronidase enzyme or by acid hydrolysis. Enzymatic hydrolysis will leave the benzodiazepine structure unchanged while the acid hydrolysis will convert the benzodiazepine into a benzophenone. Detection of the intact 7-aminoflunitrazepam can be accomplished by liquid chromatography with either an ultraviolet *(14)* or a mass spectrometric detector *(12,15)* without derivatization or by gas chromatography/mass spectrometry of trimethylsilyl or heptafluorobutyric derivatives of 7-aminoflunitrazepam. Either electron ionization or negative chemical ionization may be used. The benzophenone can also be detected by gas chromatography/mass spectrometry *(13,17)*.

## 3. DESIGNER AMINES

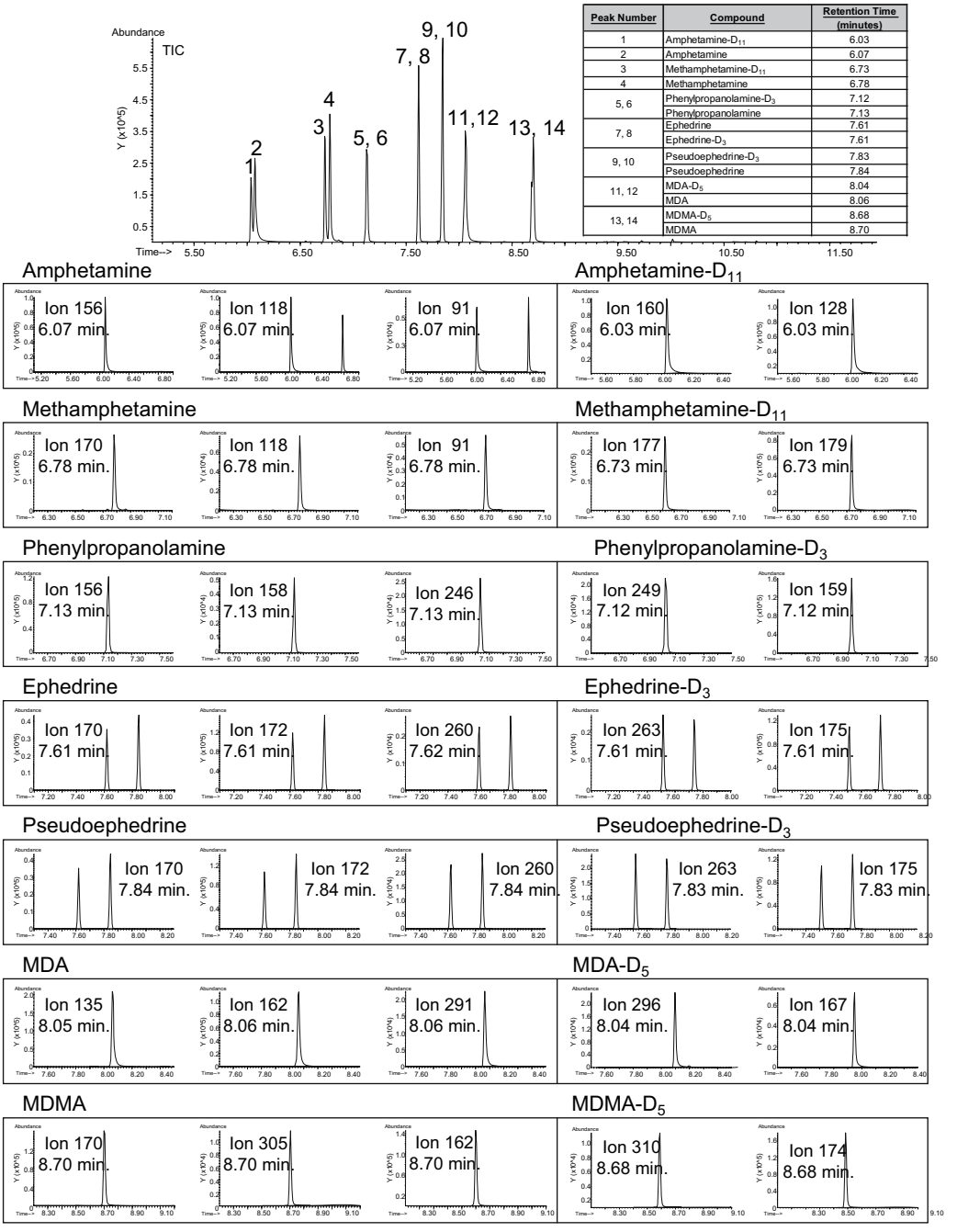
Methylenedioxymethamphetamine (MDMA) and methylenedioxyamphetamine (MDA) are ring-substituted derivatives of methamphetamine and amphetamine, respectively, and have both stimulant and hallucinogenic effects. Both compounds exist as enantiomers; both the R and S isomers of MDMA are mildly hallucinogenic while the R isomer of MDA is hallucinogenic. During the 1990s, the use of MDMA and MDA as recreational drugs increased, predominantly at all night dance parties known as "raves." MDMA has the street names of "ecstasy" or "Adam" while "Eve" is the slang name for MDA. Both drugs cause sympathomimetic activity such as peripheral vasoconstriction, tachycardia and pupillary dilatation. In addition, psychological effects such as distortions in perception, intensification of feelings and euphoria are also observed. The psychedelic effects last between 4 and 6 h *(18)*.

Human studies indicate that although MDMA is metabolized by demethylation and by breaking of the ring structure, parent drug is the primary urinary product following use. Urine concentrations above 17 mg/L have been measured in individuals following a 105 mg/70 kg dose *(19)*. Higher concentrations of MDMA have been measured in randomly collected urine specimens from individuals attending "rave" parties *(20)*.

Table 2
MDMA Cross-Reactivity in Commercially Available Amphetamine/Methamphetamine Immunoassays[a]

| *Assay* | *MDMA % reactivity* | *MDA % reactivity* | *Calibrator* | *Cutoff concentration tested* | *Intended specimen type(s)* |
|---|---|---|---|---|---|
| Abbott TDx® | 99% | 151% | *d*-Amphetamine | 100 ng/mL | Urine |
| Abuscreen® ONLINE amphetamines | 0.1% | 38% | *d*-Amphetamine | 1000 ng/mL | Urine |
| CEDIA® Amphetamines/Ecstasy Assay | 196% | 116% | *d*-Methamphetamine | 1000 ng/mL | Urine |
| CEDIA® Amphetamines Assay | 69% | 1.9% | *d*-Methamphetamine | 1000 ng/mL | Urine |
| Emit® d.a.u.® Monoclonal Amphetamine/Methamphetamine | 33% | 100% | *d*-Methamphetamine | 1000 ng/mL | Urine |
| Emit® II Plus Monoclonal Amphetamine/Methamphetamine | 11% | 47% | *d*-Methamphetamine | 1000 ng/mL | Urine |
| Immunalysis Methamphetamine Direct ELISA | 135% | <1% | *d*-Methamphetamine | 50 ng/mL | Whole blood, oral fluid, serum, plasma, urine |
| Neogen® Amphetamine | 2.2% | 0.4% | *d*-Amphetamine | Not available | Multiple matrices |
| Neogen® Amphetamine Ultra ELISA | 1.0% | 0.4% | *d*-Amphetamine | Not available | Multiple matrices |
| Neogen® Methamphetamine/MDMA | 733% | 4.0% | *d*-Methamphetamine | Not available | Multiple matrices |
| OraSure Amphetamine Specific ELISA | <1% | >100% | *d*-Amphetamine | Not available | Multiple matrices |
| OraSure Methamphetamine ELISA | 280% | 2.8% | *d*-Methamphetamine | 100 ng/mL | Multiple matrices |

[a] Data from manufacturer package inserts unless otherwise indicated.
[b] MDMA, methylenedioxymelnamphetamine.




| Peak Number | Compound | Retention Time (minutes) |
|---|---|---|
| 1 | Amphetamine-$D_{11}$ | 6.03 |
| 2 | Amphetamine | 6.07 |
| 3 | Methamphetamine-$D_{11}$ | 6.73 |
| 4 | Methamphetamine | 6.78 |
| 5, 6 | Phenylpropanolamine-$D_3$ | 7.12 |
| | Phenylpropanolamine | 7.13 |
| 7, 8 | Ephedrine | 7.61 |
| | Ephedrine-$D_3$ | 7.61 |
| 9, 10 | Pseudoephedrine-$D_3$ | 7.83 |
| | Pseudoephedrine | 7.84 |
| 11, 12 | MDA-$D_5$ | 8.04 |
| | MDA | 8.06 |
| 13, 14 | MDMA-$D_5$ | 8.68 |
| | MDMA | 8.70 |

**Fig. 1.** Chromatogram of chlorodifluoroacetyl derivatives of amine compounds.

There are numerous commercially available immunoassays for the detection of amphetamine and methamphetamine. Some companies use specific kits for amphetamine and methamphetamine. Other manufacturers use a single kit to detect both compounds. Table 2 lists the cross-reactivities of these immunoassays to MDA and MDMA. In general, immunoassays designed to detect amphetamine specifically will have good cross-reactivity to MDA, whereas immunoassays designed to detect methamphetamine specifically will have good cross-reactivity to MDMA. Single assays for amphetamine and methamphetamine will generally have cross-reactivity to MDA and MDMA.

MDA and MDMA can be confirmed using methods that confirm amphetamine and methamphetamine *(21–22)*. Each can be extracted in an alkaline drug screen, without derivatization; on a dimethylsilicone or phenylmethylsilicone gas chromatographic column, MDA and MDMA elute after amphetamine, methamphetamine and other sympathomimetic amines such as phenylpropanolamine, ephedrine and pseudoephedrine. Better chromatography is obtained if the amines are derivatized with chlorodifluoroacetic anhydride, pentafluoropropionic anhydride or heptafluorobutyric anhydride. To identify MDA and MDMA using an assay for amphetamine and methamphetamine, the chromatographic runtime must be extended for these substances to be detected. Figure 1 is a chromatogram of chlorodifluoroacetyl derivatives of the amine compounds discussed.

## 4. OPIOIDS

Opioids are a class of drugs used therapeutically to treat pain, coughs and diarrhea. They are also abused because of the euphoric effects that they produce. The prototypical opioid is morphine. From morphine, the synthetic opioid diacetylmorphine or heroin is produced. Although heroin remains the most commonly abused opioid, there are other opioids that are abused. For instance, methadone is an opioid used to treat opioid addiction and is also used to treat chronic pain. Advantages to the therapeutic use of methadone include good oral bioavailability and a much longer half-life than morphine. Oxycodone is another synthetic opioid that is used to treat pain. Recently, a sustained release form of oxycodone was produced as a way of utilizing the efficacy of oxycodone, but with a longer duration of action. Because of the abuse of this sustained release form, the higher dose form was subsequently removed from the market by the manufacturer. Another synthetic opioid, fentanyl, is used in the operating room as an adjunct to anesthesia. Fentanyl is also available in transdermal patches that are prescribed to treat chronic pain. Fentanyl and its analogs have appeared as abused drugs in various parts of the country *(23)*.

Because opioids are a common source of drug intoxication, the toxicology laboratory must be able to look for these compounds. The most common method to screen for opioids is immunoassay. The commercially available opioid immunoassays are designed to detect morphine and codeine. Most of these immunoassays do not have good detection limits for the other opioids discussed above. However, there are immunoassays that have been designed to specifically detect these compounds. As an example, Table 3 lists the cross-reactivities of oxycodone to opiate immunoassays and to specific oxycodone/oxymorphone immunoassays. The general opiate assays have cross-reactivities to oxycodone of $<21\%$; as expected, the specific oxycodone immunoassays have greater cross-reactivities to the drug.

Table 3
Oxycodone Cross-Reactivity in Commercially Available Opiate Immunoassays[a]

| *Assay* | *Oxycodone % reactivity* | *Calibrator* | *Cutoff concentration(s) tested* | *Intended specimen types* |
|---|---|---|---|---|
| Abuscreen® ONLINE Opiates | <0.3% | Morphine | 300 ng/mL | Urine |
| CEDIA® Opiate Assay | 3.1% | Morphine | 300 ng/mL | Urine |
| Emit® d.a.u.® Opiate | 6.7% | Morphine | 300 ng/mL | Urine |
| Emit® II Plus Opiate | 5.6% | Morphine | 300 ng/mL | Urine |
| Immunalysis Opiates Direct ELISA | 21% | Morphine | 25 ng/mL | Whole blood, oral fluids, serum, plasma, urine |
| Immunalysis Oxycodone Direct ELISA | 100% | Oxycodone | 10 ng/mL | Whole blood, oral fluids, serum, plasma, urine |
| Neogen® Opiate Group ELISA | 10% | Morphine | Not available | Multiple matrices |
| Neogen® Oxymorphone/Oxycodone ELISA | 400% | Oxymorphone | Not available | Multiple matrices |
| OraSure Opiates | 2.0% | Morphine | Not available | Multiple matrices |

[a] Data from manufacturer package inserts.

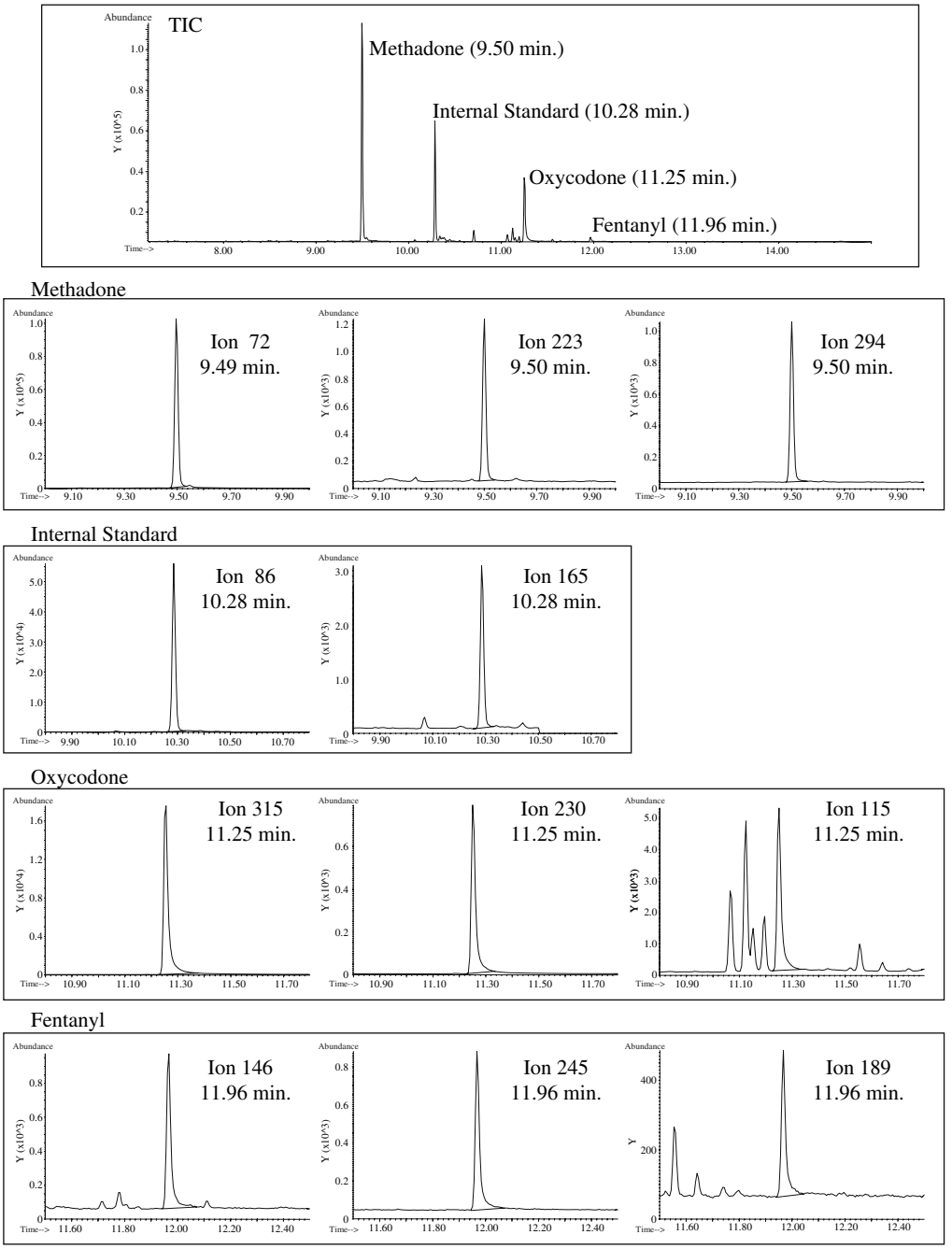



**Fig. 2.** Chromatogram of the three opioids within a single chromatographic run.

There are a number of analytical methods available to detect a larger group of opioids. A laboratory can combine a general opioid immunoassay with specific immunoassays for methadone, oxycodone and fentanyl. Alternatively, a gas chromatographic method using a methylphenylsilicone or dimethylsilicone column can simultaneously detect methadone, oxycodone and fentanyl. No derivatization is required and either a nitrogen-phosphorus or a mass selective detection system can be used. Figure 2 is a chromatogram of the three opioids within a single chromatographic run. However, the opioid immunoassay must still be included, as morphine does not elute well from these columns without derivatization *(23)*.

## 5. HALLUCINOGENS

### *5.1. Lysergic Acid Diethylamide*

Lysergic acid diethylamide (LSD) is a hallucinogenic substance that has been abused since the 1960s. The effects begin 20–80 min after oral administration and last approximately 3–4 h. Physiological effects include an increase in heart rate and blood pressure, lacrimation, salivation and pupillary dilatation. Subjective effects include mental disorientation, euphoria, altered sensory perception and hallucinations *(24)*.

The plasma half-life of LSD is about 3 h. A number of metabolites have been detected in the urine following LSD use. The major urinary metabolite appears to be 2-oxo-3-hydroxy LSD. Desmethyl LSD (nor LSD) is also a minor metabolite *(25)*.

To detect LSD use, there are a number of commercially available assays. Each is designed to target parent drug at a concentration of 0.5 ng/mL (Table 4). However, most clinical laboratories do not include LSD testing as part of their immunoassay panel. Even if an immunoassay for LSD is performed, the window of detection following last use is in terms of hours as opposed to days for most abused drugs. In addition, LSD is heat and light sensitive such that a loss of drug may occur while the specimen is in storage. As a result, a negative result does not preclude prior use of LSD *(24)*.

There are a number of chromatographic techniques available for LSD analysis, either as a screening technique or as a confirmation technique for a positive screening result. Gas chromatography without a mass spectrometer as a detector or high performance liquid chromatography (HPLC) with an ultraviolet detector lacks the sensitivity to detect LSD or its metabolites. Because of its native fluorescence, HPLC with a

**Table 4**
**LSD Commercially Available Immunoassays[a]**

| *Assay* | *% Reactivity* | *Calibrator* | *Cutoff concentration* | *Intended specimen types* |
|---|---|---|---|---|
| CEDIA® Opiate Assay | 100% | LSD | 0.5 ng/mL | Urine |
| Emit® II LSD Assay | 100% | LSD | 0.5 ng/mL | Urine |
| Immunalysis LSD Direct ELISA | 100% | LSD | 0.5 ng/mL | Whole blood, oral fluids, serum, plasma, urine |

[a] Data from manufacturer package inserts.
LSD, lysergic acid diethylamide.

fluorescence detector can be utilized. HPLC-MS can also be used without derivatization. When gas chromatography/mass spectrometry is used, a derivatization step is required; the most common derivative formed is the trimethylsilyl derivative. Tandem mass spectrometry, either attached to a gas chromatography or HPLC, offer the best chance to detect LSD, but these technologies are generally not available routinely to a clinical laboratory *(24)*.

### *5.2. Psilocin*

Psilocybin is the major psychoactive component in "psychedelic" mushrooms. The hallucinogenic effects of psilocybin occur within 30 min after ingestion and have a shorter duration of action than LSD. Upon ingestion, psilocybin is dephosphorylated to psilocin, the product detected in biological specimens.

There is no commercially available immunoassay that can detect psilocin in biological specimens. Psilocin can be detected in urine specimens following enzymatic hydrolysis of the glucuronide product. Acid hydrolysis destroys the psilocin structure and cannot be used. It can be analyzed by gas chromatography/mass spectrometry following solid-phase extraction and formation of a trimethylsilyl derivative *(26)*.

## 6. CONCLUSION

The information presented has discussed the difficulties associated with the detection of specific drugs in the clinical toxicology laboratory. Several of the drugs discussed require non-routine methods for detection; therefore, having a thorough clinical history and documentation of observed effects are crucial for their subsequent detection. In addition, some of the drugs discussed have very limited detection windows or exhibit instability that may limit or prevent their detection. The end result of a "not detected" result may be due not only to the absence of analyte, but also to the specimen being collected too late for analyte detection, a lack of sensitivity of the employed analytical methodology or the degradation of the analyte during storage.

## REFERENCES

1. Bailey DN. Results of limited versus comprehensive toxicology screening in a university medical center. Am J Clin Path 1996; 105: 572–575.
2. Abarbanel G. The victim. In: Drug-Facilitated Sexual Assault - A Forensic Handbook. Ed. Lebeau MA, Mozayani A. San Diego: Academic Press, 2004: 1–4.
3. Garriott JC, Mozayani A. Ethanol in drug-facilitated sexual assault. In: Drug-Facilitated Sexual Assault – A Forensic Handbook. Ed. Lebeau MA, Mozayani A. San Diego: Academic Press, 2004: 73.
4. Cole KA, Smith ML. χ-Hydroxybutyric acid. Analyte of the month. AACC TDM/TOX 1996; 17: 307–308.
5. Hornfeldt CS, Lothridge K, Upshaw-Downs JC. Forensic science update: gamma-hydroxybutyrate (GHB). Forensic Sci Comm 2002; 4: 1–13.
6. Brenneisen R, ElSohly MA, Murphy TP, Passarelli J, Russmann S, Salamone SJ, Watson DE. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. J Anal Toxicol 2004; 28: 625–630.
7. Andera KM, Evans HK, Wojcik CM. Microchemical identification of gamma-hydroxybutyrate (GHB). J Forensic Sci 2000; 45: 665–668.

8. Lebeau MA, Montgomery MA, Miller ML, Burmeister SG. Analysis of biofluids for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC-FID and GC-MS. J Anal Toxicol 2000; 24: 421–428.
9. McCuster RR, Paget-Wilkins H, Chronister CW, Goldberger BA, ElSohly MA. Analysis of gamma-hydroxybutyrate (GHB) in urine by gas chromatography-mass spectrometry. J Anal Toxicol 1999; 23: 301–305.
10. Negrusz A, Moore CM, Stockham TL, Poiser KR, Kern JL, Palaparthy R, Le NLT, Janicak, PG, Levy NA. Elimination of 7-aminoflunitrazepam in urine after a single dose of Rohypnol®. J Forensic Sci 2000; 45: 1031–1040.
11. Mantila MK, Larni HM. Flunitrazepam: a review of its pharmacologic properties and therapeutic use. Drugs 1980; 20: 353–374.
12. Bogusz MJ, Maier R-D, Kruger K-D, Fruchtnicht W. Determination of flunitrazepam and its metabolites in blood by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr B Biomed Sci Appl 1998; 713: 361–369.
13. Snyder H, Schwenzer KS, Pearlman R, McNally AJ, Tsilimidos M, Salamone SJ, Brenneisen R, ElSohly MA, Feng S. Serum and urine concentrations of flunitrazepam and metabolites, after a single oral dose, by immunoassay and GC-MS. J Anal Toxicol 2001; 25: 699–704.
14. Deinl I, Mahr G, von Meyer L. Determination of flunitrazepam and its main metabolites in serum and urine by HPLC after mixed-mode solid-phase extraction. J Anal Toxicol 1998; 22: 197–202.
15. Lebeau MA, Montgomery MA, Wagner JR, Miller ML. Analysis of biofluids for flunitrazepam and metabolites by electrospray liquid chromatography/mass spectrometry. J Forensic Sci 2000; 45: 1133–1141.
16. Nguyen H, Nau DR. Rapid method for the solid-phase extraction and GC-MS analysis of flunitrazepam and its major metabolites in urine. J Anal Toxicol 2000; 24: 37–45.
17. ElSohly MA, Feng S, Salamone SJ, Wu R. A sensitive GC-MS procedure for the analysis of flunitrazepam and its metabolites in urine. J Anal Toxicol 1997; 21: 335–340.
18. MDMA (Ecstasy). Executive Office of the President, Office of National Drug Control Policy, Drug Policy Information Clearinghouse Fact Sheet, NCJ-188745, April, 2002, 1–6.
19. Baselt RC. Methylenedioxymethamphetamine. In: Disposition of Toxic Drugs and Chemicals in Man. Ed. Baselt RC. Foster City, CA: Biomedical Publications, 2004: 722–725.
20. Zhao H, Brenneisen R, Scholer A, McNally AJ, ElSohly MA, Murphy T, Salamone SJ. Profiles of urine samples taken from Ecstasy users at rave parties: analysis by immunoassays, HPLC and GC-MS. J Anal Toxicol 2001; 25: 258–269.
21. Cody JT. Sympathomimetic amines. Education article, AACC TDM/TOX 1997: 18; 121–130.
22. Clark CR, Valaer AK, Noggle FT. GC-MS analysis of acylated derivatives of methamphetamine and regioisomeric phenethylamines. J Chromatogr Sci 1995; 33: 485–492.
23. Kerrigan S, Goldberger BA. Opioids. In: Principles of Forensic Toxicology. Ed. Levine B. Washington, DC: AACC Press, 2003: 187–206.
24. Paul BD, Smith ML. LSD - an overview on drug action and detection. Vol. 11. Forensic Sci Rev 1999; 158–174.
25. Pooh GK, Klette KL, Hallare DA, Manglicmot MG, Czarny RJ, McWhorter LK, Anderson CJ. Detection of metabolites of lysergic acid diethylamide (LSD) in human urine specimens: 2-oxo-3-hydroxy-LSD, a prevalent metabolite of LSD. J Chromatogr B Biomed Sci Appl 1999; 724: 23–33.
26. Grieshaber AF, Moore KA, Levine B. The detection of psilocin in human urine. J Forensic Sci 2001; 46: 627–630.

# 20 Interpretation of Amphetamines Screening and Confirmation Testing

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***Contents***



**Summary**

The interpretation of immunoassay screening and gas chromatography/mass spectrometry confirmation testing for amphetamine, methamphetamine, and designer amphetamines is complicated due to several factors including cross-reactivity from sympathomimetic amines, resolution of *d* and *l* optical isomers, generation of methamphetamine in the testing process, metabolism of other drugs to amphetamine and/or methamphetamine, and the matrix of the sample being tested. This chapter discusses interpretation of results obtained by screening and confirmation testing affected by all of these factors and when applicable describes solutions to these problems.

**Key Words:** Amphetamine; designer amphetamines; GC/MS; methamphetamine.

## 1. INTRODUCTION

Correct interpretation of testing results for amphetamines and related compounds is dependent on many factors including an understanding of the nomenclature, structure, and metabolism of these compounds. The class of phenethylamine compounds having varying degrees of sympathomimetic activity includes amphetamine, methamphetamine, and many other compounds known by several names including amphetamines (as a group) and sympathomimetic amines. The structure of many of

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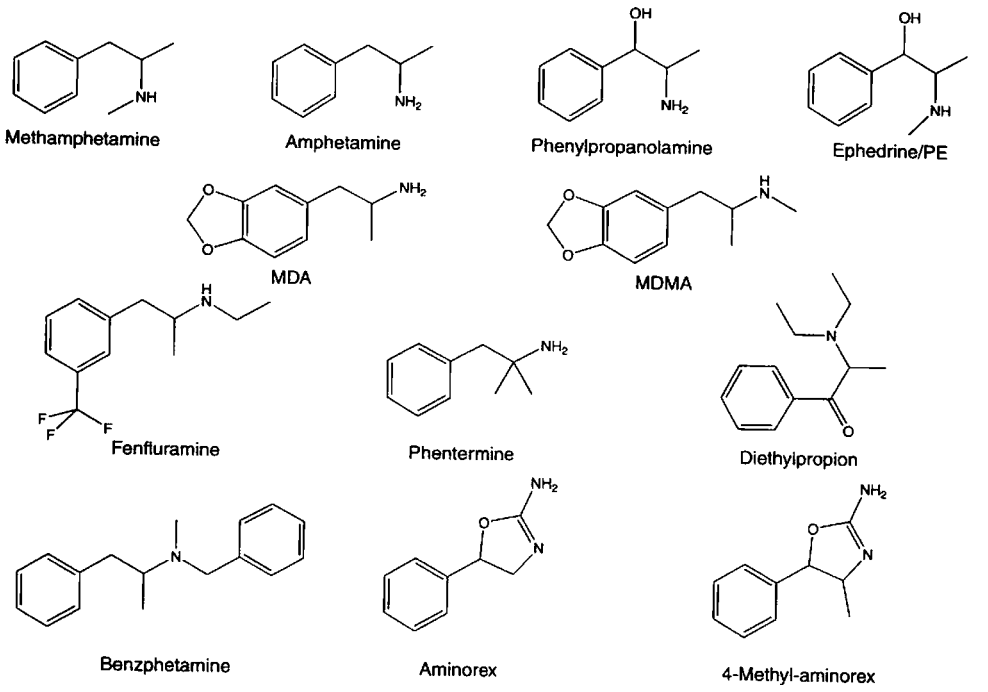


**Fig. 1.** Structures of sympathomimetic amines. Reprinted with permission from ref. *(1)*.

these compounds is shown in Fig. 1. Amphetamine is a primary amine and methamphetamine is a secondary amine. These compounds have a stereogenic center and have enantiomers or optical isomers designated *d* (or +) for dextrorotatory and *l* (or –) for levorotatory. In general, the *d* isomers are the more physiologically active compounds, but pharmaceutical preparations may consist of either isomer or a mixture of both isomers. When the mixture contains equal concentrations of the two enantiomers, it is known as a racemic mixture. The existence of enantiomers for amphetamines creates analytical and interpretative problems that will be discussed in sections 2.2 and 5.2 of this chapter. Two of the compounds, shown in Fig. 1, 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) are synthetic stimulants often called amphetamine designer drugs (see Chapter 19). Several sympathomimetic amines (ephedrine, pseudoephedrine, and phenylpropanolamine) contain a hydroxyl moiety on the carbon adjacent to the amine group (α-hydroxy) and are often ingredients in over-the-counter (OTC) drugs.

Metabolism and excretion of these compounds must be considered when discussing screening and confirmatory tests for their detection, identification, and quantification. Metabolism resulting in a phenolic hydroxyl group increases acidity of the compound and changes extraction properties. The principal urinary metabolites of methamphetamine are hydroxymethamphetamine (~15%) and amphetamine (4–7%), but approximately 40–50% is excreted unchanged *(1)*. Renal excretion is dependent on urinary pH with acidification (to a pH <5.6) decreasing the plasma half-life and alkalization increasing the plasma half-life *(1)*.

## 2. IMMUNOASSAY SCREENING

The primary screening methods for detecting amphetamines are immunoassays. The structural similarity to amphetamine or methamphetamine of the compounds (shown in Fig. 1) makes it difficult to produce antibodies specific for amphetamine, methamphetamine, or both. Based on cross-reactivity studies, most amphetamine antibodies appear to be directed toward the amino group. Both monoclonal and polyclonal antibodies have been developed and used in amphetamine immunoassays. Monoclonal antibodies result in more defined specificities but not necessarily more selectivity. In general, amphetamine assays can be classified into three general types based on antibody specificity *(2)*. One group consist of those assays highly selective for either amphetamine (and its designer counterpart MDA) or methamphetamine (and its designer drug counterpart MDMA) but not both sets simultaneously. A second group of immunoassays are those that are able to detect both amphetamine and methamphetamine to varying extent but that also exhibit higher levels of cross-reactivity to the hydroxy amine compounds found in many OTC drugs. The third group of immunoassays consist of dual assays for amphetamine and methamphetamine with low levels of cross-reactivity to OTC drugs.

Quite often antibody specificity is influenced by the intended use of the testing. The term amphetamines is typically used to denote immunoassay testing specific for the two stimulants amphetamine and methamphetamine. Government-mandated and workplace drug testing typically specifies testing for amphetamine and methamphetamine only. Laboratories performing workplace drug testing desire immunoassays specific for only those compounds specified in appropriate legislation or contracts. On the other hand, laboratories affiliated with an emergency department (ED) desire immunoassays directed toward the broad spectrum of sympathomimetic amines. This desire is articulated as a recommendation in the National Academy of Clinical Biochemistry's (NACB) Laboratory Medicine Practice Guidelines (LMPG) that the optimum immunoassays for amphetamines testing in ED patients are those directed toward phenylethyl amines as a class *(3)*. These guidelines also recommend that the name of the test should be changed from "amphetamines" to sympathomimetic amines" or "stimulant amines."

Federally mandated testing currently includes a screening cutoff of 1000 ng/mL for amphetamines, but a proposal by the Substance Abuse and Mental Health Services Administration (SAMHSA) to lower these levels to 500 ng/mL (and add MDMA) for screening is in the final stages of the regulatory process *(4)*. The proposed changes are estimated to identify 5–24% more urine specimens containing amphetamines *(4)*.

### *2.1. Immunoassay Cross-Reactivity*

Amphetamines assays are designed to target methamphetamine, amphetamine, or both, and the analyst should be aware of the targeted analyte(s) as well as the concentrations of these analytes in calibrators and controls. As previously mentioned, structural similarity to amphetamine or methamphetamine creates potential problems due to cross-reactivity of the antibodies in immunoassays. Table 1 lists the cross-reactivity of various phenethylamines in different immunoassays *(1)*. The information in this table may be used as a starting point when comparing immunoassays, but

Table 1
Cross-reactivity of Various Phenethylamines in Different Immunoassays

| Compound | *EMIT® Monoclonal* | *EMIT® Emit II* | *TDx®* | *DPC® Coat-A-Count (Meth)* | *DPC® Double Ab (Amph)* | *Roche ONLINE® (KIMS)* | *STC Microplate ELISA (Meth)* | *STC Microplate ELISA (Amph)* | *CEDIA® DAU (Amph)* |
|---|---|---|---|---|---|---|---|---|---|
| *d*-Amphetamine | 250.0 | 100.0 | 100.0 | 0.3 | 100.0 | 100.0 | 1.3 | 100.0 | 101.0 |
| *d*-Methamphetamine | 100.0 | 100.0 | 100.0 | 100.0 | 0.0 | 0.5 | 100.0 | <1 | 100.0 |
| Phenylpropanolamine | 1.3 | 0.4 | <0.1 | 0.1 | 0.5 | 0.7 | <0.1 | <0.1 | 0.3 |
| *l*-Ephedrine | 2.0 | 0.7 | <0.1 | 0.9 | 0.05 | <0.2 | 1.4 | – | 0.4 |
| Pseudoephedrine | 1.0 | 0.3 | <0.03 | 0.3 | 0.05 | <0.2 | 1.8 | <0.1 | 0.6 |
| Phentermine | – | 50.0 | 35.0 | 0.04 | 0.17 | < 0.1 | – | <0.1 | 1.9 |
| MDA | – | 33.0 | 151.0 | <0.1 | 138.0 | 32.0 | 2.8 | >100.0 | 1.9 |
| MDMA | – | 17.0 | 99.0 | >200.0 | 0 | 0.2 | 1513 | <0.1 | 69.0 |

[a] Amph, amphetamine; DAU, drugs of abuse Urine; ELISA, enzyme-linked immunosorbent assay; EMIT, enzyme multiplied immunoassay technique; KIMS, kinetic interactive microparticles in solution; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine;Meth, methamphetamine.

[b] Reprinted with permission from ref. 1.

the reader should realize that many factors influence cross-reactivity including the possibility that current assays produced by the manufacturers listed in this table may have different cross-reactivities due to changes in antibodies and reagent composition. Up-to-date cross-reactivity data are typically listed in immunoassay package inserts or may be obtained from each manufacturer. Several factors should be considered when reviewing cross-reactivity data. Studies of potential interfering substances sometimes use concentrations that are lower than those encountered in the clinical setting. Another consideration is that even though parent compounds may be shown not to cross-react in an immunoassay, it is possible for endogenous metabolites (which are often not tested by the manufacturer and may not be commercially available for testing by laboratories) to interfere with the assay *(5,6)*. Also cross-reactivity data may be instrument dependent particularly when the ratio of reagent to sample is different than those used for the manufacturers' cross-reactivity studies. There has been at least one report *(7)* of lot-to-lot variability concerning sensitivity to targeted analytes, and it is conceivable that there could be lot-to-lot variability concerning cross-reactivity. Ideally, laboratories should perform cross-reactivity studies of the most common interfering substances on their reagent/instrument system, but at a minimum they should contact the manufacturer to verify that they have the most recent applicable cross-reactivity information.

### *2.2. False-Positive Immunoassay Results*

Prescription and OTC medications (or metabolites of these medications) reported to interfere with amphetamine immunoassays include buflomedil, brompheniramine, chloroquine, chlorpromazine, ephedrine, fenfluramine, isometheptene, isoxsuprine, labetalol, mephentermine, mexiletine, N-acetylprocainamide, nylidrin, perazine, phenmetrazine, phentermine, phenylpropanolamine, promethazine, propylhexedrine, pseudoephedrine, quinacrine, ranitidine, ritodrine, tolmetin, trimethobenzamide, and tyramine *(1,5,8–18)*.

The most common cause of false-positive results with amphetamine immunoassays is cross-reactivity with α-hydroxy amine compounds and other sympathomimetic amines found in many OTC drugs. In order to decrease these false-positive incidents and thus decrease the expense and time of confirmation testing, some laboratories adopt a policy of performing a secondary screen of all positive samples following an initial immunoassay test. This secondary screen may consist of utilizing an immunoassay with different cross-reactivity to these compounds produced by a different manufacturer or reanalysis using the same immunoassay following chemical reaction or modification of the assay in some manner. Pretreatment of samples with sodium periodate in a basic solution eliminates interference from ephedrine, pseudoephedrine, and phenylpropanolamine by oxidative cleavage of the hydroxyl group, and this reaction has been utilized in the EMIT® amphetamine confirmation kit (Dade Behring, San Jose, CA). Another strategy for elimination of false-positive results is the addition of antibody to the target analyte resulting in neutralization of the signal in a true-positive sample but having no effect on the signal from a sample containing high concentrations of cross-reactive substances. In this situation, true positives are distinguished from false positives by the difference in signal before and after addition of the neutralizing antibody. This method was applied to a dual-channel neutralization procedure

for amphetamines and used as a secondary screen that was effective in reducing false positives *(19)*. Recently, Woodworth et al. *(20)* described a procedure utilizing serial dilution testing to distinguish amphetamine/methamphetamine-containing samples from samples containing cross-reacting sympathomimetic amines. Samples diluted 1:1, 1:10, and 1:20 were analyzed, and maximum slope estimates (maximum change in rate over the fractional change in concentration) were determined for each compound using the EMIT® II amphetamine/methamphetamine immunoassay. The authors were able to increase the positive predictive value of the immunoassay using optimal slope cutoffs (determined by receiver operating characteristic (ROC) analysis) to differentiate samples containing amphetamine/methamphetamine from those containing cross-reacting compounds.

The existence of *d* and *l* isomers can cause false-positive results when medication containing the *l* isomer is ingested. The most often mentioned example is that of Vicks® inhaler in which the active ingredient is *l*-methamphetamine *(21)*. Extensive use of this product could cause false-positive results for immunoassay screening and subsequent confirmation by gas chromatography/mass spectrometry (GC/MS). Tests to differentiate the isomers are discussed in Section 5.2. Although isomer resolution is required to definitively determine isomer composition, several studies have shown no false-positive results when the inhaler was used as directed and only a few false-positive results when the inhaler was used at twice the recommended frequency *(22,23)*. Conversely, other studies have shown that heavy use of the inhaler can result in methamphetamine concentrations as high as 6000 ng/mL and concentrations of the metabolite amphetamine 200 ng/mL cutoff *(24,25)*.

## 3. INTERFERENCES/ADULTERANTS

In addition to effects of adulterants mentioned in Chapter 16, the effect of some chemicals on amphetamine immunoassays has been reported. Mefenamic acid, a nonopiod analgesic, interferes with immunoassays measuring absorbance changes at 340 nm such as EMIT® assays, due to very high initial absorbance values *(26)*. In Taiwan, alum was used to interfere with the analysis of methamphetamine, and its effects were studied by Liu and Chien *(27)*. They determined that addition of alum to urine containing methamphetamine did cause false-negative or invalid results for selected immunoassays, apparently due to lowering of the pH below 4.0. In some instances, chemicals have unexpected effects on immunoassays. Tsai et al. *(28)* reported that high concentrations (1.0 M) of the adulterant nitrite actually increased the sensitivity for amphetamines with the ONLINE® (Roche Diagnostics, Somerville, NJ) immunoassay.

### *3.1. True-Positive Results*

In addition to structurally related and other cross-reacting medications, positive amphetamine results can be obtained when amphetamine, methamphetamine, or medications containing compounds metabolized to amphetamine and/or methamphetamine are ingested. Amphetamine [Dexedrine® (*d*-amphetamine), Adderall® (*d*- and *l*-amphetamine), etc.] and methamphetamine [Desoxyn® (*d*-methamphetamine)] are the active compounds of medications prescribed for narcolepsy, attention deficit disorder, and appetite suppression, and ingestion of these drugs will result in excretion

of these compounds in the urine. In addition, compounds known to be metabolized to amphetamine and/or methamphetamine include selegiline, amphetaminil, benzphetamine, clobenzorex, dimethylamphetamine, ethylamphetamine, famprofazone, fencamine, fenethylline, fenproporex, furfenorex, mefenorex, and prenylamine *(29)*. Some weight loss or nutritional supplements contain fenproporex, and use of these supplements has resulted in detection of *d*-amphetamine in the urine of users *(30)*. In January 2006, the FDA warned consumers that Brazilian dietary supplements Emagrece Sim® and Herbathin® contain active drug ingredients *(31)*.

## 4. IMMUNOASSAYS FOR BLOOD AND POST-MORTEM SPECIMENS

Detection of amphetamines in blood samples is accomplished utilizing immunoassays designed specifically for that matrix *(32)* or by modification of assays designed for a urine matrix. One such modification involves zinc sulfate protein precipitation from whole blood followed by immunoassay analysis of the supernatant *(33)*. In moderately to heavily decomposed bodies, large amounts of the putrefactive amines phenethylamine and tyramine produced by saprogenic bacteria may cause false-positive results. Techniques used to reduce or eliminate this interference include collecting blood in sodium fluoride and deproteinization using acetone or sulfosalicylic acid *(34,35)*. In one study, addition of the putrefactive bases β-phenethylamine and tyramine confirmed the manufacturer's claim of less than 2% cross-reactivity with their immunoassay, but urine stored with bladder tissue exhibited increasing concentrations of apparent amphetamine reactivity indicating other complicating factors such as possible production of increased concentrations of these or other interfering compounds *(36)*.

## 5. GC/MS CONFIRMATION PROCEDURES

Confirmation of screening results by an acceptable second method is required for SAMHSA-regulated drug testing and is recommended practice for all forensic applications. To date, GC/MS is the most frequently used confirmation procedure for the detection of amphetamine, methamphetamine, and other related compounds. Federally mandated testing currently includes confirmation cutoffs of 500 ng/mL for both amphetamine and methamphetamine, but a proposal by the SAMHSA to lower these levels to 250 ng/mL for confirmation (including MDMA, MDA, and MDEA = 3,4-methylenedioxy-N-ethylamphetamine) is in the final stages of the regulatory process *(4)*. Current regulations also require that in order to report a sample positive for methamphetamine the metabolite amphetamine must be detected at a confirmation cutoff of 200 ng/mL.

Amphetamine and related compounds are volatile and may be lost during the evaporation step of extraction or during analysis if preventative measures are not undertaken. Procedures to reduce/eliminate loss of amphetamines include lowering the temperature for evaporation, performing incomplete evaporation or adding methanolic HCl prior to evaporation in order to produce more stable hydrochloride salts *(29,37,38)*. GC/MS procedures for amphetamines include a derivatization step for many reasons including decreasing volatility, improving chromatography and quantitation, and forming higher molecular weight fragments yielding different mass ions and ion ratios than

potentially interfering compounds *(1,29,38,39)*. Derivatives used for amphetamines analysis include heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride, 4-carboxyhexafluorobutyryl chloride (4-CB), N-methyl-N-*t*-butyldimethylsilyl trifluoroacetamide, N-trifluoroacetyl-1-prolyl chloride (TPC), 2,2,2-trichloroethyl chloroformate, and propylchloroformate *(38–42)*. HFBA and PFPA are two of the more commonly used derivatives in forensic drug testing labs *(1)*. Most procedures utilize selected ion monitoring and deuterated internal standards (IS) that have similar fragmentation patterns and almost identical chromatographic properties to the non-deuterated compounds. Ideally the IS concentration is the cutoff concentration, but higher concentrations may be used to extend the linearity of the assay. If higher concentrations of IS are used, the analyst should be aware of the potential impact of small amounts of non-deuterated compound present in the IS preparation *(43)*. Monitoring the relative IS abundance of every sample and establishing acceptable limits (such as a requirement to be ≥50% and <200% of the calibrator IS area) is a recommended quality assurance measure *(43)*.

### *5.1. False-Positive Methamphetamine*

In 1993, Hornbeck et al. *(44)* demonstrated that methamphetamine can be generated from high levels of pseudoephedrine or ephedrine in injection ports at a temperature of 300°C after derivatization with 4-CB, HFBA, and TPC. They investigated the effect of changing conditions and concluded that the most important conditions for this thermal conversion are the high injector temperature and high concentrations of pseudoephedrine or ephedrine. In their experiments, the highest amphetamine concentration obtained was less than 50 ng/mL. The heptafluorobutyryl derivative of ephedrine has also been shown to give methamphetamine peak interferences because of contaminants in the derivatizing reagent *(45)*. Occurrence of false-positive results in the Federal Drug Testing Program because of generation of methamphetamine resulted in the implementation of a requirement that in order to report a positive methamphetamine the metabolite amphetamine must be present at a concentration of 200 ng/mL or higher. This concentration would be lowered to 100 ng/mL in conjunction with the proposal to lower the cutoff concentration for confirmation to 250 ng/mL *(4)*. In one study, 90% of specimens collected from volunteers ingesting methamphetamine at concentrations mimicking occasional use had amphetamine concentrations below 200 ng/mL even though most had methamphetamine concentrations above 500 ng/mL *(46)*.

Recommendations to prevent generation of methamphetamine include lowering the injector temperature and periodate pretreatment of samples. ElSohly et al. *(47)* showed that periodate treatment eliminated formation of methamphetamine by 1,000,000 ng/mL of pseudoephedrine, ephedrine, phenylpropanolamine, and norpseudoephedrine by selectively oxidizing these compounds in the presence of amphetamine and methamphetamine. They used 0.35 M sodium periodate for 10 min at room temperature. SAMHSA-certified laboratories that have an oxidation step in their amphetamine confirmation procedure are required to monitor the effectiveness of the procedure in each confirmation batch by analyzing a quality control sample containing a high concentration of sympathomimetic amines *(43)*.

### *5.2. Isomer Resolution*

As previously mentioned, many of these compounds including amphetamine and methamphetamine can exist as *d*(+) or *l*(−) isomers, and the *d* isomer is the illicit form. The GC/MS procedures previously described do not differentiate these isomers, and it is necessary to perform isomer resolution to determine that a positive result is due to the presence of the *d* isomer. This is particularly true for positive methamphetamine results because of the presence of *l*-methamphetamine in Vicks® inhaler which cannot be distinguished from use of illicit methamphetamine (*d* isomer and racemic mixture depending on method of production) with GC/MS methods using non-chiral derivatives and non-chiral columns *(41)*. Similarly, patients taking selegiline for Parkinson's will excrete *l*-methamphetamine and *l*-amphetamine.

Differentiation of *d* and *l* isomers of amphetamine and methamphetamine can be accomplished using a chiral, optically active column or chiral derivatizing reagents *(1)*. Most laboratories choose to use chiral derivatizing reagents because this allows them to perform the analysis on instrument/column systems used for other routine analyses. One disadvantage of using chiral derivatizing reagents is the possibility of obtaining four isomers instead of two if the derivatizing agent is not optically pure. For example, Hensley and Cody *(48)* found inadequate enantiomeric purity in several lot numbers of *l*-TPC from three different vendors, and they reported that the *d*-enantiomer content ranged from 2 to 12% in control samples reported to contain 0% *d*-enantiomer. Some of the chiral derivatizing agents include N-trifluoroacetyl-L-prolyl chloride [(S)-TPC], *R*(−)-α-methoxy-α-trifluoromethylphenylacetic acid chloride, and (–)-menthyl chloroformate *(37,41,49)*. The generally accepted interpretation of isomer resolution results is that greater than 80% of the *l* isomer is considered consistent with use of legitimate medication or conversely greater than 20% of the *d* isomer (and total concentration above the cutoff) is considered evidence of illicit use. SAMHSA-certified laboratories are not required to perform amphetamines isomer resolution, and if they perform it, they are allowed to perform it either to determine ratios of *d*- and *l*-methamphetamine in positive methamphetamine specimens or as the primary confirmatory test for amphetamine and methamphetamine. The test may be quantitative or qualitative (determining relative percentage of *d* and *l* isomers) and may test for only methamphetamine isomers or both amphetamine and methamphetamine isomers *(43)*.

### *5.3. GC/MS Assay Validation/Evaluation*

SAMHSA-certified laboratories are required to validate assays prior to implementation and evaluate performance characteristics annually. For non-certified laboratories, inclusion of validation and periodic evaluation of confirmation assays as part of their quality assurance plan demonstrates a commitment to professionalism and good laboratory practice. Validation studies should include determination of upper limit of linearity, limit of detection, limit of quantitation, precision/accuracy around cutoff concentrations, and carryover and interference studies *(43)*. Chromatographic evaluation of possible interferents should include potential for co-elution, presence of extraneous peaks, and similarity of mass ions and ion ratios to amphetamine and methamphetamine *(42)*. SAMHSA-certified laboratories are required to perform interference studies for amphetamine confirmation assays by analyzing samples containing interferents (phentermine at 50,000 ng/mL and phenylpropanolamine, ephedrine, and

pseudoephedrine at 1 mg/mL) in the presence of and without amphetamine and methamphetamine at 40% of the cutoff *(43)*. Other compounds with similar structures that may be tested for interference include hydroxynorephedrine, norephedrine, norpseudoephedrine, phenylephrine, and propyhexedrine *(41)*. If a certified laboratory performs isomer resolution assays, interference studies must include samples with *d*-methamphetamine, *l*-methamphetamine, *d*-amphetamine, and *l*-amphetamine at 100 ng/mL each and samples without amphetamine or methamphetamine *(43)*.

### *5.4. Ecstasy and Other Designer Drug Concerns*

Detection of designer amphetamines will be briefly discussed in this section. One street name for the designer methamphetamine MDMA is ecstasy, but this term has also been expanded to include other designer amphetamines such as MDA and MDEA. As previously mentioned, some amphetamine immunoassays cross-react with the designer amphetamines (see Table 1) *(50–58)*, but immunoassays specifically targeting these compounds have also been developed *(59)*. The proposed changes to the SAMHSA-regulated Federal Drug Testing Program shown in Table 2 include addition of testing for MDMA at a screening cutoff of 250 ng/mL, and comments in these proposals indicate that they anticipate the use of two separate initial tests, one for methamphetamine and amphetamine and a second initial test for MDMA *(4)*.

GC/MS confirmation procedures for the detection of amphetamine and methamphetamine can often be expanded to include detection of the designer amphetamines *(2,29,49,59)*, and procedures for the simultaneous measurement of all of these compounds with single sample analysis times as short as 4 min have been published *(60)*. When testing for designer amphetamines, the analyst must

**Table 2**
**Proposed Cutoff Concentrations for Amphetamine (AMP), Methamphetamine, and Designer Amphetamines in Urine and Other Matrices (*4*)**

| | *Proposed test cutoff concentrations* | | | | | |
|---|---|---|---|---|---|---|
| | *AMPS*[a] | *AMP* | *MAMP* | *MDMA* | *MDA* | *MDEA* |
| Hair initial (pg/mg) | 500 | | | 500 | | |
| Hair confirmatory (pg/mg) | | 300 | 300[b] | 300 | 300 | 300 |
| Oral fluid initial (ng/mL) | 50 | | | 50 | | |
| Oral fluid confirm (ng/mL) | | | 50[c] | 50 | 50 | 50 |
| Sweat initial (ng/patch) | 25 | | | 25 | | |
| Sweat confirm (ng/patch) | | | 25[c] | 25 | 25 | 25 |
| Urine initial (ng/mL) | 500 | | | 500 | | |
| Urine confirmatory (ng/mL) | | 250 | 250[d] | 250 | 250 | 250 |

MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine.

[a] Methamphetamine is the target analyte.

[b] Specimen must also contain amphetamine at a concentration ≥50 pg/mg.

[c] Specimen must also contain amphetamine at a concentration ≥ limit of detection.

[d] Specimen must also contain amphetamine at a concentration ≥100 ng/mL.

be aware of the metabolism and excretion patterns for these drugs. Metabolites of MDMA include MDA, 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA), 4-hydroxy-3-methoxymethamphetamine (HMMA), and 4-hydroxy-3-methoxyamphetamine (HMA). The hydroxyl methoxy metabolites (HHMA, HHA, HMMA, and HMA) are excreted as glucuronide and sulfate conjugates, and in order to obtain adequate recovery of these metabolites, a hydrolysis procedure should be included as part of the confirmation testing *(2,29,61)*. Pirnay and associates *(61)* compared acid and enzymatic (*Escherichia coli* and *Helix pomatia*) hydrolysis and concluded that optimal hydrolysis conditions for the measurement of MDMA metabolite conjugates were addition of 100 μL HCl to 1 mL urine and incubation at 120°C for 40 min.

## 6. TESTING FOR AMPHETAMINES IN ALTERNATIVE MATRICES

In addition to lowering the cutoff concentrations for amphetamine and methamphetamine and adding detection of MDMA, the proposals for changes in the SAMHSA-regulated federal program include allowance of testing for these compounds in hair, oral fluid, and sweat as well as urine. For amphetamines, the analytes targeted in these alternative matrices for screening and confirmation are the same as in urine, but cutoff concentrations are matrix dependent (see Table 2). Screening procedures for alternative matrices include adaptation of urine immunoassays as well as introduction of matrix-specific immunoassays *(62–66)*. Testing for drugs in each alternative matrix introduces the possibility of sample-handling problems unique to that matrix. For example, hair samples must be weighed and washed prior to digestion/extraction for testing. Meconium, often used for testing to determine maternal drug use during the later stages of pregnancy, presents another matrix to the analyst for which screening and confirmation procedures have been developed *(67)*. See Chapter 18 for a more comprehensive discussion of drug testing in alternative matrices.

Confirmation procedures for detection of amphetamines in alternative matrices not only include GC/MS procedures but also include development of assays combining techniques such as liquid chromatography (LC) and GC with tandem mass spectrometry (MSMS) *(68–71)*.

## 7. CONCLUSIONS

Testing for amphetamine, methamphetamine, and amphetamine-like compounds focuses primarily on detection of amphetamine, methamphetamine, and designer drugs often grouped together as ecstasy (MDMA, MDA, and MDEA). The primary concern for immunoassay screening is false-positive results due to cross-reactivity of the reagent antibodies to other sympathomimetic amines, namely, the hydroxyl amines ephedrine, pseudoephedrine, and phenylpropanolamine. For GC/MS confirmation testing, generation of methamphetamine from high levels of pseudoephedrine or ephedrine in injection ports at high temperatures after derivatization with 4-CB, HFBA, and TPC is a potential problem. Periodate treatment of samples prior to immunoassay screening or GC/MS confirmation removes the interfering sympathomimetic amines. Another problem with amphetamines testing is the existence of *d* and *l* stereoisomers that are not distinguishable by immunoassay screening and most GC/MS confirmation

procedures. Isomer resolution procedures involve separation using chiral columns or derivatization using optically pure chiral derivatizing agents. Testing for amphetamine, methamphetamine, and MDMA/metabolites in hair, oral fluid, and sweat presents matrix-specific problems and introduction of other confirmation methods involving LC and MSMS instrumentation.

## REFERENCES


1. Moore KA. Amphetamines/sympathomimetic amines in B. Levine (Ed.), Principles of Forensic Toxicology 2003; pp. 341–348. Washington, D.C.: AACC Press.
2. Butler D, Guilbault GG. Analytical techniques for ecstasy. Anal Lett 2004; 37: 2003–2030.
3. Wu AHB, McKay C, Broussard LA, Hoffman RS, Kwong TC, Moyer TP, et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines: recommendations for the use of laboratory tests to support poisoned patients who present to the emergency department. Clin Chem 2003; 49: 357–379.
4. Substance Abuse and Mental Health Services Administration: Federal Workplace Drug Testing Program; Proposed Mandatory Guidelines: Hair, sweat, oral fluid, and urine specimens testing; scientific and technical guidelines. Fed Regist 2004; 69: 19672–19732.
5. Poklis A. Unavailability of drug metabolite reference material to evaluate false-positive results for monoclonal EMIT-d.a.u. assay of amphetamine. Clin Chem 1992; 38: 2580.
6. Williams RH, Erickson T, Broussard L. Evaluating sympathomimetic intoxication in an emergency setting. Lab Med 2000; 31: 497–507.
7. Singh J. Reagent lot-to-lot variability in sensitivity for amphetamine with the Syva EMIT II monoclonal amphetamine/methamphetamine assay. J Anal Toxicol 1997; 21: 174–175.
8. Grinstead GF. Ranitidine and high concentrations of phenylpropanolamine cross react in the EMIT monoclonal amphetamine/methamphetamine assay. Clin Chem 1989; 35: 1998–1999.
9. Caplan YH, Levine B, Goldberger B. Fluorescence polarization immunoassay evaluated for screening for amphetamine and methamphetamine in urine. Clin Chem 1987; 33: 1200–1202.
10. Papa P, Rocchi L, Mainardi C, Donzelli G. Buflomedil interference with the monoclonal EMIT d.a.u. amphetamine/methamphetamine immunoassay. Eur J Clin Chem Clin Biochem 1997; 35: 369–370.
11. Olsen KM, Gulliksen M, Christophersen AS. Metabolites of chlorpromazine and brompheniramine may cause false-positive urine amphetamine results with monoclonal EMIT d.a.u. immunoassay. Clin Chem 1992; 38: 611–612.
12. Smith-Kielland A, Olsen KM, Christophersen AS. False-positive results with EMIT II amphetamine/methamphetamine assay in users of common psychotropic drugs. Clin Chem 1995; 41: 951–952.
13. Dasgupta A, Saldana S, Kinnaman G, Smith M, Johansen K. Analytical performance evaluation of EMIT II monoclonal amphetamine/methamphetamine assay: more specificity than EMIT d.a.u. monoclonal amphetamine/methamphetamine assay. Clin Chem 1993; 39: 104–108.
14. Joseph R, Dickerson S, Willis R, Frankenfield D, Cone EJ, Smith DR. Interference by nonsteroidal anti-inflammatory drugs in EMIT and TDx assays for drugs of abuse. J Anal Toxicol 1995; 19:13–17.
15. Nice A, Maturen A. False-positive urine amphetamine screen with ritodrine. Clin Chem 1989; 35: 1542–1543.
16. Jones R, Klette K, Kuhlman JJ, Levine B, Smith ML, Watson CV, Selavka CM. Trimethobenzamide cross-reacts in immunoassays of amphetamine-methamphetamine. Clin Chem 1993; 39: 699–700.
17. Kozer E, Verjee Z, Koren G. Misdiagnosis of a mexiletine overdose because of a nonspecific result of urinary toxicology screening. N Engl J Med 2000; 343 (26): 1971.
18. Schmolke M, Hallbach J, Guder WG. False-positive results for urine amphetamine and opiate immunoassays in a patient intoxicated with perazine. Clin Chem 1996; 42: 1725–1726.
19. Shindelman J, Mahal J, Hemphill G, Pizzo P, Coty WA. Development and evaluation of an improved method for screening of amphetamines. J Anal Toxicol 1999; 23: 506–510.
20. Woodworth A, Saunders AN, Koenig JW, Moyer TP, Turk J, Dietzen DJ. Differentiation of amphetamine/methamphetamine and other cross-immunoreactive sympathomimetic amines in urine samples by serial dilution testing. Clin Chem 2006; 52: 743–746.

21. Solomon MD, Wright JA. False-positive for (+)-methamphetamine. Clin Chem 1977; 23:1504.
22. Poklis A, Moore KA. Stereoselectivity of the TDxADx/FLxamphetamine/methamphetamine II immunoassay: response of urine specimens following nasal inhaler use. J Toxicol Clin Toxicol 1995; 33: 35–41.
23. Poklis A, Moore KA. Response of EMIT amphetamine immunoassays to urinary dexoyephedrine following "Vicks inhaler use. Ther Drug Monit 1995: 17: 89–94.
24. Fitzgerald RL, Ramos JM, Bogema SC, Poklis A. Resolution of methamphetamine stereoisomers in urine drug testing: urinary excretion of R(-)-methamphetamine following use of nasal inhalers. J Anal Toxicol 1988; 12: 255–259.
25. Hornbeck CL, Czarny RJ. Retrospective analysis of some L-methamphetamine/L-amphetamine urine data. J Anal Toxicol 1993; 17:23–25.
26. Crane T, Badminton MN, Dawson CM, Rainbow SJ. Mefenamic acid prevents assessment of drug abuse with EMIT assays. Clin Chem 1993; 39: 548.
27. Liu C-Y, Chien C-S. Interference of alum on analysis of methamphetamine in urine specimens. Clin Chem 1996; 42: 338–340.
28. Tsai SCJ, ElSohly MA, Dubrovsky T, Twarowska B, Towt J, Salamone SJ. Determination of five abused drugs in nitrite-adulterated urine by immunoassays and gas chromatography-mass spectrometry. J Anal Toxicol 1998; 22: 474–480.
29. Kraemer T, Maurer HH. Determination of amphetamine, methamphetamine and amphetamine-derived designer drugs or medicaments in blood and urine. J Chromatogr B Biomed Sci Appl 1998; 713: 163–187.
30. Jemionek J, Bosy TJ, Jacobs A, Holler J, Magluli J, Dunkley C. Five cases of D-amphetamine positive urines resulting from ingestion of "Brazilian nutritional supplements" containing fenproporex have been reported. ToxTalk SOFT Newsletter 2006; 30(2): 11.
31. FDA News P06-07, January 13, 2006.
32. Kerrigan S, Phillips WH. Comparison of ELISAs for opiates, methamphetamine, cocaine metabolite, benzodiazepines, phencyclidine, and cannabinoids in whole blood and urine. Clin Chem 2001; 47: 540–547.
33. Simonick TF, Watts VW. Preliminary evaluation of the Abbott TDx for screening of D-methamphetamine in whole blood specimens. J Anal Toxicol 1992; 16: 115–118.
34. Hino Y, Ojanpera I, Rasanen I, Vuori E. Performance of immunoassays in screening for opiates, cannabinoids and amphetamines in post-mortem blood. Forensic Sci Int 2003; 131: 148–155.
35. Moriya F, Hashimoto Y. Evaluation of Triage screening for drugs of abuse in postmortem blood and urine samples. Nihon Hoigaku Zasshi 1997; 51: 214–219.
36. Kintz P, Tracqui A, Mangin P, Lugnier A, Chaumont A. Specificity of the Abbott TDx assay for amphetamine in post-mortem urine samples. Clin Chem 1988; 34: 2374–2375.
37. Holler JM, Vorce SP, Bosy TZ, Jacobs A. Quantitative and isomeric determination of amphetamine and methamphetamine from urine using a nonprotic elution solvent and *R*(−)-α-methoxy-α-trifluoromethylphenylacetic acid chloride derivatization. J Anal Toxicol 2005; 29: 652–657.
38. Blandford DE, Desjardins PRE. Detection and identification of amphetamine and methamphetamine in urine by GC/MS. Clin Chem 1994; 40: 145–147.
39. Melgar R, Kelly RC. A novel GC/MS derivatization method for amphetamines. J Anal Toxicol 1993; 17: 399–402.
40. Meatherall R. Rapid GC-MS confirmation of urinary amphetamine and methamphetamine as their propylchloroformate derivatives. J Anal Toxicol 1995; 19: 316–322.
41. Goldberger BA, Cone EJ. Confirmatory tests for drugs in the workplace by gas chromatography—mass spectrometry. J Chromatogr A 1994; 674: 73–86.
42. Dasgupta A, Spies J. A rapid novel derivatization of amphetamine and methamphetamine using 2,2,2-trichloroethyl chloroformate for gas chromatography electron ionization and chemical ionization mass spectrometric analysis. Am J Clin Pathol 1998; 109: 527–532.
43. National Laboratory Certification Program Manual for laboratories and inspectors. November 1, 2006.
44. Hornbeck CL, Carrig JE, Czarny RJ. Detection of a GC/MS artifact peak as methamphetamine. J Anal Toxicol 1993; 17: 257–263.

45. Wu AHB, Wong SS, Johnson KG, Ballatore, Seifert WE. The conversion of ephedrine to methamphetamine and methamphetamine-like compounds during and prior to gas chromatographic/mass spectrometric analysis of CB and HFB derivatives. Biol Mass Spectrom 1992; 21: 278–284.
46. Valentine JL, Kearns GL, Sparks C, Letzig LG, Valentine CR, Shappell SA, et al. GC-MS determination of amphetamine and methamphetamine in human urine for 12 hours following oral administration of dextro-methamphetamine: lack of evidence supporting the established forensic guidelines for methamphetamine confirmation. J Anal Toxicol 1995; 19: 581–590.
47. ElSohly MA, Stanford DF, Sherman D, Shah H, Bernot D, Turner CE. A procedure for eliminating interferences from ephedrine and related compounds in the GC/MS analysis of amphetamine and methamphetamine. J Anal Toxicol 1992; 16: 109–111.
48. Hensley D, Cody JT. Simultaneous determination of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and methylenedioxyethylamphetamine (MDEA). J Anal Toxicol 1999; 23: 518–523.
49. Paul BD, Jemionek J, Lesser D, Jacobs A, Searles DA. Enantiomeric separation and quantitation of (±)-amphetamine, (±)-methamphetamine, (±)-MDA, (±)MDMA, and (±)-MDEA in urine specimens by GC-EI-MS after derivatization with (*R*)(−)- or (*S*)-(+)α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA). J Anal Toxicol 2004; 28: 449–455.
50. Cody JT. Cross-reactivity of amphetamine analogues with Roche Abuscreen radioimmunoassay reagents. J Anal Toxicol 1990; 14: 50–53.
51. Ramos JM, Fitzgerald RL, Poklis A. MDMA and MDA cross reactivity observed with Abbott TDx amphetamine/methamphetamine reagents. Clin Chem 1988; 34: 991.
52. Cody JT. Detection of D,L-amphetamine and D,L-methamphetamine, and illicit amphetamine analogs using diagnostic products corporation's amphetamine and methamphetamine radioimmunoassay. J Anal Toxicol 1990; 14: 321–324.
53. Kunsman GW, Manno JE, Cockerham KR, Manno BR. Application of the Syva EMIT and Abbott TDx amphetamine immunoassays to the detection of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethamaphetamine (MDEA) in urine. J Anal Toxicol 1990; 12: 149–153.
54. Ruangyuttikarn W, Moody DE. Comparison of three commercial amphetamine immunoassays for detection of methamphetamine, methylenedioxyamphetamine, methylenedioxymethamphetamine, and methylenedioxyethylamphetamine. J Anal Toxicol 1988; 12: 229–233.
55. Zhao H, Brenneisen R, Scholer A, McNally AJ, ElSohly MA, Murphy TP, Salamone SJ. Profiles of urine samples taken from ecstasy users at rave parties: analysis by immunoassays, HPLC and GC-MS. J Anal Toxicol 2001; 25: 258–269.
56. Ledskulchai V, Mokkhavesa C. Evaluation of Roche Abuscreen ONLINE amphetamine immunoassay for screening of new amphetamine analogues. J Anal Toxicol 2001; 25: 471–475.
57. Kunsman GW, Levine B, Kuhlman JJ, Jones RL, Hughes RO, Fujiyama CI, Smith ML. MDA-MDMA concentrations in urine specimens. J Anal Toxicol 1996; 20: 517–521.
58. Cody JT, Schwarzhoff R. Fluorescence polarization immunoassay detection of amphetamine, methamphetamine, and illicit amphetamine analogues. J Anal Toxicol 1993; 17: 26–30.
59. Stout PR, Klette KL, Wiegand R. Comparison and evaluation of DRI methamphetamine, DRI ecstasy, Abuscreen ONLINE amphetamine, and a modified Abuscreen ONLINE amphetamine screening immunoassays for the detection of amphetamine (AMP), methamphetamine (MTH), 3-4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) in human urine. J Anal Toxicol 2003; 27: 265–269.
60. Klette KL, Jamerson MH, Morris-Kukoski CL, Kettle AR, Snyder JJ. Rapid simultaneous determination of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, and 3,4-methylenedioxyethylamphetamine in urine by fast gas chromatography-mass spectrometry. J Anal Toxicol 2005; 29: 669–674.
61. Pirnay SO, Abraham TT, Lowe RH, Huestis MA. Selection and optimization of hydrolysis conditions for the quantification of urinary metabolites of MDMA. J Anal Toxicol 2006; 30: 563–569.
62. Miki A, Katagi M, Tsuchihashi H. Application of EMIT d.a.u. for the semiquantitative screening of methamphetamine incorporated in hair. J Anal Toxicol 2002; 26: 274–279.
63. Tang K, Anne L. Automated homogeneous enzyme immunoassays for the detection of amphetamines in human oral fluid (abstract). Clin Chem 2006; 52: A74.

64. Cooper G, Wilson L, Reid C, Hand C, Spiehler V. Validation of the Cozart amphetamine microplate EIA for the analysis of amphetamines in oral fluid. Forensic Sci Int 2006; 159: 104–112.
65. Miki A, Katagi M, Shima N, Tsuchihashi H. Application of ORAL-screen saliva drug test for the screening of methamphetamine, MDMA, and MDEA incorporated in hair. J Anal Toxicol 2004; 28: 132–134.
66. Sweeney SA, Kelly RC, Bourland JA, Johnson T, Brown WC, Lee H, Lewis E. Amphetamines in hair by enzyme-linked immunosorbent assay. J Anal Toxicol 1998; 22: 418–424.
67. ElSohly MA, Stanford DF, Murphy TP, Lester BM, Wright LL, et al. Immunoassay and GC-MS procedures for the analysis of drugs of abuse in meconium. J Anal Toxicol 1999; 23: 436–445.
68. Musshoff F, Junker HP, Lachenmeier DW, Kroener L, Madea B. Fully automated determination of amphetamines and synthetic designer drugs in hair samples using headspace solid-phase microextraction and gas chromatography-mass spectrometry. J Chromatogr Sci 2002; 40: 359–364.
69. Villamor JL, Bermejo AM, Fernandez P, Tabernero MJ. A new GC-MS method for the determination of five amphetamines in human hair. J Anal Toxicol 2005; 29: 135–139.
70. Miki A, Katagi M, Tsuchihashi H. Determination of methamphetamine and its metabolites incorporated in hair by column-switching liquid chromatography-mass spectrometry. J Anal Toxicol 2003; 27: 95–102.
71. Wood M, DeBoeck G, Samyn N, Morris M, Cooper DP, Maes RAA, DeBruijn EA. Development of a rapid and sensitive method for the quantitation of amphetamines in human plasma and oral fluid by LC-MS-MS. J Anal Toxicol 2003; 27: 78–87.

# 21 Clinical False-Positive Drug Test Results

*Tai C. Kwong, PhD*

***Contents***



**Summary**

A confirmed positive drug test reassures all the parties involved in the drug testing process that the reported positive result is an analytical true positive and as such is evidence that the individual has been exposed to the drug. That individual may not be a drug abuser and may have a valid alternative explanation for the positive result. In this context, an analytical true positive result may be a clinical false positive. There are many causes other than illicit drug use which can produce positive analytical results. These include environmental exposure, ingestion of medications containing the drugs or medications which metabolize to the target drugs, and consumption of food products containing the drug. Additional laboratory tests and a thorough medical review will ascertain the source of the drug detected and eliminate misinterpreting a clinical false-positive result to implicate that individual as an illicit drug user.

**Key Words:** Amphetamine; cocaine; false positive; GC-MS; marijuana; opiate.

## 1. INTRODUCTION

The testing for drugs of abuse in the urine is an objective means to document a person's drug exposure. This procedure has been used effectively in a variety of settings including emergency departments for the diagnosis of drug overdose and in other clinical services such as pediatrics, obstetrics, psychiatry, addiction medicine, as well as in organ transplant for documentation and management of drug use or exposure.

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The positive finding of a drug test is evidence of exposure to the drug but it does not, however, provide direct information on the person's history of use in terms of the nature of the exposure, when the exposure took place, the level of exposure ("dose"), and if the patient was under the influence of the drug at the time of urine collection.

## 2. ANALYTICAL TRUE POSITIVE VERSUS CLINICAL FALSE POSITIVE

The assumption underlying interpretation of a drug test result is the accuracy and reliability in the analysis and identification of the drug or drug metabolite present in the urine. The standard urine drug test protocol involves an initial test using a battery of immunoassays. The accuracy of an immunoassay is determined by the immunospecificity of the assay antibody, and immunoassays in general do not have strict specificity for the target drug or drug metabolites. Many immunoassays have demonstrable reactivities with structurally similar compounds, some of which are not illicit drugs or are not abused. Moreover, some immunoassays may even detect substances that are structurally unrelated to the target analyte (e.g., PCP assay detects dextromethorphan) *(1,2)*. This limitation of specificity of immunoassays is well recognized, and this awareness has led to two important tenets of urine drug testing programs: (a) the positive result of an immunoassay is only a presumptive positive result and (b) definitive identification of the drug or the metabolite must be based on a second test, the confirmation test. Laboratorians and clinicians are familiar with the concepts that an initial positive result by immunoassay could be a false positive and that confirmation testing resolves the uncertainty surrounding the first test and definitively identifies the drug or its metabolites.

Healthcare providers who make clinical or management decisions based on drug test results do so because of the confidence they have that a positive result confirmed by the laboratory is a true positive. But a confirmed positive result is only an analytical true positive, while it can be a clinical false positive. This is because the presence of a drug or drug metabolite in the urine documents only that the individual has been exposed to the drug but warrants no inference about the nature of the exposure or the reason for the positive test. The person may very well have a valid explanation for producing the positive drug test, for example, he or she is on a prescription medication containing codeine, which can account for the positive codeine and morphine results. In the context of the reason for drug testing, this analytical true positive result may wrongly implicate the person as an illicit drug user. Hence, this gas chromatography-mass spectrometry (GC-MS) confirmed analytical true positive result is a clinical false positive case. The converse of this is when the individual cannot give a credible explanation for the analytical true positive result, in which case the test result is not only an analytical true positive, but it is also a clinical true positive in the sense that the drug test has identified illicit drug use. Thus, recognizing a positive result to be a clinical false positive eliminates the wrongful implication, with grave consequences to the person, that the individual is an illicit drug user. This can be accomplished, in some cases, by additional laboratory testing (e.g., 6-acetylmorphine testing for morphine positive results), and in every instance with a thorough medical review conducted by a qualified physician.

**Table 1**
**Reported Causes and Examples of Clinical False Positive Results**[a]

| *Causes of clinical false positive* | *Examples of clinical false-positive results* |
|---|---|
| Environmental: passive inhalation/secondary smoke | Δ9 THC-COOH |
| Use of prescription medications containing target drug(s), e.g., | |
| Acetaminophen with codeine | Codeine and morphine |
| Adderall® | Amphetamine |
| Marinol® | Δ9 THC-COOH |
| Use of prescription medication which are metabolized to target drug(s), e.g., | |
| Selegiline | *l*-Methamphetamine, *l*-Amphetamine |
| Clobenzorex | *d*-Ampheatmaine |
| Consumption of food products which contain or are contaminated with target drug(s), e.g., | |
| Poppy seeds contaminated with morphine | Morphine (and codeine) |
| Hemp products | Δ9 THC-COOH |

THC-COOH, Δ9-tetrahydrocannabinoid carboxylic acid.

[a] Medical Review Officer Manual for Federal Agency Workplace Drug Testing Programs http://dwp.samhsa.gov/DrugTesting/Level_1_Pages/HHS%20MRO%20Manual%20(Effective%20November%201,%202004).aspx (accessed 11/05/2006).

## 3. CLINICAL FALSE-POSITIVE RESULTS

Many causes for clinical false-positive results have been published in the scientific literature or reported anecdotally. The most common causes can be grouped into four categories: (a) environmental exposure—passive inhalation or secondary smoke; (b) use of prescription medications containing the target drug(s); (c) use of prescription medications which are metabolized to the target drug(s); (d) consumption of food products which contain or are contaminated with the target drug(s). These reported causes and examples of the clinical false-positive results are listed in Table 1. In the following sections, clinical false-positive results of individual drug classes will be discussed in detail.

## 4. AMPHETAMINES

Amphetamine and methamphetamine exist in two optical isomeric forms (enantiomers) designated as *d*- (dextro) or *l*- (levo). In another nomenclature, the two enantiomers are designated as S(+)- and R(−)-, respectively. The *d*-isomer has much stronger central nervous system (CNS) stimulant effect and has high abuse potential; the *l*-isomer exerts its vasoconstrictive effect peripherally. For example, *l*-methamphetamine has much lower potency as a CNS stimulant than *d*−methamphetamine and is available as a nonprescription nasal inhalant (Vicks Inhaler®).

Clinical false-positive amphetamines result can come from one of the following causes.

### 4.1. l-Methamphetamine

The Vicks Inhaler® is the only over-the-counter drug that contains *l*-methamphetamine. Most amphetamines immunoassays are designed to detect *d*-methamphetamine. Despite being relatively stereospecific for *d*-methamphetamine, some amphetamines immunoassays can produce a positive result if *l*-methamphetamine is present in high concentrations *(1,3)*. Standard GC-MS confirmation methodology cannot distinguish between the two enantiomers and will confirm the presence of methamphetamine that was first detected by the initial test and a clinical false-positive result will be reported. Enantiomeric analysis using chiral derivatization and chromatography is needed to verify that the confirmed methamphetamine result was due to Vicks Inhaler® *(3,4)*. In this special procedure using optically active derivatizing reagents, *d*- and *l*-enantiomers are converted into diasteriomers, which then can be chromatographically separated prior to mass spectrometric analysis *(5)*. If there is greater than 80% *l*-methamphetamine (not 100% because there may be a trace contaminant of *d*-methamphetamine in Vicks Inhaler®), the result is considered to be consistent with Vicks Inhaler® use *(6)*.

### 4.2. Prescription Medications Containing Amphetamine or Methamphetamine

Drug Enforcement Agency listed amphetamine and methamphetamine as Schedule II controlled substances. Clinical uses include treatment of attention deficit disorder with hyperactivity, narcolepsy, and obesity. Pharmaceutical methamphetamine is *d*-methamphetamine. Amphetamine, however, is available as *d*-amphetamine as well as a mixture of *d*- and *l*-isomers. Medications containing d-amphetamine (Dexedrine®, Destrostat®), racemic amphetamine (Adderall®), and *d*-methamphetamine (Deoxsyn®) are listed in Table 2. Illicit methamphetamine and amphetamine products consist mostly of the *d*-isomer but, depending on the starting materials used by clandestine laboratories, significant amounts of the *l*-enantiomer may be present. All these products will give positive amphetamines results by immunoassay as well as analytical true positive results by the standard GC-MS confirmation tests. Enantiomeric analysis can distinguish between the two enantiomers and determine their relative percentages to aid in determining whether the results are clinical false positives *(5)*.

### 4.3. Substances Known to Metabolize to Methamphetamine and Amphetamine

Many medications and substances are known to metabolize to methamphetamine or amphetamine, thus giving analytical true positives (Table 2). If a patient is on one of these medications, the positive result is a clinical false positive. Enantiomeric analysis may be useful in verifying that the positive result was due to the use of a prescription drug. For example, selegiline, a drug used in the treatment of Parkinson's

**Table 2**
**Examples of Amphetamines-Containing products**[a]

| | *Amphetamines-containing Products* |
|---|---|
| Substances known to contain *d*-amphetamine or *d*,*l*-amphetamine | Adderall®<br>Dexedrine®<br>DextroStat® |
| Substances known to contain *d*-methamphetamine | Desoxyn® |
| Substances known to contain *l*-methamphetamine | Vicks Inhaler® |
| Substances known to metabolize to methamphetamine (and amphetamine) | Benzphetamine (Didrex®)<br>Dimethylamphetamine<br>Famprofazone<br>Fencamine<br>Furfenorex<br>Selegiline (Eldepryl®) |
| Substances known to metabolize to amphetamine | Amphetaminil<br>Clobenzorex<br>Ethylamphetamine<br>Fenethylline<br>Fenproporex<br>Mefenorex |

[a] Medical Review Officer Manual for Federal Agency Workplace Drug Testing Programs http://dwp.samhsa.gov/DrugTesting/Level_1_Pages/HHS%20MRO%20Manual%20(Effective%20November%201,%202004).aspx (accessed 11/05/2006).

disease, is metabolized to *l*-methamphetamine (and *l*-amphetamine) without any racemization during metabolism *(7)*. Given the immunospecificity of most amphetamines immunoassay is mostly directed toward *d*-methamphetamine, and given the fact that *l*-methamphetamine and *l*-amphetamine concentrations derived from selegiline metabolism are relatively low, urine specimens of patients on selegiline most likely would not test positive. Should there be a positive initial test, enantiomeric analysis should show the presence of only the *l*-isomer if the patient has taken selegiline *(5)*. Clobenzorex is metabolized to *d*-amphetamine. Therefore, enantiomeric analysis should show only *d*-amphetamine; the presence of the *l*-amphetamine is inconsistent with the sole use of clobenzorex *(8)*.

## 5. COCAINE

Clinical false-positive results for the cocaine metabolite benzoylecgonine have been reported due to cocaine exposure following ear, nose, and throat (ENT) surgery, ophthalmological procedures, skin suturing, and drinking of coca leaf tea.

### 5.1. Extemporaneous Preparations Containing Cocaine

There are no prescription medications that contain cocaine. Extemporaneous preparations containing cocaine, however, are used as local anesthetics in ENT surgery and in ophthalmological procedures. Patients who had had these ENT and ophthalmological procedures tested positive for benzoylecgonine up to 2–3 days following the procedure *(9,10)*.

TAC (tetracaine, adrenalin, and cocaine) is a topical preparation used for surface anesthesia during various surgical procedures. It was used extensively on patients during skin suturing in the emergency department although the popularity of this preparation has waned in recent years. In one report, 78% of the patients who were exposed to TAC tested positive for benzoylecgonine the morning after and some continued to test positive 36 h after the use of TAC *(11)*.

### 5.2. "Health Inca Tea"

In the early 1980s, imported coca leaf tea was marketed under the name "Health Inca Tea." The tea was "de-cocainized coca leaves" but it contained a detectable amount of cocaine *(12)*. An individual who consumed one cup of "Health Inca Tea" had (peak) benzoylecgonine concentration of 1250 ng/ml in the urine specimen collected 7 h after consumption and 117 ng/ml at 29 h *(13)*. In another study by Jackson et al., four males each ingested one cup of "Health Inca Tea" which contained 1.87 mg of cocaine. Positive immunoassay results for benzoylecgonine were observed even 26 h post-ingestion. The maximum benzoylecgonine concentrations in urine ranging from 1400 to 2800 ng/ml were obtained between 4 and 11 h post-ingestion *(14)*. The US Food and Drug Administration has banned the import of this tea into the United States. Therefore, any current "Health Inca Tea" product should not contain any cocaine. South American countries such as Peru and Bolivia have a tradition of drinking medicinal tea made from coca leaf. Public markets there sell loose coca leaves and commercial tea bags made from coca leaves. Therefore, such product may come to other countries illegally or through travellers. A recent British report showed positive benzoylecgonine results in persons 24 h after ingestion of 250 ml of Mate de Coca tea. In the 1980s, de-cocainized Mate de Coca tea was sold extensively in the United States as "Health Inca Tea" *(15)*.

### 5.3. Passive Exposure to Cocaine Smoke

Passive inhalation of cocaine smoke leading to a positive test for benzoylecgonine in adults has not been reported in the literature. Passive exposure to cocaine smoke can result in absorption of cocaine and excretion of a detectable amount of benzoylecgonine into the urine *(16)*. In this study, all urine specimens collected from six individuals following passive exposure to 100 or 200 mg of vaporized cocaine tested negative for benzoylecgonine using the standard 300 ng/ml cutoff. Peak benzoylecgonine concentrations ranged from 22 to 123 ng/ml, and the amount of inhaled cocaine was calculated to be 0.25 mg. In a parallel study, 1 mg of cocaine delivered intravenously to the same study subjects produced cocaine-positive urine specimens. It was estimated that the amount of cocaine absorbed from secondary smoke exceeding 1 mg could result in a cocaine-positive urine specimen *(16)*.

## 6. MARIJUANA

Clinical false-positive results for marijuana can be due to use of prescription Δ9-tetrahydrocannabinoid (THC), secondary inhalation of marijuana smoke, and ingestion of hemp products.

### *6.1. Prescription THC*

Although medical use of marijuana has been approved by two states (California and Arizona), marijuana remains a Schedule 1 drug. A synthetic THC, dronabinol, is available under the trade name Marinol®. It is prescribed for the treatment of nausea and vomiting associated with cancer chemotherapy, appetite stimulation in AIDS patients, and the management of glaucoma. Because Marino® is THC, it is metabolized to △9 THC carboxylic acid (THC-COOH), and the patient if given a drug test will have a clinical false-positive result for marijuana use. The standard drugs of abuse test for marijuana use, based on the detection of THC-COOH, cannot determine whether a positive drug test is the consequence of marijuana or Marinol® use. It has been proposed that Δ9-tetrahydrocannabivarin (THCV, the C3 homolog of THC), a natural component of most cannabis products, can be used as a marker for marijuana use. Because Marinol® is a synthetic product, it does not contain THCV *(17)*. Therefore, the presence of THCV (and its metabolite THCV-COOH) in a urine specimen is an indication that the patient has used marijuana, with or without Marinol®.

### *6.2. Passive Inhalation*

Passive inhalation or exposure to marijuana smoke can produce detectable concentrations of THC-COOH in urine specimens *(18)*. Clinical studies have shown that it is unlikely that passive inhalation taking place in typical social settings could result in a high enough concentration of THC-COOH in urine to produce a positive drug test for marijuana *(19,20)*. The study that showed that passive inhalation indeed resulted in THC-COOH concentrations exceeding standard drug test cutoff was conducted under "unrealistic" conditions *(19)*. Exceeding the standard cutoff limit of marijuana tests is difficult to achieve through passive inhalation *(20)*.

A more recent study indicated that risk of positive test for marijuana metabolite in oral fluid after passive inhalation of marijuana lasted approximately 30 min after exposure *(21)*. Niedbala et al. *(22)* reported in a later study the effect of passive inhalation of marijuana on both urine and oral fluid testing. The authors used high marijuana-content cigarettes. In study 1, four subjects smoked THC mixed with tobacco (39.5 mg THC) in an unventilated eight-passenger van and four volunteers were exposed to passive smoke. In study 2, the four subjects smoked cigarettes containing only marijuana (83.2 mg THC). Oral fluids were collected from both passive and active smokers. In study 1, oral fluid specimens from active and passive subjects were collected for the first hour inside the van and then up to 72 h (passive) or 8 h (active) outside the van. For study 2, all oral fluid collections were made outside the van immediately after cessation of smoking to 8 h. In study 2, all oral fluid samples collected outside the van were negative in passive smokers (THC values, ranging from 0 to 1.2 ng/ml by GC-MS-MS, were below the 2 ng/ml oral fluid THC cutoff proposed by SAMHSA). In study 1, however, positive THC concentrations were detected (peak

value of 7.5 ng/ml) in oral fluid specimens of passive smokers collected inside the van, but THC concentrations quickly declined to below cutoff within 30–45 min. Because in study 2 all oral fluid specimens from passive smoker were collected outside the van and they tested negative, the authors concluded that the positive results in study 1 were due to contamination of oral fluid collection devices by marijuana smoke because the specimens were collected inside the van. None of the urine specimens from passive smokers (both study 1 and study 2) showed any positive result for THC metabolite using immunoassay cutoff at 50 ng/ml or by GC-MS-MS using 15 ng/ml cutoff (THC-COOH concentrations ranged from 2.9 to 14.7 ng/ml) *(22)*. This study further validates that it is unlikely to observe a positive test for urinary THC metabolite after passive exposure to marijuana smoke.

### *6.3. Hemp Products*

Hemp and marijuana belong to the same species, *Cannabis sativa L*, but differ in their cannabinoid content. Therefore, seeds and oil prepared from the hemp plant contain some THC. Several reports have shown that ingestion of food products containing hemp seeds or oil can produce THC-COOH concentrations in urine which cause confirmed positive test results *(23–26)*. Most of these studies were conducted before 1998 when THC content in hemp oil routinely exceeded 50 μg/g. The majority of THC is located on the outside of the seed hulls. Since 1998, the implementation of more thorough seed drying and cleaning before seed processing has considerably reduced THC levels in seeds and oil available in the United States (as low as 5 μg/g in hemp oil) *(27)*. At this low THC residue level, repeated daily ingestion of hemp product did not produce any positive drug test. In one study, Leson et al. reported that consumption of 125 ml of hemp oil (0.6 mg THC) produced THC metabolite level of only 5.2 ng/ml. It would require ingestion of unrealistically high amounts of such products to obtain the amount of THC necessary to produce a positive test *(27)*. In another study, Gustafson et al. *(28)* used seven volunteers (double blind and placebo control) who received 0, 0.39, 0.47, 7.5, and 14.8 mg THC per day. THC doses (hemp oils with various THC concentrations and the drug Marinol®) were administered three times daily for 5 days and urine voids were collected over 10 weeks. The authors reported that at cannabinoid immunoassay cutoff of 50 ng/ml, the mean detection rate was <0.2% during ingestion of two low doses typical of current hemp oil (0.39 and 0.47 mg THC/per day). The authors concluded that, at the 50 ng/ml cannabinoid cutoff, it is possible but unlikely for a urine specimen to test positive after ingestion of manufacturer's recommended dose of low-THC hemp oil. However, with Marinol® therapy, there is a higher likelihood of urine specimen being positive for THC metabolite *(28)*.

## 7. OPIATES

Clinical false-positive results for morphine or codeine can occur due to the use of a medication that contains morphine or codeine, or consumption of food products that contain poppy seeds. A clinical false-positive interpretation also may occur if the presence of hydrocodone or hydromorphone is not recognized as a minor metabolite of codeine and morphine, respectively.

### *7.1. Drug Products Containing Morphine or Codeine*

Many drug products contain morphine or codeine, some of which are non-prescription medications. Some products containing opiates are listed in Table 3.

### *7.2. Poppy Seeds*

It has been well documented that some batches of poppy seeds are contaminated with morphine and codeine and that consumption of poppy seed-containing food items can lead to a positive urine drug test for morphine and codeine. The morphine content, which varies with the source of the poppy seeds, ranges from non-detectable to 965 μg/g of poppy seeds; the codeine content is usually much lower *(29)*. Various studies on the effect of poppy seed ingestion on urine drug test results generally showed that maximum urine morphine and codeine concentrations usually occur during the first 6 h following ingestion, and the maximum morphine concentration in urine specimens collected 12–24 h post-ingestion was <2500 ng/ml. Codeine concentrations were generally <300 ng/ml *(29)*. In one study, the effect of ingesting poppy

**Table 3**
**Examples of Opiates-Containing Products**[a]

| *Drug* | *Prescription products* | *Non-prescription products*[b] |
|---|---|---|
| Codeine | Ambenyl with Codeine®<br>Codimal PH7 Syrup®<br>Fioricet and Codeine®<br>Fiorinal with Codeine®<br>Guiatuss A.C.®<br>Phenaphen with Codeine®<br>Robitussin-DAC®<br>Triacin-C®<br>Tylenol with Codeine | Kaodene with Codeine® |
| Morphine | Avinza®<br>Astramorph PF®<br>Depodur®<br>Duramorph®<br>Kadian®<br>MS Contin Tablets®<br>Oramorph SR®<br>Roxanol®<br>Pareforic® [d] | Donnagel-PG® [c]<br>Infantol Pink® [c]<br>Kaodene with Paregoric® [c,d]<br>Quiagel PG® [c] |

[a] Medical Review Officer Manual for Federal Agency Workplace Drug Testing Programs http://dwp.samhsa.gov/DrugTesting/Level_1_Pages/HHS%20MRO%20Manual%20 (Effective%20November%201,%202004).aspx (accessed 11/05/2006).

[b] Non-prescription products are anti-diarrheal medications. Non-prescription sale is prohibited in some states.

[c] Contain opium.

[d] Paregoric alone is a Schedule III prescription drug, but in combination with other substances is a Schedule V over-the-counter product.

seeds on oral fluid testing for opiates was investigated *(30)*. Four volunteers ate three poppy seed bagels each. Neither morphine nor codeine was detected in any oral fluid specimens. Urine morphine concentrations, however, were found and ranged from 312 to 602 ng/ml. When three volunteers ate one poppy seed bagel and then unlimited amount of poppy seeds in 1 h (volunteer 1, 14.82 g; volunteer 2, 9.82 g; and volunteer 3, 20.82 g), oral fluid specimens tested positive up to 1 h after ingestion at 40 ng/ml cut off (highest morphine concentration being 205 ng/ml). Urine specimens were positive for 8 h *(30)*. In Germany, blood free morphine cutoff in drivers suspected of driving under the influence is 10 ng/ml. Moeller et al. *(31)* measured blood and urine morphine concentrations after consumption of poppy seed products. All five volunteers showed positive opiates urine drug tests (up to 2079 ng/ml morphine by the semi-quantitative Abbott assay, and 147–1300 ng/ml by GC-MS). No blood specimen tested positive for free morphine, but following hydrolysis, total morphine concentration as high as 24 ng/ml was detected *(31)*.

In order to minimize the occurrence of poppy seed-induced clinical false-positive results, the Mandatory Guidelines for Federal Drug Testing Programs increased the initial and confirmation tests cutoff for opiates from 300 to 2000 ng/ml and also required testing of 6-acetylmorphine, the heroin-specific metabolite *(32)*. Additional interpretative criteria of opiates results are included in the Federal Mandatory Guidelines to distinguish positive results due to opiates abuse from ingestion of food items. The proof of heroin use requires the codeine and/or morphine concentration to be $\geq 15,000$ ng/ml, if there is no legitimate medical explanation for the presence of morphine or codeine ($< 15,000$ ng/ml if there is clinical evidence of illegal drug use), or the presence of 6-acetylmorphine. The consumption of poppy seed-containing food items cannot be the explanation for the high morphine or codeine concentration $\geq 15,000$ ng/ml *(6)*.

ElSohly and Jones *(29,33)* have suggested a guideline for interpreting opiate-positive results in order to differentiate sources of the opiates in urine. It has been suggested that the presence of thebaine in urine may be used as a marker for ingestion of poppy seeds. Cassella et al. *(34)* reported that thebaine was detected in the urine of poppy seed eaters, and the concentrations varied from 2.0 to 81.0 ng/ml. However, Meadway et al. *(35)* reported that elimination of thebaine after ingestion of poppy seed products varied widely between different subjects, and absence of thebaine in a urine specimen is not an indication of opiate abuse.

### *7.3. Minor Metabolites of Opiates*

It is important to understand the metabolism of the opiates for proper interpretation, particularly of the minor metabolites: morphine is a minor metabolite of codeine; hydromorphone and dihydrocodeine are minor metabolites of hydrocodone; oxymorphone is a metabolite of oxycodone; and hydrocodone and hydromorphone are minor metabolites found in the presence of very high codeine and morphine concentrations, respectively *(36,37)*. Detection of one of these minor metabolites is an analytical true positive result. These metabolites are themselves opiates that are also drugs of abuse. Therefore, without the full understanding of opiates metabolism, the presence of a minor metabolite (e.g., hydromorphone) in addition to the prescribed medication (morphine) will be perceived as an unexpected finding. The positive result may be mistakenly interpreted as evidence of illicit use of hydromorphone (Dilaudid®), a clinical false positive.

## 8. CONCLUSION

A clinical false-positive result is an analytical true positive result produced by a patient who has been exposed to the drug and who has a valid reason other than illicit drug use for having the drug in the urine. As this is an analytical true positive, successful identification of this positive result as a clinical false positive will have to rely on additional testing (e.g., enantiomeric analysis of amphetamines) by the laboratory and a thorough medical review by a qualified physician.

## REFERENCES

1. Magnani B. Concentrations of compounds that produce positive results. In: Shaw L, Kwong T, eds. The Clinical Toxicology Laboratory, Contemporary Practice of Poisoning Evaluation. Washington, DC: AACC Press, 2001:482–497.
2. Green KB, Isenschmid DS. Medical review officer interpretation of urine drug test results. In: Liu RH, Goldberger B, eds. Handbook of Workplace Drug Testing. Washington, DC: AACC Press, 1995:321–354.
3. Fitzgerald RL, Ramos JM, Jr, Bogema SC, Poklis A. Resolution of methamphetamine stereoisomers in urine drug testing: urinary excretion of R(-)-methamphetamine following use of nasal inhalers. J Anal Toxicol 1988;12:255–259.
4. Cody JT. Determination of methamphetamine enantiomer ratios in urine by gas chromatography-mass spectrometry. J Chromatogr 1992;580:77–95.
5. Cody JT. Important issues in testing of methamphetamine enantiomer ratios in urine by gas chromatography-mass spectrometry. In: Liu RH, Goldberger B, eds. Handbook of Workplace Drug Testing. Washington, DC: AACC Press, 1995:239–288.
6. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration. Medical Review Officer Manual for Federal Agency Workplace Drug Testing Programs. http://dwp.samhsa.gov/DrugTesting/Level_1_Pages/HHS%20MRO%20Manual%20(Effective%20November%201,%202004).aspx. Accessed 11/05/2006.
7. Mahmood I. Clinical pharmacokinetics and pharmacodynamics of selegiline. An update. Clin Pharmacokinet 1997;33:91–102.
8. Cody JT, Valteria S. Amphetamine, clobenzorex, and 4-hydroxyclobenzorex levels following multidose administration of clobenzorex. J Anal Toxicol 2001;25:158–165.
9. Patrinely JR, Cruz OA, Reyna GS. The use of cocaine as an anesthetic in lacrimal surgery. J Anal Toxicol 1994;18:54–56.
10. Bralliar BB, Skarf B, Owens JB. Ophthalmic use of cocaine and the urine test for benzoylecgonine. N Engl J Med 1989;320:1757.
11. Altieri M, Bogema SC, Schwartz RH. TAC topical anesthesia produces positive urine tests for cocaine. Ann Emerg Med 1990;19:577–579.
12. Siegel RK, ElSohly HN, Plowman T, Rury PM, Jones RT. Cocaine in herbal tea. JAMA 1986;**255**:40.
13. ElSohly MA, Standford DF, ElSohly HN. Coca tea and urinalysis for cocaine metabolites. J Anal Toxicol 1986;10:256.
14. Jackson GF, Saddy JJ, Poklis A. Urinary excretion of benzoylecgonine following ingestion of Health Inca Tea. Forensic Sci Int 1991;49:57–64.
15. Turner M, McCrory P, Johnston A. Time for tea anyone? Br J Sports Med 2005;39:e37.
16. Cone E, Yousefnejad D, Hillsgrove MJ, Holicky B, Darwin WD. Passive inhalation of cocaine. J Anal Toxicol 1995;19:399–411.
17. ElSohly MA, Dewit H, Wachtel SR, Feng S, Murphy T. $\Delta^9$-Tetrahydrocannabivarin as a marker for the ingestion of marijuana versus Marinol® : results of a clinical study. J Anal Toxicol 2001;25:565–571.
18. Huestis MA, Cone E. Drug test findings resulting from unconventional drug exposure. In: Liu RH, Goldberger B, eds. Handbook of Workplace Drug Testing. Washington, DC: AACC Press, 1995:289–320.

19. Cone E, Johnson RE, Darwin WD, Yousefnejad D, Mell LD, Paul BD. Passive inhalation of marijuana smoke: urinalysis and room air level of delta-9-tetrahydrocannabinol. J Anal Toxicol 1987;11:89–96.
20. Mule SJ, Casella GA. Active and realistic passive marijuana exposure tested by three immunoassays and GC/MS in urine. J Anal Toxicol 1988;12:113–116.
21. Niedbala S, Karodos K, Salamone S, Fritch D, Bronsgeest M, Cone EJ. Passive cannabis smoke exposure and oral fluid testing. J Anal Toxicol 2004;28:546–552.
22. Niedbala RS, Karodos KW, Fritch DF, Kunsman KP, Blum KA, Newland GA, Waga J, Kurtz L, Bronsgeest M, Cone EJ. Passive cannabis smoke exposure and oral fluid testing II: two studies of extreme cannabis smoke exposure in a motor vehicle. J Anal Toxicol 2005;29:607–615.
23. Alt A, Reinhardt G. Positive cannabis results in urine and blood samples after ingestion of hemp food products. J Anal Toxicol 1998;22:80–81.
24. Costantino A, Schwartz RH, Kaplan P. Hemp oil ingestion causes positive urine tests for $\Delta^9$-tetrahydrocannabinol carboxylic acid. J Anal Toxicol 1997;21:482–485.
25. Fortner N, Fogerson R, Lindman D, Iversen T, Armbruster D. Marijuana-positive urine test results from consumption of hemp seeds in food products. J Anal Toxicol 1997;21:476–481.
26. Struempler RE, Nelson G, Urry FM. A positive cannabinoids workplace drug test following ingestion of commercially available hemp seed oil. J Anal Toxicol 1997;21:283–285.
27. Leson G, Pless Petra, Grotenhermen F, Kalant H, ElSohly MA. Evaluating the impact of hemp food consumption on workplace drug tests. J Anal Toxicol 2001;25:691–698.
28. Gustafson RA, Levine B, Stout PR, Klette KL, George MP, Moolchan ET, Huestis MA. Urinary cannabinoid detection times after controlled oral administration of delta9-tetrahydrocannabinol. Clin Chem 2003;49:1114–1117.
29. ElSohly MA, Jones AB. Origin of morphine and codeine in biological fluids. In: Liu RH, Goldberger B, eds. Handbook of Workplace Drug Testing. Washington, DC: AACC Press, 1995:225–238.
30. Jackson GF, Saddy JJ, Poklis A. The determination of morphine in urine and oral fluid following ingestion of poppy seeds. J Anal Toxicol 2003; 27: 449–452.
31. Moeller MR, Hammer K, Engel O. Poppy seed consumption and toxicological analysis of blood and urine samples. Forensic Sci Int 2004;143:183–186.
32. Department of Health and Human Services, Substance Abuse and Mental Health Services Administration. Federal Register Notice Changing the Opiate Testing Cutoff Concentrations (effective December 1, 1998). http://dwp.samhsa.gov/FedPgms/Files/FR_Notice_Opiate_Testing_Cutoff_Concentrations.aspx. Accessed 11/05/2006.
33. ElSohly HN, ElSohly MA, Standford DF. Poppy seed ingestion of opiate urinalysis: a closer look. J Anal Toxicol 1990;14:308–310.
34. Cassella G, Wu AQH, Shaw BR, Hill DW. The analysis of thebaine in urine for the detection of poppy seed consumption. J Anal Toxicol 1997;21:376–383.
35. Meadway C, George S, Braithwaite R. Opiate concentrations following the ingestion of poppy seed products-evidence for poppy seed defense. Forensic Sci Int 1998;96:29–38.
36. Oyler JM, Cone E, Joseph RE, Jr, Huestis MA. Identification of hydrocodone in human urine following controlled codeine administration. J Anal Toxicol 2000;24:530–535.
37. Cone E, Heit HA, Caplan Y, Gourly D. Evidence of morphine metabolism to hydromorphone in pain patients chronically treated with morphine. J Anal Toxicol 2006;30:1–5.

# 22 Providing Expert Witness for Alcohol and Positive Drugs of Abuse Test Results

*Andrea Terrell, PhD, William Clarke, PhD, Michael Evans, PhD, and Jennifer Collins, PhD*

***Contents***



**Summary**

Often in judicial trials, employment arbitration or administrative hearings positive drug tests are a factor in the proceedings. In these cases, it is important to consider the validity, or lack of validity, of the drug testing results. An expert witness in these instances is a laboratory professional who has the expertise to render an opinion regarding the validity of test results based on the information provided to them as well as information from the scientific literature. Depending on the situation, the expert's background will be in forensic or workplace drug testing. It is important for the expert witness to be familiar with testing procedures and potential interferences, the various settings where their testimony may occur, possible alternative explanations for positive results and how their testimony may be used. This chapter will discuss venues for expert testimony, provide a description of what an expert witness is, discuss preparation for proceedings where expert testimony is needed and review potential alternative explanations for positive drug test results.

**Key Words:** Drugs of abuse; expert witness; MRO; judicial trials; validity.

## 1. INTRODUCTION

As an expert witness, one may encounter a variety of courtroom situations depending on the type of trial and the type of case. These will be discussed in this chapter along with what is expected from an expert witness in a trial or administrative hearing.

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## *1.1. Forensic Testing*

### 1.1.1. Types of Trials

There are three basic types of trials: jury trials, bench trials and administrative hearings. In a jury trial, the attorneys present their case before a panel of individuals in the presence of a judge. When making their cases, attorneys often attempt to appeal to the emotions of the jury members, and as a result trials can tend to get dramatic. Bench trials do not involve a jury. Rather, the judge is the jury, eliminating the need for a layperson audience. Bench trials are more straightforward than jury trials, as judges do not allow for dramatization on the part of the attorneys. Administrative hearings are more informal and are often utilized in family services and employment termination proceedings.

### 1.1.2. Types of Cases

There are two types of court cases: criminal cases and civil cases. In a criminal case, the government (prosecution) is attempting to convict an individual (defendant) for committing wrongful and illegal acts against another party. If the defendant is found guilty, the judge's sentence may include probation, community service, psychiatric/medical treatment and/or prison time. In order to be convicted, guilt must be proven beyond a reasonable doubt. Reasonable doubt refers to the level of certainty that the judge or jury needs to have before finding an individual guilty *(1)*. This doubt may have arisen from the evidence or lack of evidence. If, after carefully considering all of the evidence or lack of it, a reasonable person still has doubt about the defendant's guilt, then that individual cannot be considered guilty.

Civil cases are reserved for situations in which two parties (a plaintiff and defendant) are tangled in a dispute and rely on the courts to resolve the issue. The burden of proof is lower. Neither party face the risk of jail time, and the outcome is typically financial.

## *1.2. Workplace Drug Testing*

Providing expert testimony in workplace drug testing cases bears many similarities to that described for forensic and criminal cases; however, there are some important differences. While workplace cases can be tried in civil proceedings, they are more often than not adjudicated in a less formal setting. The two most common formats are arbitration and administrative hearings.

### 1.2.1. Arbitration

Arbitration or "alternative dispute resolution" is defined as the submission of a dispute to one or more impartial persons as an alternative to the judicial system (judge or jury). The arbiter is presented with evidence at a formal hearing and a decision is rendered based on the evidence presented. The resulting decision is usually final and binding *(1)*. Arbitration may be used to resolve many types of employment disputes including wrongful termination and sexual harassment and discrimination. In the context of workplace drug testing programs, they are most often encountered as a part of grievance procedures defined in collective bargaining agreements. There are defined federal rules for conducting arbitration proceedings, and some states have specific statutes in that regard *(2)*.

While less formal than courtroom proceedings, arbitrations are conducted in accordance with defined protocols. The arbitrator is agreed upon by the parties. The participants may be represented by attorneys or designated representatives and witnesses relevant to the dispute may appear. Like trials, whether or not the witnesses are present during the entire proceeding or are sequestered varies and may not be determined until just prior to initiation of the hearing. Generally, the format follows standard courtroom protocol in that each side presents evidence and testimony from relevant witnesses who are subject to direct questioning and cross-examination. Witnesses testify under oath, and the proceedings are recorded. Rules for discovery and presentation of evidence are not as rigid as litigation, and the arbitrator may allow presentation of any information deemed relevant to the issues under dispute. Arbitration decisions are rendered by the arbitrator at a later date.

As an expert witness in an arbitration proceeding, one must be "qualified" as having specialized knowledge by experience, education and/or skill that is necessary to the understanding of the issues in the case. Most often, the curriculum vitae (CV) serves as documentation of experience and qualifications of the expert and will be offered as evidence. As in court proceedings, it is extremely important that the CV is up-to-date and information presented is accurate, because if accepted as an expert this document will be entered into the permanent record and may be discoverable in future cases.

#### 1.2.2. Administrative Hearings

Administrative hearings are also common in workplace drug testing cases, in the context of both wrongful termination and unemployment compensation proceedings. Like arbitrations, administrative hearings tend to be less formal proceedings, and the format and content varies depending on the employer and the locale. It is not uncommon, particularly in unemployment compensation hearings, for the witnesses to appear by telephone rather than in person. This is generally determined by rules of the jurisdiction, the discretion of the hearing officer and the willingness of both parties to accept testimony over the telephone.

Administrative hearings are generally structured in a manner similar to arbitration, witnesses testify under oath, relevant evidence is presented and the hearing officer renders a decision at a later date. In unemployment compensation hearings, it is relatively unusual for the individual to have legal representation, and questions may be posed to the expert witness by the individual challenging the denial of compensation.

## 2. WHAT IS AN EXPERT WITNESS?

### *2.1. Participants in the Judicial System (Forensic Testing)*

In the case of a trial, there are two stories: one told by the prosecution and another as interpreted by the defense. Attorneys from both sides present their respective stories in a persuasive manner. This is done by enlisting witnesses to bring the details of each story to life. At the end of the trial, it is often up to the jury but sometimes a judge to determine the outcome of a case.

### 2.1.1. Attorneys

In a typical court case, there are two types of attorneys: prosecution and defense (in civil cases there will be a plaintiff's attorney instead of a prosecutor). Prosecutors are elected or appointed on behalf of the state to convict an individual or organization (known in court as the defendant), and defense attorneys serve as advocates for the defendant. In criminal cases, the prosecution is charged with meeting the burden of proof, that is, proving beyond a reasonable doubt that the defendant is guilty of committing the crime in question. They must provide evidence confirming the defendant's motive, means and/or opportunity to carry out the crime.

By comparison, a defense attorney's goal is to prove that reasonable doubt exists. The defense attempts to create reasonable doubt by raising doubts through the prosecution's argument, discrediting evidence and testimony and highlighting inconsistencies that indicate the innocence of the defendant or even the guilt of another person.

### 2.1.2. The Jury

In the United States, the US constitution guarantees that every citizen has the right to a fair trial. This includes being judged by a jury of one's peers. In a criminal trial, it is the jury that weighs the evidence, testimony and general arguments in order to reach a decision about the defendant's guilt or innocence. The members of a jury are unbiased; therefore, attorneys from both sides spend a great deal of time interviewing potential candidates, weeding out those with prior knowledge or preconceived opinions about any aspect of the case.

It is the job of an expert witness to educate the jury about the science involved in a case and provide an educated opinion as to what the science implies. The expert witness must also ensure that neither party twists or misconstrues scientific data for their own gain. Attorneys are advocates for their own agenda; thus, the expert witness is the only advocate of science. The expert witness should keep in mind that jury members come from a variety of social, economic and educational backgrounds and most of them will possess little more than a basic understanding of grade school science. Therefore, testimony should be presented in a manner that can be understood and used by the general public.

### 2.1.3. Witnesses

In every case, both the prosecution and defense will enlist a variety of witnesses to support their arguments; the order of the witnesses depends on how their testimony relates to the logistics of the case. There are two different types of witness: fact witness and expert witness. Fact witnesses are called to testify to actions they personally performed or observed and are often the first witnesses to be called to the stand, as their testimony lays the foundation for an attorney's case. Laboratory technicians or bench-level chemists are often called into court as fact witnesses to testify on how they personally handled a specimen, especially if the chain of custody for a specimen is called into question.

Fact witnesses should not offer opinions based on the results of laboratory analysis. When the data require more explanation or interpretation than a fact witness is qualified to provide, an expert witness is necessary. Expert witnesses are called to provide

opinions based on their scientific training, knowledge and experience and are essential in helping interpret complex technical information.

### *2.2. Witnesses for Workplace Drug Testing*

In workplace drug testing cases, the arbitration or hearing is usually a result of punitive employment action taken as a result of a positive finding. It is the employers' responsibility to provide evidence that the action taken was consistent with their routine policies and procedures. The expert witness provides testimony in support of the drug testing results, that the testing was performed accurately and in accordance with laboratory standard operating procedure as well as in accordance with any applicable regulatory requirements.

### *2.3. Who is an Expert?*

An expert is defined as an individual having special skill or knowledge in a particular field, a specialist or authority *(3)*. Based on that definition, individuals with a solid educational background and a reasonable amount of specialized training and experience in toxicology can qualify as expert witnesses. In addition to education and training, an understanding of applicable regulatory requirements, relevant peer-reviewed literature and familiarity with industry standards is required in workplace drug testing cases.

## 3. HOW TO PREPARE AS AN EXPERT WITNESS

At the heart of every case or hearing is the evidence as presented in the proceeding. An attorney or arbiter will draw upon witness testimony, business records, physical objects and other tangible items that corroborate with his/her interpretation of the case. In forensic cases, if any such item is not accepted into court as evidence, it ceases to exist, at least in the eyes of the judge and jury. Therefore, the easiest way to damage a case is to block the admission of evidence. Preparation for a workplace hearing should include a full review of all data and documentation associated with the sample or samples in question. It is common to submit a copy of the laboratory "litigation package" as evidence in the hearing. While individual laboratories have different formats for these data packages, they generally contain accurate copies of external and internal chain of custody documents, analytical data generated and the final report with any associated correspondence.

Preparatory review for a proceeding should include tracking of the chain of custody to ensure that it is complete, review of all data and any corrective actions taken, if any, and review of the standard operating procedures in effect in at the laboratory when the sample(s) was processed. Depending on the proceeding, the expert witness may be asked to explain the litigation package page by page before it is tendered and/or accepted as evidence.

In addition to the credentials of an expert, the credentials of the laboratory are also important. This includes the current licensure and certifications as well as participation in external proficiency programs. The expert witness should be aware of any state-specific requirements that apply, both in the state where the laboratory is located and in the state where the employer is located. This information may be obtained from the attorney or employer contact, published state statutes or published summaries of state

drug testing regulations *(4)*. Because many states adhere to or model programs after the federal workplace drug testing guidelines, familiarity with those documents and applicability is necessary. The most current federal workplace drug testing guidelines can be accessed over the internet *(5)*.

### *3.1. Business Records*

Business records are categorized as documents generated during the normal course of a corporation's work day; for a laboratory or hospital, key business records include chain-of-custody forms, raw data and laboratory reports. The testimony provided by expert witnesses is often used as a means for attorneys to enter these records into evidence. There is a common perception that records provided by forensic laboratories fare better in court than those produced by hospital laboratories due to stricter chain-of-custody processes in the forensic laboratory. While it is true that the two facilities follow different processes for handling important specimens and documents, each course of action is specifically tailored to meet the needs of its business and is valid in its own right. Hospitals are charged with making life-and-death decisions every day. There is no reason to think their standards are less rigorous than the standards of a forensic laboratory.

### *3.2. Qualification of the Expert*

The testimony of an expert witness plays a crucial role in the outcome of a trial; therefore, it is important that an attorney distinguishes the witness as an expert in their field of interest. Experts whose credentials are not properly established in court run the risk of having their entire testimony stricken from the record and the collective mind of the jury.

It is important for the expert to discuss his or her qualifications with the attorney before the trial. The CV should be reviewed beforehand so that notable projects, coursework, published works, teaching experience, professional activities, awards and so on can be highlighted for the attorney. It is important to remember that jury members are not the only ones who lack in scientific training. This is often true of the attorney. It is the responsibility of the expert to educate the attorney and to make sure the right questions are being asked. The expert should never testify to facts that fall outside the realm of the individual's training.

### *3.3. Qualification of Opinion*

One of the biggest distinctions between fact witnesses and expert witnesses is literally a matter of opinion. While a fact witness's testimony is bound to actions and observations the individual personally performed or observed, an expert witness is allowed and even encouraged to use scientific data to form educated opinions about what the data might indicate—such as whether a potential interference in the assay is relevant to the case or hearing. However, even the opinion of an expert may be tightly regulated in an attempt to keep "junk science" out of the courtroom. The Daubert Standard was set by the US Supreme Court in 1993 to exclude the presentation of unqualified evidence to a jury, ruling all testimony must be relevant to the facts of the case and reliable, that is, grounded in the scientific method. Prior to the Daubert ruling,

criteria against which to measure scientific evidence were outlined in *Frye v. United States* cite in 1923. In this case, the court upheld a prior court's decision to refuse the acceptance of a test and the expert testimony relating to the test. The court concluded that new or novel scientific evidence, or the novel application of scientific principles, must have been generally accepted in the relevant scientific community before it can be admitted into evidence. The test in question in *Frye v. United States* was determined not to have been generally accepted in the relevant scientific community.

### *3.4. Basis for Opinions*

There is a variety of literature available to assist the expert witness in formulating a professional opinion. When offering opinion in a proceeding, the opposing counsel or party will challenge the expert to base that opinion on fact. Therefore, it is important to understand the type of materials available and how they may be of service to the expert in preparing to be a witness. Relevant literature review should include technical inserts or fact sheets provided by assay and/or instrument vendors, peer-reviewed journal articles about analysis, and interpretation of results as well as trade publications. The latter are useful for tracking trends and current issues such as specimen validity testing and cases of interest. Knowledge of assay limitations, cross-reactivity, and potential interferences is essential.

#### 3.4.1. Case Reports and Controlled Studies

Oftentimes in forensic cases, case reports are crucial in determining the cause of death. Such reports are based on data for a single individual and provide information from a retrospective point of view. This is different from a controlled study, which can take a prospective or retrospective approach to generate statistically relevant data. A number of participants are recruited for carefully controlled experiments that attempt to identify current sociological and/or scientific patterns and trends.

Both documents can be equally important to a case, though in the forensic realm controlled studies may be few and far between. Case reports may be all an expert witness can rely on. A laboratory can test a newborn's meconium to determine if the mother abused drugs during pregnancy and provide a case report stating that fact, but it is obviously unlawful and unethical to conduct a controlled study on expecting women in hopes of quantifying the harm cocaine inflicts on fetuses in utero.

#### 3.4.2. Peer-Reviewed and Non-Peer-Reviewed Documents

At one point or another during their career, many scientists publish their original work in peer-reviewed scientific journals. These publications are held in high esteem due to rigorous review and approval processes set in place by the editors and reviewers of the respective journal. Once the scientific findings are deemed sound by a panel of peers, the manuscript is accepted for publication. The processes alone are not enough to guarantee truth, but they do provide a strong foundation for labeling an author's work as valid.

When it comes to court proceedings, it is in the expert's best interests to rely exclusively on peer-reviewed works if possible over those lacking in similar checks and balances. While www.wikipedia.com offers a wealth of information about a number of scientific topics, there are no safeguards to ensure that the information is credible.

However, it should not be written off as "junk science," either. A much broader body of work exists in non-peer-reviewed material, and such articles often reflect the current state of the science as seen through the eyes of the writer, usually an expert in the field. But when participating in a trial, the expert is providing testimony that will be used to sway the outcome, it is imperative the information provided be scientifically sound.

**3.4.2.1. A Note on Peer-Reviewed Works.** When referring to a peer-reviewed work in court, the expert must be sure they are referencing the official results of a study and not the author's discussion of those results. While it is perfectly acceptable to agree with an expert's opinion, it is unacceptable to base an opinion entirely on somebody else's opinion.

#### 3.4.3. Literature Totality

It is the duty of the expert to study literature from a variety of sources and weigh each piece against a common body of knowledge to ensure the concluding opinion formed is based in legitimate science. This is especially true when the expert testifies in court. In order for the expert's opinion to be taken seriously, the expert must be able to support it with articles and data that are held in high regard by expert's colleagues, peers, and discipline in general. In other words, if 20 studies were conducted on the harmful effects of propoxyphene and 19 of them reach the same conclusion, the expert must take that into consideration when forming their opinion, even if an attorney wants them to argue for the results of the single contradictory study.

#### 3.4.4. Impeachment

Expressing an expert opinion in court is not always easy. Just as scientists are trained to manipulate molecules and chemicals, attorneys are trained to manipulate words. Opposing counsel will do its best to confuse the expert witness with clever rephrasing and added emphasis. They will put words in the expert's mouth and compare current statements to testimony provided 10 years ago.

In these situations, comprehensive experience is the saving grace of an expert. Expert opinion is based on the totality of expertise. Data can be wrong, articles can be wrong, but expert opinion can *never* be wrong. Opposing counsel should be reminded that expert opinion is based on materials provided to the witness about the case. If that material includes inaccurate or incomplete information, it will obviously affect the expert's opinion. If new facts are presented, the expert is well within their rights to request time to examine the evidence and re-evaluate their stance.

### *3.5. Pre-Trial Documents for Experts in Forensic Testing*

The job of an expert witness begins before the person even appears in a courtroom, as they prepare and review paperwork that will be entered into evidence. Here are just some of the activities in which an expert may be asked to participate.

#### 3.5.1. Written Reports

An expert may be asked to prepare written reports of his or her opinions, which may be useful in settling a case in lieu of going to trial. In it, the expert will explain

his or her credentials and experience as it relates to the case and summarize significant facts that contributed to the forming of expert opinion regarding the case. Official documents that relate to their training and the specifics of the case should also be included, including but not limited to a CV for the expert witness, police and autopsy reports, depositions, test results, and letters and e-mails divulging important updates or developments.

It is important to find out whether or not the opposing party has an expert witness and if that witness has prepared a written report. If the answer is yes, the expert should request a copy and allow plenty of time to analyze it prior to submitting his or her own report (if possible), or before the start of the trial or hearing. Anticipation of possible questions or issues that might be raised during the hearing is also useful in preparation. The attorney or employer representative may have information in that regard that might assist the expert witness.

In addition, it is common to be asked questions about test reliability and accuracy and possible human errors leading to erroneous results. These questions are not always easy to answer; while the testing process is reliable, one cannot ensure that no one will ever make an error. When an expert witness formulates answers to these questions, it is important to keep in mind the difference between what is possible (anything) versus what is likely or probable. Multiple tests on separate aliquots are performed to minimize the possibility of error. As is true in any proceeding, the opinions rendered by the expert witness should be based on facts and scientific evidence rather than conjecture or anecdotal information.

### 3.5.2. Discovery Depositions

Often ordered by the opposing counsel, a discovery deposition is designed to present both sides of a dispute with the information that will be presented in a trial, thus eliminating the potential for any *Perry Mason*-like surprises. Depositions are taken under oath, generally in the presence of both attorneys and a court reporter and should be prepared for in the same manner that one prepares for court. If an expert witness is called to give a deposition, the person should be sure to bring any documents in his or her possession that relate to the case.

### 3.5.3. Affidavits

An affidavit is a written statement of the facts that is confirmed under oath or by affirmation and is used to document the personal observations, recollections, and actions but not opinions or expert witnesses. It may be executed before a person such as a notary public and can often be used in lieu of courtroom testimony. There are many types of affidavits, one of the most common being the "custodian of records" affidavit, in which the executor acknowledges to being the custodian of the physical evidence (such as a blood or urine specimen) in question.

### 3.5.4. Written Interrogatories

Written interrogatories are the written equivalent of discovery depositions, as both features a series of questions presented by opposing council. In this instance, however, the attorney is the author and will rely on the written report from the witness to answer

questions relating to experience and opinion of the witness. The witness may be asked to review the document before it is submitted to the court.

#### 3.5.5. Subpoenas and Subpoena Duces Tecum

Subpoenas are official summons issued by the court and provides notification that the expert is expected to appear in court. It includes general information about the case, when and where the expert will testify, and the contact information of the attorneys who requested the subpoena. Experts are often presented with an additional subpoena called the *subpoena duces tecum*, which requires the witness to produce the documents that support the expert opinion. Failure to respond to a subpoena is considered contempt of court. If an expert is unable to testify on the date or time indicated, the expert must contact the counsel and the court immediately to inquire about rescheduling. Failure to comply without the court's approval may result in fines, community service, or even a warrant for arrest.

## 4. DEFENSES OF POSITIVE RESULTS

In an employment-related matter, it is likely that the individual has denied any recent illegal drug use. The explanations for laboratory results that infer otherwise are varied and range from laboratory and/or collector errors to creative means by which the compounds were unknowingly ingested. An expert witness should be prepared to provide scientific data to support the accuracy of the results as well as to counter common perceptions and misconceptions about the testing process and interpretation of results.

As the testing process begins at the collection site, suggestions of sample mix-ups may be directed both at the collection site and at the laboratory. A representative of the collection process may be called as a witness to attest to the validity of the collection. As the expert witness from the laboratory, you can only attest to the process followed after the specimen is received at the laboratory. Recalling specific details about handling and testing individual specimens is not required because utilization of laboratory automation, bar coding of specimen labels and written standard operating procedures are routinely utilized by laboratory personnel to ensure the integrity of the overall process of laboratory operation.

### *4.1. Marijuana*

A commonly encountered area for confusion is the correlation between initial screening cutoffs, confirmation cutoffs, and positive results, particularly when the specimen is positive for cannabinoids (THC). It is not unusual to hear the assertion that a laboratory result of 35 ng/ml Carboxy-THC should not have been reported as positive because the screening cutoff is 50 ng/ml. A brief description of cannabinoid metabolism and the differing specificities between immunoassay (total cannabinoids) and gas chromatography/mass spectrometry (GC/MS) (the carboxy-THC metabolite) provide the basis for an appropriate response to these concerns.

Passive exposure is probably the most commonly proposed explanation for a positive urine drug test result. As is true with tobacco products, inhalation of secondary marijuana smoke can produce measurable concentrations of Carboxy-THC in the urine

of non-smokers. This phenomenon was the subject of several studies in the 1970s and 1980s, a comprehensive review of the literature was published by Cone and Huestis in 1989 *(6)*. Those studies established the following data useful in refuting the "passive inhalation defense": the amount ingested depends on the concentration achieved in the room air, that is, large venues such as concerts and parties are not consistent with production of positive urine samples, and use of a 50 ng/ml initial immunoassay cutoff practically eliminates passive inhalation as a reasonable explanation for a positive test result.

While the data produced by these studies have been fairly well circulated, "passive inhalation defenses" are still commonly encountered by Medical Review Officers (MROs) in their conversations with donors and are still presented during arbitrations and administrative hearings. In some cases, there are some interesting "twists" to the story. For example, a landlord who rents property to marijuana-smoking tenants is passively exposed to marijuana smoke while performing household repairs or an individual is passively exposed to cannabis via "exchange of bodily fluids" with a spouse.

In addition to passive exposure which may also be classified as "knowing but passive," unwitting exposure is sometimes used as a defense. This would include consumption of foodstuffs containing marijuana (e.g., brownies or cookies), dietary supplements (hemp oil), and cocaine in herbal teas. Oral bioavailability and activity of these drugs varies by compound and the vehicle by which they are ingested, but the consumption of drugs in food products can lead to pharmacological effects and excretion of detectable amounts of drugs/metabolites in urine *(6)*. The Controlled Substances Act makes it unlawful to possess or distribute controlled substances without a license, and the Drug Enforcement Agency has issued rules clarifying the status of hemp products that cause THC to enter the human body as illegal, making active ingestion of these products a violation of federal law *(7,8)*. For the expert witness, there is scientific data available regarding the pharmacokinetics and pharmacodynamics of these compounds after oral administration or consumption that can be used in formulating an opinion. Whether the ingestion was "unknowing" and whether unwitting ingestion is an acceptable defense is a question subject to the arbiter's or hearing officer's decision.

### *4.2. Methamphetamine*

Anecdotal information about so-called false positive test results is easy to access using the internet. While much of the information is unreliable, some is based in fact, for example, known cross-reactivity of over-the-counter sympathomimetic amines with immunoassays for amphetamine and methamphetamine. However, the information is incomplete and usually fails to point out that interference in initial tests is resolved by confirmation testing. In addition, immunoassay specificity varies by manufacturer and product line. Review of current package inserts and cross-reactivity data will assist in clarifying these issues (see Chapter 20).

Use of nasal inhalers is another commonly encountered explanation for a positive result that is accurately based but rarely applicable. L-methamphetamine has been demonstrated to exhibit weak central nervous system action but significant peripheral sympathomimetic effects and is thus a component in several over-the-counter nasal

inhalers. Unlike its mirror-image isomer, d-methamphetamine, it is not a scheduled drug. While most initial immunoassay tests have only limited cross-reactivity to the l-isomer, it is possible for immunoassay positives to result from inhaler use. In addition, conventional GC/MS assays for amphetamines do not separate the optical isomers of amphetamine and methamphetamine. As a part of routine verification of positive methamphetamine results, many MROs request chiral methamphetamine analysis prior to issuing a final determination. Greater than 99% of the time, the results indicate that the methamphetamine present is predominately d-methamphetamine.

### *4.3. Opiates*

Poppy seed consumption leading to positive morphine results is another "false positive" issue described by the lay press. From a scientific perspective, however, these are true positive results. Poppy seed paste and seeds are derived from the opium poppy, and detectable levels of codeine and morphine may be found when seeds are analyzed and in urine samples after consumption of poppy seed-containing food (19–12). Many workplace drug testing programs currently utilize a 2000 ng/ml initial immunoassay cutoff for opiates to reduce the frequency of low level opiates potentially attributable to poppy seed consumption. There is ample scientific literature that describes both analytical and interpretive issues that arise that can be reviewed and utilized to clarify this issue if it arises.

### *4.4. Alcohol*

Microorganisms existing in blood or urine samples may produce ethanol via fermentation of sugars that are present in the specimen. The mechanism of post-collection fermentation in a urine or blood specimen is the same mechanism that is exploited to produce alcoholic beverages. The microorganisms may be introduced to the sample through non-sterile collection procedures or if the donor has a genitourinary infection, commonly *Candida albicans*. The likelihood of post-collection formation of ethanol through fermentation goes up during warm summer months, as samples may sit at outdoor ambient temperatures during their transport to the laboratory. Similar to poppy seed ingestion resulting in a positive test for morphine, a positive alcohol result in a sample that underwent fermentation is a true positive result. Samples can be checked for glucose by a simple "dipstick" test. The medical history of the donor should also be investigated.

## 5. CONCLUSIONS

In both forensic and workplace drug testing, an expert witness is needed to render an opinion regarding the validity or lack of validity for laboratory results. In summary, there are seven important things regarding a trial and expert witness.

1. Establish expertise in the field: The point of sharing an expert's background with a judge or jury is to inform them how the expert is qualified to advocate for the science involved in a particular case.

2. Recognize the limits of expertise: Equally important as sharing expert qualifications with a judge, jury, or arbiter is narrowing the scope of the expert witness. It is important to avoid offering opinions on subjects that fall outside the realm of the expertise of an expert witness.
3. "Garbage in, Garbage out": If the expert witness is presented with inaccurate or incomplete information during the course of a case, it is undoubtedly going to affect the opinion of the expert witness. If opposing counsel tries to dismiss the opinion as faulty, it is important to remind them the opinion is based on the totality of expertise. If the defense wants to introduce new facts for consideration, the expert should be given the proper time to review the new documents.
4. Avoid being referred to as a "hired gun": The last thing an expert wants to do is sacrifice his or her professional reputation to make a quick profit. There are a number of experts who will modify their opinions to fit the needs of an attorney, but it is not wise to do it because of possibility of loss of reputation as an expert witness for future cases.
5. Stick to the science and the expert opinion will never be impeached: When caught in the middle of cross-examination, it is important to remember that opposing counsel's sole objective is to damage the expert's credibility in the eyes of the jury. It is vital to carefully listen to each question before providing an answer, and let the science be the only guide.
6. Stay one step ahead of opposing expert witnesses: An expert witness should know the experts hired by opposing council and the other plans to discredit his or her testimony. Preparation to effectively address any argument that is introduced is an important part of rendering and effective opinion.
7. Consultation fee for an expert: It is expected that experts are compensated for the time spent preparing for trial. If the opposing attorney asks if the expert is being paid for their services, it is important to be honest. However, equally important is to make the distinction that it is the expert's time being paid for, not the actual testimony.

In addition, there are several challenges for expert witnesses on the horizon. In workplace proceedings where drug testing results are the basis for employment action, there are often administrative factors that are being challenged as well, the drug testing result is only a part of the process. The regulatory aspects of the federal workplace urine drug testing program provide a widely accepted basis for testing, and there is substantial precedent validating the testing methodologies and commonly applied administrative cutoffs. If the technical aspects are sound, there may be only a perfunctory challenge to the results. However, as newer, alternative matrices and technologies become more widely utilized in drug testing, there will likely be a renewed challenge to the expert witness to establish the sound scientific and technical basis for acceptance of the new matrix and technology.

## REFERENCES

1. American Arbitration Association, http://www.adr.org.
2. Federal Arbitration Act – Title 9, U.S.C.
3. Random House Unabridged Dictionary, Random House, Inc. 2006.
4. de Bernardo, MA, Nieman, MF. Guide to State and Federal Drug-Testing Laws, 14th Edition. Institute for a Drug Free Workplace, 2006.
5. http://dwp.samhsa.gov, http://www.dot.gov/ost/dapc/.

6. Cone, EJ, Huestis, MS. Urinary excretion of commonly abused drugs following unconventional means of administration. Forensic Science Review 1989; 1(2):121–138.
7. Controlled Substances Act – 21 U.S.C. 811.
8. 21 CFR Part 1308, March 21, 2003
9. ElSohly, MA, Jones, AB. Morphine and codeine in biological fluids: Approaches to source differentiation. Forensic Science Review 1989; 1(1):13–22.
10. Struempler, RE. Excretion of codeine and morphine following ingestion of poppy seeds. Journal of Analytical Toxicology 1987; 11:97–99.
11. Zebelman, AM, Troyer BL, Randall GL, Batjer JD. Detection of morphine and codeine following consumption of poppy seeds. Journal of Analytical Toxicology 1987; 11:131–132.
12. Trafkowski, J, Madea, B, Musshoff, F. The significance of putative urinary markers of illicit heroin use after consumption of poppy seeds products. Therapeutic Drug Monitoring 2006; 28: 552–558.

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