Cell Tissue Res (2015) 360:87–107
DOI 10.1007/s00441-014-2093-4

REVIEW

Autoradiography techniques and quantification of drug distribution

Eric G. Solon

Received: 12 September 2014 / Accepted: 11 December 2014 / Published online: 22 January 2015

Abstract The use of radiolabeled drug compounds offers the most efficient way to quantify the amount of drug and/or drug-derived metabolites in biological samples. Autoradiography is a technique using X- ray film, phosphor imaging plates, beta imaging systems, or photo-nuclear emulsion to visualize molecules or fragments of molecules that have been radioactively labeled, and it has been used to quantify and localize drugs in tissues and cells for decades. Quantitative whole-body autoradiography or autoradioluminography (QWBA) using phosphor imaging technology has revolutionized the conduct of drug distribution studies by providing high resolution images of the spatial distribution and matching tissue concentrations of drug-related radioactivity throughout the body of laboratory animals. This provides tissue-specific pharmacokinetic (PK) compartmental analysis which has been useful in toxicology, pharmacology, and drug disposition/patterns, and to predict human exposure to drugs and metabolites, and also radioactivity, when a human radiolabeled drug study is necessary. Microautoradiography (MARG) is another autoradiographic technique that qualitatively resolves the localization of radiolabeled compounds to the cellular level in a histological preparation. There are several examples in the literature of investigators attempting to obtain drug concentration data from MARG samples; however, there are technical issues which make that problematic. These issues will be discussed. This review will present a synopsis of both techniques and examples of how they have been used for drug research in recent years.

Keywords Quantitative autoradiography · Microautoradiography · Imaging · ADME · Drug concentration

E. G. Solon (✉)
QPS, LLC, 110 Executive Drive, Suite 7, Newark, DE, USA
e-mail: eric.solon@qps.com

Introduction

The determination of drug concentration, pharmacokinetics (PK), and distribution to tissues and cells is of critical importance for researchers developing drugs to treat various diseases. International drug regulatory authorities also require pharmaceutical companies to describe and characterize the disposition of their drug entities in humans before they can be used to treat diseases. Currently, the use of radiolabeled drug compounds and autoradiography in animal studies offers a high-resolution solution to determine drug distribution at the macro and micro levels. Autoradiography tissue distribution studies, which are part of a set of experiments known as absorption, distribution, metabolism and excretion (ADME) studies, are performed to understand the disposition of small (<1000 kD) and large molecule (>1000 kD) drugs after administration to animals and humans. In the past (pre-1990s), organ dissection and analysis of organ homogenates by liquid scintillation counting (LSC) provided pharmaceutical companies with quantitative organ distribution information. In the 1990s, quantitative phosphor imaging technology revolutionized the conduct of drug distribution studies by providing true tissue-level quantitative data using autoradiography, or more precisely autoradioluminographic methods. Today, quantitative whole-body autoradiography, or autoradioluminography (QWBA), is the standard method for conducting tissue distribution studies, and regulatory authorities have come to expect these studies as part of innovative drug applications (IND) and/or new drug applications (NDA). QWBA provides high resolution images of the spatial distribution of drug-related radioactivity throughout the body of laboratory animals, and matching highly reproducible quantitative tissue concentration data in regions as fine as 50–100 μm. The main advantages of QWBA are minimal sample processing/alteration and provision of true tissue-level (as opposed to organ-level) concentrations from an in situ preparation. The main

limitation, which is shared by all studies relying on a radiolabeled compound, is that the technique provides data on total radioactivity only and not specifically of the parent compound. In other words, the concentration of radioactivity does not always equate with the identity of the original compound that was labeled, and it may also include radioactivity associated with metabolites and/or degradation products.

Sven Ullberg pioneered the technique of whole-body autoradiography, which relied on the use of x-ray film, in 1954 (Ullberg 1954, Ullberg 1977). The technique was made more easily and robustly quantifiable by Luckey in 1975, when he developed phosphor imaging technology or radioluminography (Luckey 1975). The QWBA technique begins when each animal is euthanized at a time point after administration of a radiolabeled test compound, and the carcass is quickly frozen. The frozen carcass is then embedded in a frozen block of supporting media and cryosectioned to obtain representative in situ samples of all organs, tissues, biological fluids, ingesta, and excreta. Whole-body sections (20–50 μm thick) are then dehydrated, exposed to phosphor imaging plates along with radioactive image calibration standards, and the resulting digital images are analyzed to determine tissue concentrations and to show detailed spatial distribution patterns. Tissue concentration versus time profiles can then be constructed to provide data for organ- or tissue-specific pharmacokinetic (PK) compartmental analysis, enabling the construction and examination of complex whole-body and/or specific tissue kinetic models. These models are used to help evaluate toxicology, pharmacology, and drug disposition/patterns, and to predict human exposure to drugs and metabolites, and also radioactivity, when a human radiolabeled drug study is necessary. It is important to note that this very brief description of QWBA methods omits many of the detailed steps that it is necessary to follow if reliable concentration data are to be obtained. Unless a scientist has been specifically trained by a professional and/or experienced autoradiographer, there is a high chance that critical pitfalls will be overlooked during the QWBA process, and the resulting data will be open to criticism, or, worse yet, the results will mislead the investigators using the data.

Microautoradiography (MARG), another autoradiographic technique, which was first developed in 1924 (Lacassagne and Lattes 1924), provides the ability to visually localize radiolabeled compounds to the cellular level in a histological preparation. In these studies, a radiolabeled test drug is administered to laboratory animals, which are later euthanized at different time points, and samples of the animal's organs and tissues are then removed, cryopreserved, and very thinly cryosectioned. Tissue sections (4–10 μm thick) are thaw-mounted onto microscope slides that have been pre-coated with a photographic/nuclear emulsion, which is sensitive to the radiation emitted from the radiolabeled compound. Silver grains in the emulsion are exposed to radioactivity for a period of time, and are developed in a similar manner to film, using developer and fixer, such that they precipitate and appear as black specks directly under or nearby the source of radioactivity in the tissue section. The resolution of how closely the location of the deposited silver grain is to the radioactive compound source depends on the energy of radioisotope used for the radiolabel. Low-energy isotopes such as ^{3}H and ^{14}C produce an image on the tissue section that is closer to its site of origin than a higher-energy isotope like ^{125}I, which can expose silver grains located much farther away. Because this is a histological sample, MARG can sometimes be coupled with immunohistochemical staining methods to co-localize drug entities with cellular targets in the various biological matrices. There are several examples in the literature of investigators attempting to obtain quantitative data from MARG preparations; however, MARG is prone to many artifacts, and section thickness and the variable thickness of the emulsion detection media, which are applied manually, are not well controlled. Furthermore, and perhaps most importantly, internal calibration standards have not been applied, which makes true quantitation problematic, if not impossible, using the current methods. This review will present a synopsis of both techniques and a few examples of how they have been used by investigators in recent years.

The radiolabeled test article

Before presenting a further description of quantitative autoradiographic techniques, it is important to understand the characteristics of the radiolabeled material being quantified. The quality and stability of the radiolabeled drug is one of the most important considerations whenever it is to be used for determinations of drug concentrations by any radiological techniques. This includes LSC, QWBA, and MARG, as well as in vivo autoradiography techniques, such as positron emission topography (PET), single-photon emission computerized tomography (SPECT) and gamma radiation imaging. The radiolabel provides a method for tracking the location and quantitation of total radioactivity which represents the parent molecule and metabolites that retain the radiolabeled portion of the molecule. The radiolabel also enables the specific identification and quantification of metabolites that contain the radiolabel, by using radio-high performance liquid chromatography (HPLC) techniques and mass spectroscopy. In general, small organic molecules are labeled with ^{14}C or ^{3}H (Hesk et al. 1997), and larger molecules, such as proteins, and peptides, are commonly labeled using ^{125}I (and sometimes ^{35}S).

Selection and stability of the appropriate radiolabel

In all cases, the location of the radiolabel must be in a known and stable position on the test molecule and the compound must have high radiopurity so that investigators are sure that the matching quantitative data (i.e., disintegrations of

radioactivity per minute) reflect the presence of the drug and/or metabolite of interest and not a degradent or impurity. Small molecular entities (chemical compounds that have molecular weights of <1000 kD) and large molecules (e.g., peptides, proteins, enzymes, oligonucleotides) need to be radiolabeled with an appropriate isotope [most often carbon-14 (^{14}C), hydrogen-3 or tritium (^{3}H), sulfur-35 (^{35}S), or iodine-125 (^{125}I)] so that the compound can be accurately imaged/identified, localized, and reliably quantified in various tissues, fluids, and excreta. Investigators need to ensure that the radioactivity they are measuring accurately reflects the parent test molecule and/or its derived metabolites, and so a radiopurity of 97 % or higher is recommended.

In most cases, ^{14}C is the best alternative when labeling small organic compounds because they can be located at specifically known and metabolically stable positions on the molecule, so that investigators can best track and quantify the parent molecule and its metabolites (Solon and Kraus 2002). Other radioisotopes, such as ^{3}H, ^{125}I, ^{111}In, are sometimes used for labeling large molecules for autoradiography, but the stability of the label is usually not as good as ^{14}C unless it can be well integrated into the test compound. ^{125}I is perhaps the most widely used radioisotope for QWBA analyses to study tissue distribution and pharmacokinetics of large molecule drugs in the biotechnology arena. Increasingly, regulatory agencies are asking for this information, as data gleaned from these studies have helped them to better understand and select their compounds in drug development. Most iodinated large molecules are absorbed intact through the intestinal tract, but once in the blood stream de-iodination can occur to varying degrees depending on the labeling. The in vivo stability of ^{125}I labeling on large molecules is subject to biodehalogenation (Venturi and Venturi 1999), which often results in relatively high concentrations of free ^{125}I in circulation. Although the in vivo stability of ^{125}I on most large molecule xenobiotics is difficult to assure, the data generated from these studies can be useful, and semi-quantitative data can be obtained with confidence when the in vivo stability and amount of free ^{125}I circulating in the body is characterized. The determination of drug concentrations in tissues using ^{125}I-labeled molecules must be considered as semi-quantitative due to the inevitability of measuring free ^{125}I along with the test article. Iodide has a volume of distribution that is about 38 % of body weight, is mostly extracellular, and free iodide is eliminated from the plasma primarily by glomerular filtration in the kidney and by organification by the thyroid (Venturi and Venturi 1999). If these tissues are of therapeutic interest, then care must be used when interpreting results. Caution must also be used when interpreting tissue concentrations of ^{125}I-labeled compounds for the stomach, mammary gland, salivary gland, thymus, epidermis, and choriod plexus, which also contain a sodium-iodide symporter that is involved in the organification and/or elimination of free ^{125}I (Venturi and Venturi 1999). Administration of non-radiolabeled sodium-iodide to test animals prior to giving the ^{125}I test compound is one way to reduce the uptake of free ^{125}I by tissues by shunting free ^{125}I to the kidneys to increase the rate of clearance and reduce the effect on tissue quantitation (Solon 2007). Although administration of non-radiolabeled iodide can help reduce the background "noise" of free ^{125}I on quantitation, it does not eliminate the effect. To characterize the effect, the amount of free versus protein-bound (and presumably test article-bound) ^{125}I can be evaluated using trichloroacetic acid (TCA) protein precipitation of plasma (and sometimes tissues) to determine the ratio of protein-precipitable ^{125}I versus free ^{125}I (Motie et al. 1998). That ratio can be used to correct tissue concentrations obtained using QWBA, but it does not account for the further possibility of circulating free ^{125}I to endogenous animal proteins and/or distinguishing between parent drug, metabolites, and degradation products. To address those questions, further analysis of plasma and/or tissues may be performed using extraction techniques followed by gel-electrophoresis, thin-layer chromatography, ELISA, and/or specialized mass spectroscopy techniques. A more definitive method to confirm the identity of the test compound in tissues is to analyze tissue/organ homogenates using polyacrylamide gel electrophoresis (PAGE) separation followed by autoradiography, which will provide information regarding the size of the iodinated test compound and/or its metabolites and/or degradation products. A similar approach could also be adopted for ^{3}H-labeled test articles. Thus, tissue quantitation of drug-derived radioactivity using ^{125}I and QWBA or gamma counting techniques carry the caveat of being semi-quantitative at best.

^{3}H is often used in drug discovery because it is relatively quick and easy to synthesize. However, it is important to verify the stability of the ^{3}H label on the compound whenever it is used in vivo, because the ^{3}H label is known to undergo hydrogen-exchange with endogenous water. This will in turn provide misleading quantitative data when the ^{3}H-water is lost during the dehydration steps used in autoradiography and/or erroneously included and/or omitted during other radiodetection/counting techniques (Kim et al. 2002). It is therefore crucial to characterize the extent of in vivo ^{3}H stability on the test molecule. ^{3}H-exchange can be monitored by determining the concentration of radioactivity in fresh (wet) and evaporated samples of plasma, urine, and/or tissue samples obtained from the animals being studied. The radioactive counts obtained from the wet and dried samples are then compared to see if there is a difference that would indicate loss of ^{3}H from the labeled test compound. In summary, the in vivo stability and radiopurity of the radiolabel on the test compound are important to know about whenever they will be used for determining the concentration of a drug and/or its metabolites in biological samples, especially when the compound is labeled with ^{3}H or ^{125}I, which are known to be unstable when used in vivo.

Quantitative whole-body autoradiography

Study design for QWBA

After approximately 10 years of intensive validation and education of the techniques of QWBA, it is now commonly accepted that QWBA (Solon et al. 2010) provides the most reliable and precise tissue concentration data, but it is worth mentioning here the importance of the study design, especially when a goal is to determine the tissue pharmacokinetics. Drug tissue distribution studies are routinely conducted in rodents, rabbits, canines, and non-human primates, and the choice of the animal model depends on the purpose of the study. In most cases, the animal model will mimic the expected PK in humans, but animal models, such as genetically and/or surgically modified animals, may be used to answer specific questions (e.g., toxicology, pharmacology, oncology, genetic modifications). Various dose routes can also be used for administration of the radiolabeled compound depending on the goals of the study. These include but are not limited to: oral, intravenous, intramuscular, intravesicular, intraocular, subcutaneous, topical, intranasal, and by inhalation. The number of time points used for sample collection and the overall duration of the study are also important variables to consider. Time points should be designed to obtain useful and reliable tissue concentration–time profile data. This can be difficult to determine even when the terminal half-life (T1/2) of the drug in plasma is known, because the T1/2 of the compound in the various tissues will undoubtedly be different and are almost impossible to predict. A suggested rule to determine the length of a tissue distribution study is to set the last time to be at least 10 plasma T1/2 values, but this is often not long enough to determine the clearance from all the different tissues in the body, especially if the compound has the potential to associate with melanin, where it might remain for several weeks. This is discussed further later on in this report. Another suggestion is to include as many time points as possible to increase the likelihood of capturing a reliable T1/2 for as many tissues as possible. For many studies, using 10 time points spread across a 2-week period will provide enough data to capture reliable PK parameters for many tissues. Tissue PK parameters such as maximal drug concentration reached (Cmax); time of maximal concentration (Tmax); area under the time–concentration curve (AUC), which describes tissue exposure to a drug; and the T1/2, which describes how long it takes for a tissue to eliminate a drug, can be determined. Statistically speaking, the portion of the time–concentration curve used to determine the T1/2 for each tissue must have an r^2 value of 0.8 or higher, and there should be at least 3 or more time points in that part of the curve. If reliable animal tissue PK parameters are not obtained by using enough time points, then predictions of human tissue PK parameters must be treated with caution.

Advantages of QWBA over LSC analyses

A key advantage of QWBA is that the controlled processes preserve tissue concentrations that are reflective of the in vivo condition at the time point of euthanasia, which no other technique currently offers. With QWBA, animals are quickly frozen, which eliminates the possibility of cross-contamination and variable effects of exsanguination of radioactivity that undoubtedly occur during a tissue excision procedure. Figure 1 shows a pictorial summary of the procedures used to obtain QWBA data. The frozen carcass is embedded in a frozen block of supporting media, and then cryosectioned (usually in a sagittal orientation and at a uniform thickness of 40–50 μm) using a special large format microtome to obtain representative in situ samples of all organs, tissues, biological fluids, ingesta, and excreta. Currently, Leica Microsystem is the main distributor of such large format cryomicrotomes; however, rebuilt used large-format microtomes made by former companies such as Vibratome and Hacker Instruments can still be found for sale through various distributors. Approximately 6–10 whole-body sections at various depths through the animal are necessary to collect the typical set of 40–50 tissues; however, any tissue of the body may be visually identified and obtained depending on the goals of the study. These whole-body sections, which are collected on large-format adhesive tape, are then dehydrated (~2 days in a cryomacrotome or more rapidly using a lyophilizer) and exposed to phosphor imaging along with a set of internal quality control (QC) standards to assure uniform section thickness, and external radioactive calibration standards that are used for assuring reliable and reproducible quantitation. (Technical note: commercially available radioactive autoradiography calibration standards require re-calibration before use because they are well-known to have inaccurate concentration values assigned to them; Solon and Kraus 2002.) After an appropriate exposure period (1–14 days depending on the isotope and/or amount of radioactivity present in the sample), the imaging plates are scanned by a phosphor imaging device, and gray-scaled digital images of the radioactivity in the sections are obtained. Digital QWBA images typically have pixel resolutions of 100 μm^2, therefore accurate measurements can be obtained even for small tissues; however, today's phosphor and direct nuclear imagers often offer pixel resolutions down to 10 and/or 25 μm^2, which are among the highest resolving powers available. Phosphor imaging is highly reproducible and is capable of quantification in the linear range of approximately 0.0001 to 10 μCi/g tissue in regions of interest as small as 0.5 mm^2 (Kolbe and Dietzel 2000). This kind of sensitivity is unmatched even by LSC technology. Phosphor imaging is also able to image the relatively weak energy of ^{14}C and ^{3}H, which fortunately are also long-lived isotopes so that drugs and metabolites with very long half-lives can be tracked in the body of animals over years. This is

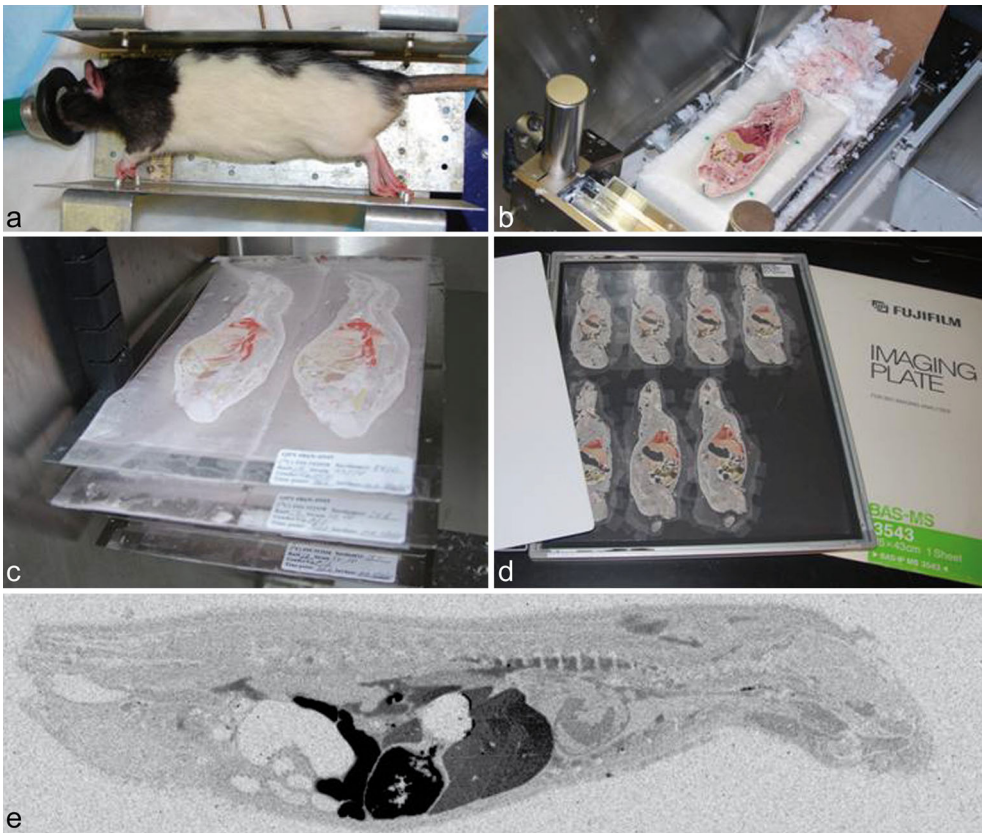

Fig. 1 Whole-body autoradiography methods. **a** The carcass is frozen in a hexane-dry ice bath; **b** whole-body sections are dehydrated; **c** sections are exposed to phosphor imaging plates along with radioactive image calibration standards; **d** the imaging plates are scanned by a phosphor imaging plate scanner; **e** gray-scaled digital images of the radioactivity in the sections are obtained for image analysis to determine tissue concentrations

not possible using the relatively short-lived isotopes used for in vivo positron emission topography (PET) and single photon emission computed topography (SPECT) imaging, which utilize isotopes with half-lives generally much less than 1 day.

In addition to phosphor imaging, direct nuclear imaging technologies are also available. These instruments, which were first developed in the 1980s (Jeavons et al. 1983; Charpak et al. 1989), utilize ionization chambers consisting of a parallel plaque avalanche chamber. Two such instruments (Beta and Micro-Imagers), which are currently sold by Biospace Lab (Paris, France), also image radioactivity in whole-body and individual tissue sections, and have the ability to acquire quantitative images in real-time. These instruments have proved most useful in drug discovery, because they are capable of dual isotope analysis and they provide data relatively quickly. However, relatively few sections can be analyzed at one time and the instruments require regular and careful maintenance.

Tissue concentrations determined by phosphor image analysis

Quantification using phosphor imaging is performed by image densitometry analysis. A standard curve is constructed from the integrated responses of densitometry values/mm^2 of the pixels of each image of the calibration standards (that were co-exposed with the sections imaged) with the known concentrations of radioactivity of each standard [in μ-Curies (μCi) or mega-Becquerels (MBq) of radioactivity/g of standard] that were determined using an LSC assay. The concentrations of radioactivity in each standard, and subsequently each tissue, are determined as μCi/g or MBq/g, which can then be converted to μg equivalents of drug per gram sample (μg equiv/g) using the specific activity of the administered radiolabeled drug (expressed as μCi/mg, or MBq/mg). Response curves determined for image analysis calibration can be evaluated and controlled. An image analysis software, such as MCID (InterFocus, Linton, UK) offers such control, and users of this software often use a weighted, first-degree, polynomial, linear equation (1/densitometry value/mm^2) and a numerical estimate of goodness of fit of the standard values, where the absolute value for the relative error of each calibration standard is ≤0.250 (Solon and Kraus 2002). An example of a standard curve calculation is provided below.

$$\text{Response} = a_1 x\ \text{Concentration} + a_0$$

Where:

Response	densitometry value as provided by the software/area of tissue mm^2
Concentration	standard concentration (in μCi/g or MBq/g)

a_1	slope
a_0	y-intercept

The relative error for each standard is calculated using the standard curve according to:

$$\text{Relative Error} = \frac{\text{knownconcentration } (\mu\text{Ci/g}) \text{minuscalculatedconcentration}(\mu\text{Ci/g})}{\text{knownconcentration}(\mu\text{Ci/g})}$$

Individual tissue sample concentrations are then calculated according to:

$$\text{Concentration of drug } (\mu\text{g equivalents /g of tissue}) = \frac{\text{Concentration from std. curve } (\mu\text{Ci/g})}{\text{Specific Activity of drug}(\mu\text{Ci/g})}$$

Lower (LLOQ) and upper limits of quantitation (ULOQ) can be based on the lowest (e.g., 0.0001 μCi/g), and highest (e.g., 10 μCi/g) standards used in the calibration curve such as:

$$\text{LLOQ} = \frac{\text{Density−Standard } \left(\text{e.g., } 0.0001\ \mu\text{Ci}/\text{g}\right)}{\text{Specific Activity } \left(\text{in } \mu\text{Ci}/\mu\text{g}\right)}$$

$$\text{ULOQ} = \frac{\text{Density−Standard } (\text{e.g., } 10\ \mu\text{Ci/g})}{\text{Specific Activity } (\text{in } \mu\text{Ci}/\mu\text{g})}$$

The LLOQ may also be determined as a function of the background where 2× and 3× background are used; however, this may affect the determination of tissue pharmacokinetic data due to the acceptance or rejection of data that is considered to be below the LLOQ (Solon and Lee 2002).

Tissue concentrations are obtained by selecting regions (actual pixels) of interest of the autoradiograph that correspond to each tissue of interest, and therefore it relies on the use of an image analysis system that is capable of precisely sampling regions of an autoradiograph that corresponds to each tissue of interest. The person performing the image analysis needs to have a very good knowledge of animal anatomy, interpreting tissue distribution patterns and artifacts, and possess a complete knowledge of the entire process so that they can recognize such things as improper dosing, freezing, sectioning, section melting or faulty dehydration, and section thickness inconsistencies.

At this point, it is worth discussing the image analysis software used for QWBA. To date, the three most popular software systems being used for QWBA are: AIDA™ (Raytest Isotopenmessgeräte, Straubenhardt, Germany), Seescan 2™ (LabLogic Systems, Sheffield, UK) and MCID™ (InterFocus). These software packages offer good image sampling tools, region of interest (ROI) tracking, and powerful algorithmic calibration features that lend themselves to use for regulatory purposes. Image contrasting features also help to identify tissues with very low or very high concentrations during image analysis. One recent improvement made by Raytest in their AIDA™ software is that which easily co-registers the autoradioluminograph with a color image of the actual whole-body sections (obtained from a conventional scanner). This enables the user to identify regions to quantify on the color image of the actual section while the software samples the autoradioluminographic image. This is useful when tissue concentrations are very low, and for beginner autoradiographers, who may be less experienced in sampling autoradiographs. However, it can lead the analyst into a false sense of security where artifactual radioactivity can contaminate adjacent regions of the specimen and, if careful attention is not paid, the analyst can easily and unknowingly sample regions that have been contaminated with high radioactivity, thus obtaining inaccurate results. Another feature offered by AIDA™ and Seescan2™ are extra features that help address regulatory compliance issues with the capability to add notes and secure audit trails to aid in documentation. All three systems have procedures in place to maintain data integrity to match quantitative values to specific regions of the images sampled.

Limitations of QWBA

QWBA has several limitations that need to be considered. One of the major limitations of QWBA is its inherent non-selectivity, which is true for all radiolabeled studies, as previously discussed. In addition, QWBA tissue sections are typically dehydrated to facilitate further processing, which results in the loss of volatile metabolites. Another limitation of QWBA is that it can be difficult to evaluate short-lived isotopes, like those used for radiopharmaceuticals, due to the processing time required. For example, when using ^{90}Y ^{11}C, or ^{18}F as the radiolabel, which have half-lives of 2.67 days, 21 min, and 60 min, respectively, the frozen 'wet' sections may need to be exposed to imaging plates immediately and while under freezer conditions. Despite these technical challenges, QWBA has been used recently to validate the performance of a modified PET scanner for small animal imaging (Walker et al. 2014).

Although not an autoradiographic technique, it is worth mentioning that new developments in mass spectroscopy

imaging have resulted in new ways to determine drug and drug metabolite distribution at the tissue and cellular level. Research over the last 15 years has brought improved utility to the techniques of matrix-assisted laser desorption mass spectroscopy imaging (MALDI-IMS) and single ion mass spectroscopy imaging (SIMS) (Solon et al. 2010). Further information on these techniques has been widely published in current literature and readers are encouraged to review the literature for descriptions and applications.

QWBA cannot adequately provide data at the microscopic level (i.e., cellular resolution) due to the relatively slow freezing technique that disrupts cellular morphology, which renders histological evaluation useless. To that end, MARG is the alternative technique for examining drug distribution at the cellular level.

Microautoradiography

Microautoradiography methods

Micro-autoradiography (MARG) is a high resolution qualitative tool to investigate spatial localization of radiolabeled drugs at the cellular level in histological samples. MARG is especially good at providing insight regarding localization of soluble and receptor-bound drug-derived radioactivity in situ in various cell types and has predictive value for specific drug targeting. In this respect, it has been used widely to provide important information on cellular mechanisms. MARG has applications in all areas of science, but this report will discuss examples in drug metabolism, pharmacology, toxicology, and molecular biology. The methods used by the author, which are described in this section, are based on the methods of Appleton and Stumpf, but it is important to realize that there are many variations of the method presented in the literature (Appleton 1964; Stumpf 2003). To begin, an animal is dosed with a radiolabeled substance (typically ^{3}H, ^{14}C, ^{35}S, or ^{125}I), the animal is exsanguinated, and tissues are dissected and snap-frozen in isopentane that is chilled with liquid nitrogen. The tissue is then cryosectioned at −20 °C (or the optimal cutting temperature for a given tissue/organ), to obtain 4- to 10-μm-thick sections. Then, under darkroom conditions, sections are thaw-mounted onto dry glass microscope slides that have been pre-coated with nuclear photographic emulsion. The slides are placed into a light-tight box with desiccant and allowed to expose for an appropriate length of time. Figure 2 presents a pictorial summary of the methods used for MARG, and Fig. 3 is an example of MARG that shows the location of ^{14}C-azidothymidine in rat brain choroid plexus after an IV administration. The collection of cryosections onto dry, pre-coated slides, while under darkroom conditions, is a key step developed by Appleton (1964) and it eliminates the possibility of diffusion of soluble compounds, which can happen during slide and section dipping into an aqueous emulsion (Baker 1989). In contrast, the original Stumpf and Roth (1964) method involved collection of the section into vials for freeze-drying, which required very careful section handling and was very time consuming and prone to sample destruction. Following exposure of the section to the emulsion, the slides are developed in a manner similar to that used for developing photographic film. Slides are then stained using conventional histological staining protocols, which may include immunohistochemical (IHC) staining techniques that can provide positive co-localization of drug-derived radioactivity to known cell types, receptors, and/or other structures/markers (Stumpf 2003). However, IHC staining protocols may require some modification because the emulsion, and the chemicals used to develop and fix the samples, can affect the IHC process and it may not work in some cases.

Limitations of microautoradiography

Several limitations have impeded the progress and wider use of MARG in drug discovery and development. These include: the processing time required to obtain results; the inability of the technique to provide quantitative results (which will be discussed further below); the high rate and ease of artifact production; and difficulties in collecting tissue sections under darkroom conditions. The processing time to obtain results from MARG is a difficult thing to gauge because each tissue must be treated and evaluated differently, depending on how much radioactivity is present. The exposure time can take anywhere from days to weeks, and even months, to obtain optimal results. This often discourages its use in drug discovery where investigators often work under much shorter timelines. It may also be impractical for scientists in the development area, who may require many more samples to be included in the evaluation, and who may be challenged by regulators to use validated procedures. Currently, the following types of artifacts must be controlled: (1) effects on emulsion by slight variations in light, humidity, temperature, tissue characteristics, fixation, freezing, chemicals, pH, developer, fixer, and miscellaneous debris in developer solutions; (2) tissue condition (e.g., freezing technique, fixation, autolysis, sectioning temperature, improper section mounting); (3) light leaks; (4) latent image fading; (5) reticulation of emulsion; (6) positive chemography; (7) negative chemography; (8) deviations of pH in processing fluids; (9) pressure artifacts; (10) ice crystals on knife; and (11) crystalline deposits from the developing process (Stumpf 2003). Some of these are more easily controlled than others, but together they require a high level of skill by the analyst to overcome, and the presence of any can invalidate months of work.

Fig. 2 A pictorial summary of the methods used for MARG. **a** The snap-freezing apparatus and *inset* the fresh tissue sample on a cryostat sample holder for mounting and freezing. **b** A Leica CM3050 Cryostat for cryosectioning of sample and collection onto slides pre-coated with emulsion (note sections must be collected onto glass slides that have been pre-coated with photo-emulsion while under darkroom conditions, thus all equipment lights have been covered with black tape). **c** Dip Miser slide coating cup (Electron Microscopy Sciences), Kodak emulsion, black slide exposure box, Drierite desiccant and a coated slide. Slides are dipped into liquid emulsion heated in the Dip Miser and allowed to dry. Sections are collected onto the precoated slides and tissue sections are exposed in a sealed black slide box with desiccant at 4 °C. **d** Slides are developed using Kodak Developer and Fixer. **e** Slides may be stained as usual. **f**. Examples of slides that have been stained; note the bottom slide shows how emulsion picks up stain, while the upper slide has no emulsion coating. Taken from Solon (2012) Chapter 6, Autoradiography in Pharmaceutical Discovery and Development. In: Braddock M (ed) Biomedical Imaging, RSC Drug Discovery Series No. 15, Royal Society of Chemistry

Drug concentrations in tissues/cells by microautoradiography?

Nevertheless, there are several examples in the literature of investigators attempting to obtain quantitative data from MARG preparations. A few methods have been proposed, which date back to the late 1960s, and have been presented in books by Stumpf (2003), and Baker (1989). The methods claim sensitivity to enable the determination of the actual number of molecules of drug present in cells, but this has not withstood the scrutiny of bioanalytical expertise in numerous pharmaceutical companies (unpublished accounts and anecdotal observations of the author over the past 20 years). One of the earliest methods, which was known as the "Restricted Method" was based on the analysis of structures of similar shape and size (Salpeter et al. 1969) and was used to describe the distribution of silver grains in sections of ^{3}H-noradrenalin labeled nerve terminals. A second "Restricted Method" was also based on the analysis of structures of similar shape, but dissimilar sizes (Downs and Williams 1984). This method established a "universal curve" and required that the 2-dimensional structures associated with radioactivity were of similar shape. An "Unrestricted Method" or "Circle Method" was developed by Williams (1969). This tested the hypothesis that radioactivity was randomly distributed and it ascribed values for relative concentrations. It also attempted to account for a lack of precision of the restricted models. A second "Unrestricted Method" known as the

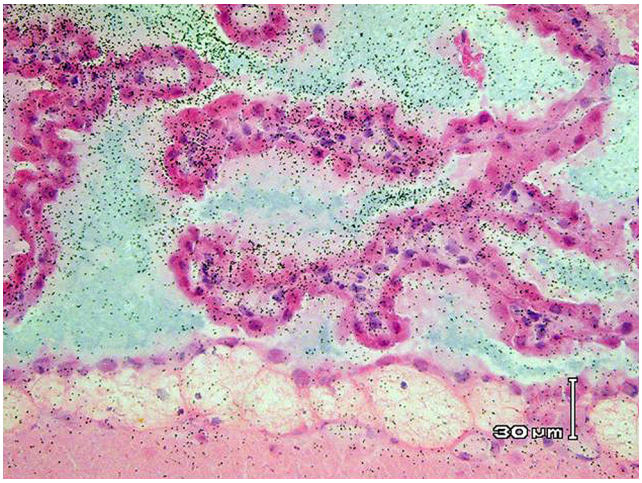

Fig. 3 Microautoradiograph of ^{14}C-azidothymidine in rat brain choroid plexus after an intravenous administration. Brain parenchyma proximal to the CP is shown at the *bottom* of the image. The location of the drug-derived radioactivity is made apparent by the *black specks* that appear over the section

"Hypothetical Grain" method was developed by Blackett and Parry (1973). This method has five stages that include: (1) overlay screen preparation; (2) collection of "hypothetical grains" and construction of a "cross-fire" matrix; (3) collection of real-grain data; (4) fitting of hypothetical and real grain data using chi-square; and (5) modification of the matrix until an "acceptable fit" is obtained.

Each of these methods claimed to enable an estimate of the number of molecules in subcellular regions if the specific silver grain yield (average number of disintegrations to produce one silver grain) was determined. The proposed calculations consider exposure time, specific activity, silver grain yield (dependent on sample uniform cut method conditions), section thickness, volume of the compartment, and "other parameters", which are very difficult to control due to uncontrolled section thickness, uncontrolled application of emulsion, and various artifacts inherent in this very sensitive technique. High sensitivity techniques require extremely consistent and careful preparation, especially because the current MARG techniques rely on many manual and "artful" steps. The uniformity of the detection media (i.e., the production and use of manually emulsion-coated slide) is unknown and not characterized in all examples reviewed in the literature. Tissue absorption of radioactivity is also rarely considered or characterized, and the number of cells counted/quantified must be sufficient for robust statistical analysis. Variable background and background subtraction can also invalidate results. Changes in daily cosmic and background radiation can adversely affect the results of an entire study, which can take weeks to months to obtain. Furthermore there are no internal standards, and/or calibrators to assure the quantitative results are valid, so the technique is often dismissed when scrutinized by scientists in other bioanalytical disciplines. In short, most pharmaceutical researchers do not have the resources available to develop routine MARG procedures to enable and/or validate MARG as a quantitative technique; thus, its use in the pharmaceutical industry has been limited.

In the future, technology may help to solve some of these problems if the detection media (e.g., emulsions) can be more uniformly produced and made to have inherently linear quantitation. Technology may also help to develop easier methods of collecting uniformly thick tissue sections that can be automatically mounted onto slides for processing. Dependable micro-sized calibration and quality control standards that can be co-exposed with every section would also need to be developed to assure reproducibility of quantitation. New methods would need to enable a significant reduction in the amount and types of artifacts that are produced, as previously mentioned. Some of these are more easily controlled than others, but together they require a high level of skill by the analyst to overcome, while the presence of any can invalidate months of work. Until methods and/or technologies can be developed that can better control tissue section and emulsion uniformity, and also reduce the sources of and occurrence of artifacts, the current technique will remain strictly qualitative and will prove to be daunting for routine use in pharmaceutical discovery and development. The lack of new developments in MARG methods have continued to make MARG an underutilized technique in drug discovery and development, but, when performed correctly, the results can be of utmost value in promoting a drug candidate and in answering some pivotal questions for pharmaceutical investigators.

Electron microscopy and autoradiography

Just as MARG is used to examine the distribution of radiolabeled substances at the cellular level, it is worth mentioning that autoradiography has been used along with electron microscopic (EM) techniques to examine the distribution at the subcellular level. Soon after the introduction of the electron microscope, Liquier-Milward (1956) proposed the idea of using EM and autoradiography to more specifically study the distribution of compounds in subcellular structures. Shortly after that, investigators realized that grain counting could provide a quantitative way of characterizing the distribution observed (Flitney 1969). Various techniques have been developed over the years, and one application has been for the quantitative study of neurotrophin transport, which requires the careful use of the ^{125}I-labeled neurotrophic proteins (von Bartheld 2001) and image analysis techniques. One such example utilized quantitative EM autoradiography to examine the fate of retrogradely transported neurotrophins in hypoglossal motorneurons of neonatal rats (Rind et al. 2005). These researchers compared the transsynaptic transfer and accumulation of tetanus toxin, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF),

and cardiotrophin-1 (CT-1). They found that BDNF and GDNF were transported not only to hypoglossal cell bodies but also to multivesicular bodies of dendrites, where they can be transferred across synapses of neighboring neurons. They also found that tetanus toxin rapidly accumulated in afferent synapses presynaptically, but that synaptic accumulation of GDNF occurred more slowly and CT-1 was not found at the synapse. Ultimately, this research and the approach suggested that quantitative EM autoradiography can provide a good way of studying the targeted delivery of therapeutic neurotrophic proteins.

Current studies using autoradiography

The current literature included in this review reports the various application of old (such as x-ray film) and new (such as phosphor imaging) autoradiographic methods being used to study the distribution of new small and large molecule drugs to support characterization of ADME and toxicity properties, receptor binding, diseases, and as a technique to validate new models. The studies include the use of various radioisotopes, such as ^{3}H, ^{14}C, ^{125}I, ^{131}I, ^{111}In, $^{67/68}$Ga, and ^{177}Lu.

Autoradiography studies in support of efficacy

It may be argued that QWBA has become the new standard for evaluating tissue distribution to support the regulatory filing of new drugs, and the literature is full of examples. This is also partly because the use of a radiolabel also enables the profiling, identification, and quantitation of metabolites and the determination of mass balance of drug derived compounds in the body of laboratory animals and humans. A prime example of this was demonstrated by scientists at Bayer Healthcare who reported on the tissue distribution and cardiorenal organ protection of finerenone as compared to eplerenone using two disease rat models (Kolkhof et al. 2014). In this study, the investigators used QWBA and found that efficacious amounts of ^{14}C-finerenone distributed equally into rat heart and kidney tissues, which were important target organs (Fig. 4). Upon further investigations into the animals' vital signs, and histopathological and electrolyte evaluations, they also found that finerenone prevented tissue damage at doses that did not also reduce blood pressure. They concluded that finerenone may offer a better alternative for treating end-organ damage with a low risk of electrolyte disturbance. Scientists at Bayer Healthcare have also reported the use of both QWBA and histopathology in their investigations on the effects of glucose transporter inhibition to treat malignant cancers (Héroult et al. 2014). Malignant cancer cells are known to have increased glucose uptake through the overexpression of the glucose transporter GLUT 1 and/or GLUT3, and inhibition of those have become a target for cancer treatment. These scientists used QWBA in mice xenografted with NCI-H460 human tumor cells and given a single dose of a GLUT1 selective inhibitor, a multi-GLUT inhibitor, or vehicle (control) followed by a single dose of ^{14}C-deoxyglucose ([^{14}C]-2-DG) given 60 min after the inhibitor or vehicle (Fig. 5). QWBA revealed "Selective GLUT1 inhibitor inhibited the uptake of [^{14}C]-2-DG in brain and tumor only; the concentrations in brown adipose tissue and heart were similar to control; brain concentration recovered after 2 h; tumor and heart concentration remained consistently low; the quantitative data support the qualitative assessment above; treatment with the multi-GLUT inhibitor compound inhibited [^{14}C]-2-DG uptake in brain, tumor, brown adipose tissue, and heart by more than 50 %; only brain concentration recovered after 2 h; the effect of selective GLUT1 inhibition was similar for brain, but it did not affect [^{14}C]-2-DG concentration in the heart." (Fig. 6) The histopathological findings extended the QWBA findings and they concluded that "The [^{14}C]-2-DG findings were in parallel with histopathological findings present in the brain after treatment with the multi-GLUT inhibitor. The etiology of the observed lesions may be related to the decrease in the glucose content. A disturbed glucose homeostasis may lead to damage of tissue and organs, especially in those with high-energy demands; however, in a similar study, comparable histopathological findings were not observed in the brain after treatment with the selective GLUT1 inhibitor".

Applications for metabolism study

In a different study, the tissue distribution and metabolism of GDC-0152, which is a small molecule peptidomimetic antagonist of inhibitor apoptosis proteins (IAP) with anti-tumor activity, was studied using a strategy where GDC-0152 was radiolabeled with ^{14}C at two different positions [one at the terminal phenyl ring (A), or at the carbonyl of the 2-amino-2-cyclohexylacetyl moiety (B)] (Yue et al. 2013). The mass balance, QWBA and metabolite analyses of samples obtained from rats given each version of the different radiolabeled GDC-0152 enabled the improved spatial localization and identification of the various metabolites that resulted from the extensive metabolism of this compound. This showed that 62.3 and 25.1 % of A was excreted in urine and feces, respectively, but 27.2, 32.2, and 27.5 % of B was found in urine, feces, and expired air, respectively. GDC-0152 underwent extensive metabolism and <9 % of the dose recovered as parent in excreta. QWBA (Fig. 7) showed that radioactivity in tissues derived from B remained longer than for A, which was consistent with the T1/2 of the total radioactivity in circulation, which was 21.2 h for B and 4.59 h for A. A commonly asked question about this type of study strategy is: can a test compound be dual-labeled, i.e., labeled in one position using ^{3}H and in another position using ^{14}C, and then imaged to track and quantify the different resulting metabolites? At first,

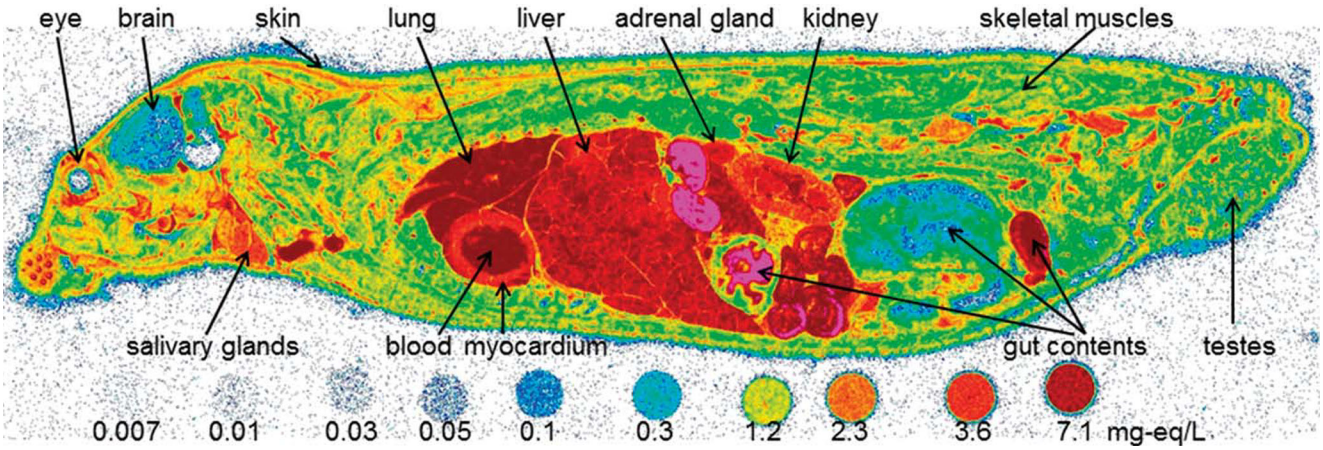

Fig. 4 Distribution of radioactivity in a male Wistar rat 1 h after oral administration of 3 mg/kg [^{14}C]-labeled finerenone. *Blue* lowest detectable concentration; *red* highest detectable concentration of radioactivity; *pink* above the upper detection limit. [^{14}C]-radiation standards spiked with stated concentrations are given at the *bottom*. Reproduced with permission from Kolkhof et al. (2014)

one may answer 'yes', but practically it is very difficult to adequately image and/or quantify these using QWBA and/or LSC due to the inability to entirely eliminate the detection and/or account for the beta energy of one isotope influencing the quantitation of the other. Mostly, this effect is from ^{14}C on the detection of ^{3}H, which is hard to subtract from the total. However, a better strategy would entail the use of a shorter-lived and higher-energy isotope such as ^{35}S. In this scenario, the ^{35}S and ^{14}C would be imaged by QWBA together and the image analysis completed to determine the total concentration of radioactivity (i.e., ^{35}S + ^{14}C). Then, the QWBA sections would be stored away to allow for the total decay of the ^{35}S, which has a radioisotopic T1/2 of about 60 days. After which time, a QWBA image analysis would be conducted again to determine tissue concentrations of only ^{14}C, and then the values obtained using the total radioactivity would be subtracted from the radioactivity detected from ^{14}C alone to determine tissue concentrations derived from both isotopes. The drawback is the time it would take to get these data due to the decay of the ^{35}S signal.

QWBA is often the first technique to offer pharmacologists, toxicologists and pharmacokinetic scientists a broader view of specific tissue exposure than that provided by plasma concentration and in vitro data alone. In an ADME study of AZD7903, which is being developed by Astra-Zeneca as a non-narcotic analgesic, QWBA revealed the presence of ^{14}C-AZD7903-derived radioactivity in the stomach of rats after a single intravenous administration (Haglund and Borg 2013). This indicated an unforeseen route of elimination and

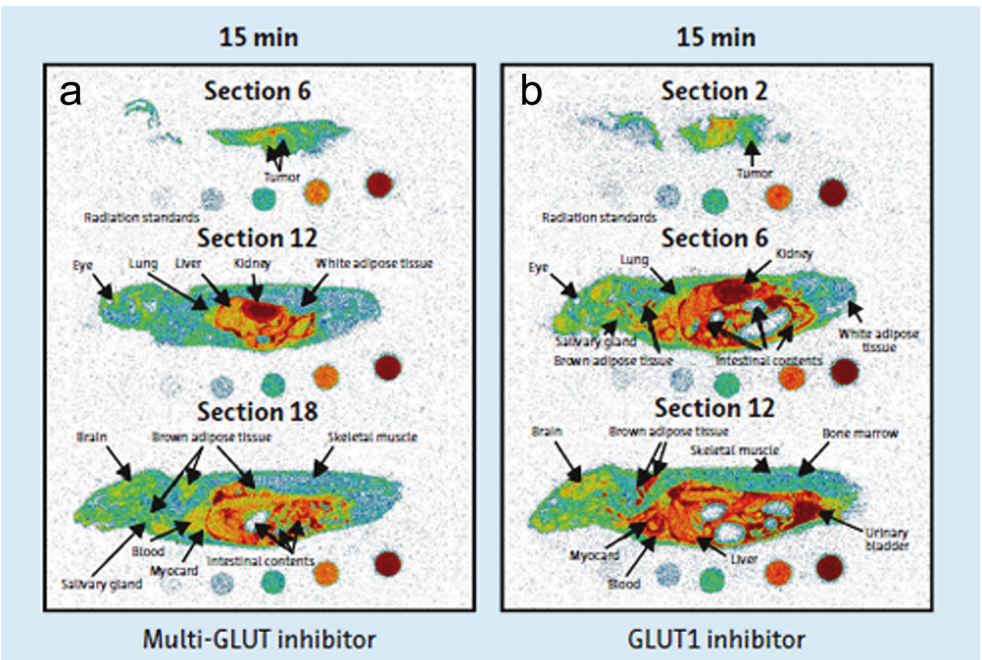

Fig. 5 Distribution of [^{14}C]-2-deoxyglucose in rats after administration of GLUT inhibitors. Reproduced with permission from Héroult et al. (2014)

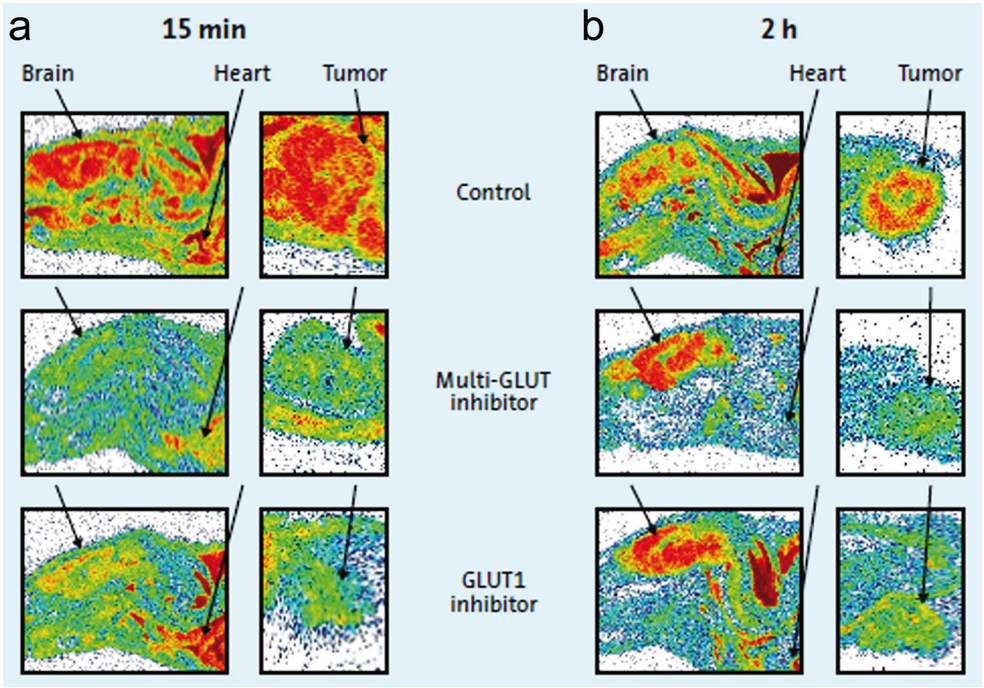

Fig. 6 Effect of tissue profiles of GLUT inhibitors on distribution of [^{14}C]-2- deoxyglucose in target tissues. Reproduced with permission from Héroult et al. (2014)

exposure of the stomach to concentrations of AZD7903 that were higher than observed in blood and plasma. The investigators also performed excretion and PK evaluations and concluded that there were sex-related differences in metabolism and that AZD7903 was a substrate for a transporter protein. QWBA was also recently used to evaluate the testicular toxicity of JNJ 40929837, which is a novel leukotriene A4 hydrolase inhibitor (Ward and La 2014) used to treat

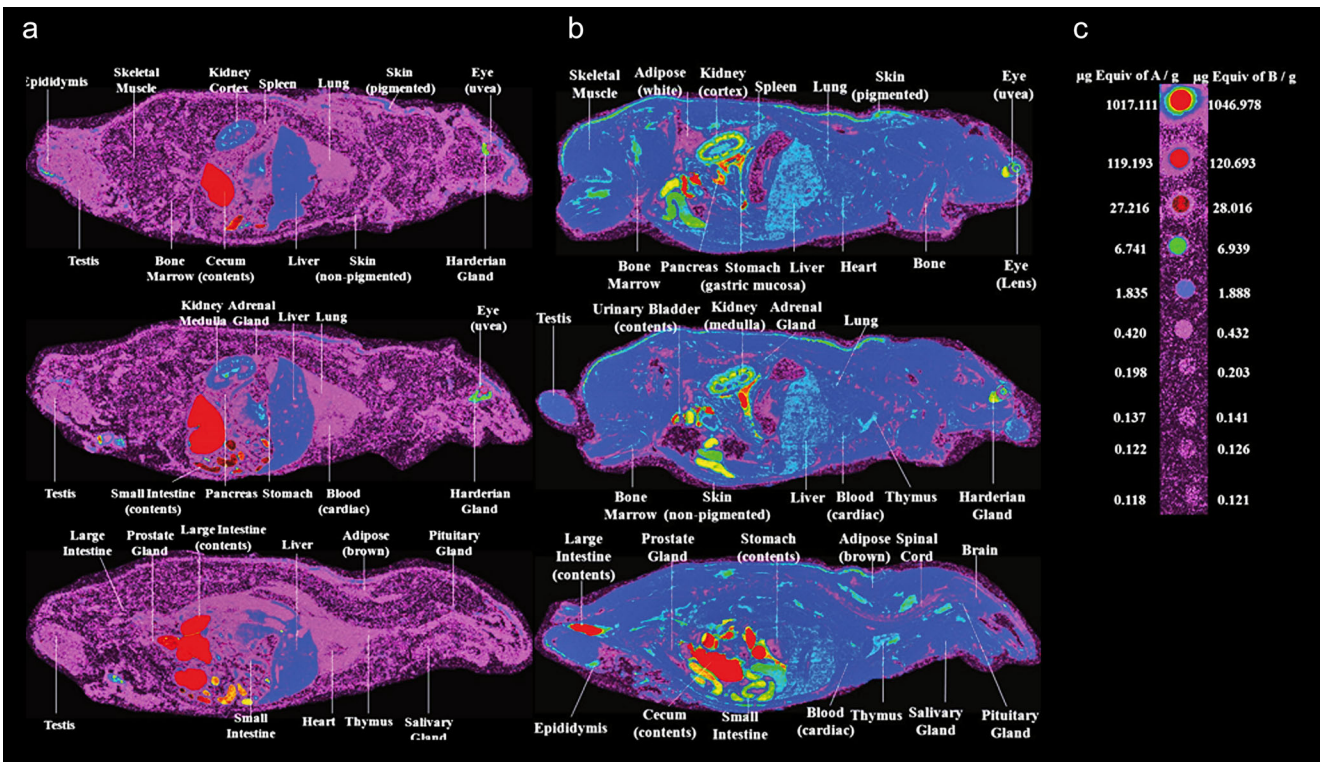

Fig. 7 A representative whole-body autoradiogram of the ^{14}C-GDC-0152-derived radioactivity distribution in male Long-Evans rats at 12 h post-dose following IV administration of ^{14}C-GDC-0152 that was radiolabeled in the A position or B position of the parent molecule. Reproduced with permission from Yue et al. (2013)

inflammatory conditions, such as rheumatic diseases. QWBA clearly demonstrated retention of ^{14}C-JNJ 40929837-derived radioactivity in the testes, which was consistent with the site of toxicity, and a follow-up multi-dose study, which included an evaluation of metabolites in the testes, suggested a large accumulation of two metabolites. The study supports the importance of using QWBA studies early in development to identify potential sites of toxicity due to the presence of large amounts of drug-derived compounds in sensitive tissues. QWBA was also used recently in a similar study of testicular drug distribution to examine the unexplained fetal malformations observed in rats and rabbits after treatments with teriflunomide (Davenport 2013). A QWBA study was performed in adult male rats after a single administration of ^{14}C- teriflunomide to see if there was any possible effect on male reproduction through effects on sperm. Results showed ^{14}C- teriflunomide-derived radioactivity was lower in testes and epididymis than in blood at 6 h post-dose, and were below the limit of quantitation by 14 days post-dose. This suggested that teriflunomide did not irreversibly bind to sperm, which takes 56 days to develop in the testes and epididymis, and there were no external malformations in the offspring of male rats given teriflunomide prior to mating with untreated females. After also conducting in vitro genotoxicity and in vivo clastogenic tests, this investigator concluded that there was "no evidence that teriflunomide would produce male-mediated embryo-fetal toxicity by damaging sperm DNA". The accumulation of drugs and/or their metabolites in tissues has also been a concern for pharmaceutical companies developing new inhaled corticosteroids, which tend to be highly lipophilic in systemic tissues, which may lead to adverse systemic effects.

Scientists at AstraZeneca and ActivBiotech used QWBA to compare tissue accumulation of the active metabolite of ciclesonide (des-CIC), which is being developed for pharmaceutical use and is highly lipophilic, and budesonide, which is moderately lipophilic (Märs et al. 2013). Male albino mice were given 1, 3, or 14 daily subcutaneous doses of ^{3}H-des-CIC or ^{3}H-budesonide and then the distribution of radioactivity was examined by QWBA. Results showed that the patterns of distribution were similar for both drugs after single and multiple doses, but that des-CIC accumulated significantly more in tissues than budesonide and that "systemic accumulation may lead to increased risk of adverse side effects during long-time therapy".

Ocular studies using autoradiography

This is not to imply that tissue retention always presents a risk, and QWBA has been used to support studies on how in vivo tissue binding/associations can be used to the advantage of drug developers. Robbie et al. (2013) investigated the use of known melanin binding properties of the pigmented uveal tract of eye as a depot for the slow release of vascular endothelial growth factors/platelet-derived growth factor receptor tyrosine kinase inhibitors to treat ocular neovascular disease. These investigators examined "uveal-retention" of ^{14}C-pazopanib as a positive control for melanin binding, as compared to their test article, ^{14}C-GW771806. Their QWBA experiment showed that both ^{14}C-pazopanib and ^{14}C-GW771806 were present in the melanin-containing uveal tract of pigmented rats for up to 35 days after a single oral dose. They improved the dosing regimen using a single oral loading dose of ^{14}C-pazopanib or ^{14}C-GW771806 followed by repeat topical ocular administrations of each compound in pigmented and non-pigmented rats that had laser-induced choroidal neovascularization. At 3 days after the last topical treatment, LC/MS/MS analysis of retinal and choroid homogenate samples showed that the ocular T1/2 of pazopanib and GW771806 were 439 and 442 h, respectively. Their study also showed that topical GW771806 treatment in rats was more effective than intravitreal injections to treat choroidal neovascularization, and they concluded that their data supported the need for further research into using the known properties of melanin binding as a depot for extended drug delivery to treat ocular diseases.

Many lipophilic drugs with pKa values above 7 bind to melanin; however, the toxicological consequence of this binding has been debated (LeBlanc et al. 1998). Structure activity relationship studies using whole-body autoradiography (WBA) for melanin binding as a function of various other physicochemical characteristics showed a correlation with volume of distribution, log P, pK_a and binding energy, in pigmented rats. Strongly basic structures, such as piperidine and piperazine moieties and other amines, showed potential for retention in the ocular melanin (Zane et al. 1990). Covalent binding of drugs to melanin is rare; however, its polyanionic nature and high content of carboxyl and semiquinone subunits facilitates non-covalent binding with many drugs. Although melanin binding of new chemical entities (NCEs) is not predictive of toxicity, it may prohibit the conduct of human radiolabeled studies where prolonged exposure of pigmented tissues, especially the eye, to radioactivity could pose a health risk (Dain et al. 1994). QWBA in pigmented rats often provides the first evidence of melanin binding of new drugs because melanin binding is not routinely assayed by other means during drug discovery and development. Figure 8a shows a Sprague Dawley rat autoradiograph obtained at 2 h after a single administration of a ^{14}C-labeled compound, and shows wide tissue distribution of the drug. Figure 8b, which is an autoradiograph of an albino rat that was prepared for WBA at 96 h after a single dose, shows the absence of drug-derived radioactivity in the ocular tissues. Figure 8c shows the binding of drug-derived radioactivity in the pigmented skin and uveal tract of the eye of a pigmented Long-Evans rat at 72 h after a single oral dose. Although this compound did not produce any toxicity related to melanin binding, the predicted exposure to

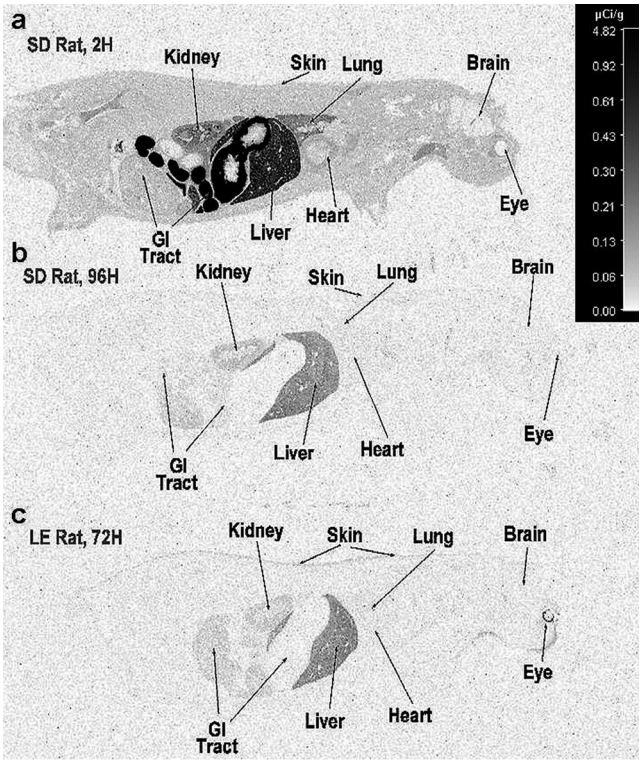

Fig. 8 Melanin binding. Whole-body autoradiographs reveal tissue distribution of a ^{14}C-labeled drug in albino and pigmented rats. **a** Sprague-Dawley rat at 2 h post-dose shows wide distribution of drug-derived radioactivity. **b** Sprague-Dawley rat at 96 h post-dose shows limited distribution of drug-derived radioactivity and, notably, no radioactivity in the eye. **c** Pigmented Long-Evans rat at 72 h post-dose shows limited tissue distribution of drug-derived radioactivity, but a high concentration of radioactivity in the eye, which is due to melanin binding

drug-derived radioactivity in humans during a proposed human ^{14}C metabolite identification study was shown to be a cause for concern.

Large molecule tissue distribution studies by autoradiography

As the need to characterize the ADME characteristics of large molecule, biological drugs has increased, QWBA and MARG have been used to determine their unique disposition in tissues. Owing to the complexity of their molecular structures, it is often not chemically feasible or economically practical to radiolabel large molecules, such as therapeutic proteins, peptides, antibodies, and small interfering RNAs, with ^{14}C in a stable and known location on the molecule. However, it is possible, and ^{14}C-labeling was used in the study of the tissue distribution of Peginesatide, a polyethylene glycol (PEG)ylated peptide-based erythropoiesis stimulating agent (ESA), in monkeys (Woodburn et al. 2013). In this series of studies, Peginesatide was labeled with ^{14}C, which was located on the lysine linker between the peptide dimer and the two 20-kDa PEG chains. ^{14}C-Peginesatide was used to localize and quantitate the concentration of Peginesatide in tissues of monkeys after a single IV bolus dose using QWBA at 48 h, 1 week, and 3 weeks after dosing (Fig. 9). MARG was used to examine cellular distribution of liver, spleen, thymus, mesenteric lymph nodes, axillary lymph nodes, sternum, bone marrow (femur), kidney cortex, kidney medulla, and kidney papilla at 48 h post-dose. The QWBA results showed that radioactivity remained at 3 weeks post-dose with the levels in most tissues <1 μg equiv/g of tissue. It also showed that radioactivity levels in the spleen and lymph nodes remained increased compared to 48 h, and that levels in the bone marrow, adrenal gland, and pituitary gland were lower by approximately 35–55 % at week 3 compared to week 1. The MARG results from 48 h post-dose revealed uniform distribution of radioactivity in the bone marrow with no specific cell-associated accumulation of radioactivity, while hard bone had no radioactivity. Radioactivity in the liver and thymus was low and diffusely scattered across cellular and non-cellular compartments. There was differential distribution observed between red and white pulp of the spleen, which was consistent with the QWBA data. Radioactivity in lymph nodes was also low and diffusely scattered with no cell-specific localization. Radioactivity in the glomeruli, convoluted tubules, collecting ducts, blood vessels, and interstitial cells of the renal cortex appeared similarly distributed, but was higher (Fig. 10a) in the medulla, and there was less association of radioactivity with the lumen of the ducts compared to cells in the interstitium (Fig. 10b). The paper concluded that "Peginesatide biodistribution data may potentially provide insight into the biodistribution of ESAs and perhaps even erythropoiesis receptor (EPOr) sites. The data suggest that peginesatide is localized to known EPOr sites (bone marrow), sites that may have functional EPOr (e.g., splenic red pulp), and putative EPOr sites such as the renal medulla and papilla"

Last year, the Center for Biologics Evaluation and Research of the US Food and Drug Administration (FDA) released a Pharmacology and Toxicology Primary Discipline Review of the Biological License Application for the Novo Nordisk drug NovoEight® (N8) (Brown 2013). This report reviewed a submission that included a QWBA study (conducted in 2008) using ^{125}I-labeled N8. N8 is a recombinant antihemophilic factor for the prophylaxis and/or on demand treatment of hemophilia A patients during and/or after major surgery or other life-threatening events. The QWBA study examined the tissue distribution of ^{125}I-N8-derived radioactivity in wild-type and von Willebrand factor (vWF) knockout (KO) mice, which enabled a comparison of the distribution of N8 in the absence of vWF that prevents the degradation of inactive FVIII, which is the essential blood-clotting protein, in circulation. This study examined distribution at a very limited set of data obtained at 15, 30 and 90 min after a single dose, and the FDA reviewer concluded that "It appears that there is a time- and dose-dependent distribution of ^{125}I-N8 in mice following dosing. As time progresses, radioactivity

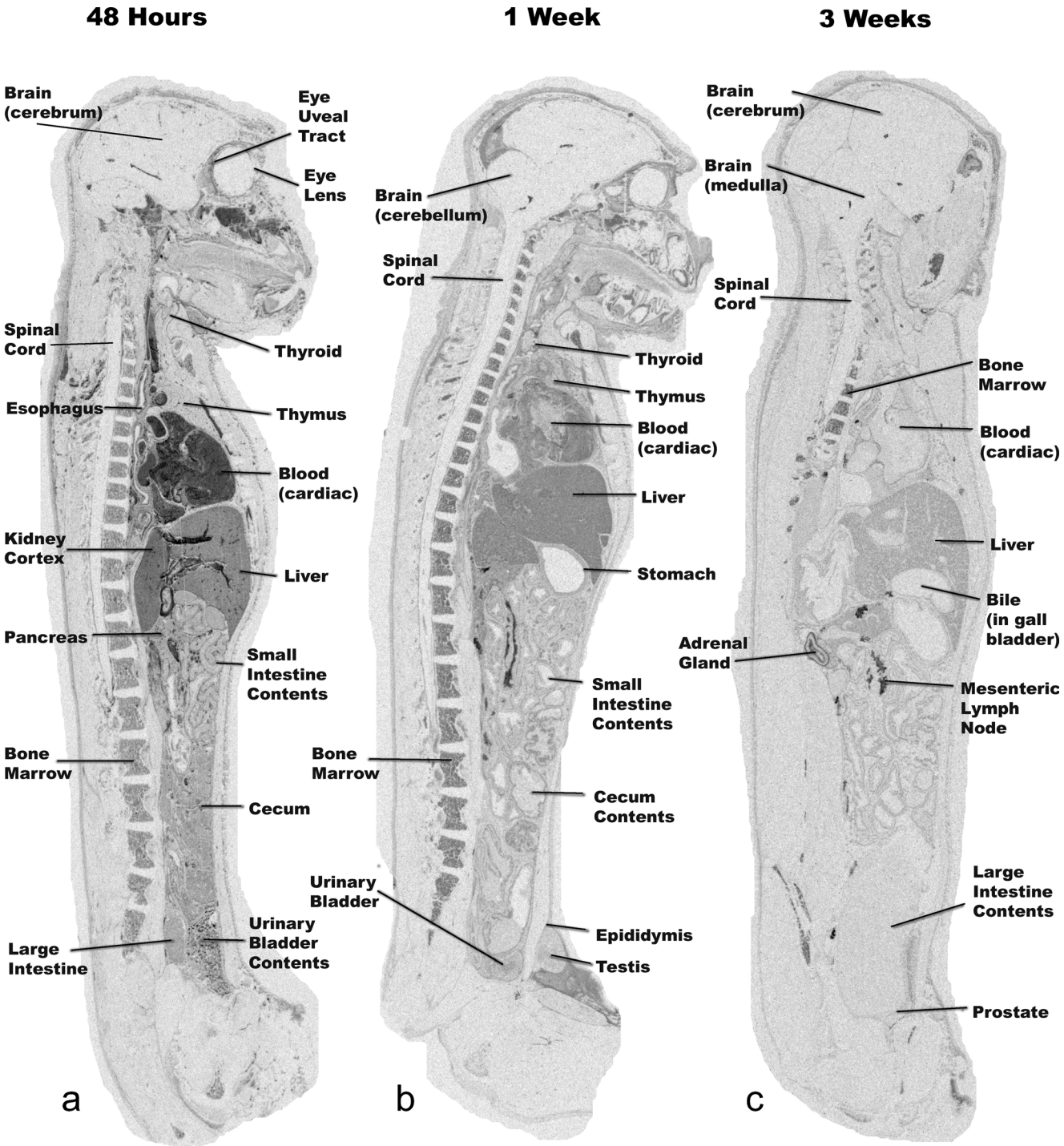

Fig. 9 Whole-body autoradiograms of male *Cynomolgus* monkeys at 48 h, 1 week and 3 weeks post-dose following a single IV administration of [^{14}C]-peginesatide at 2.1 mg/kg. Reproduced with permission from Woodburn et al. (2013)

levels decrease and are allocated to red blood cell-rich depots or high blood flow areas such as the spleen, liver, and thyroid gland". In experiments on the ADME characteristics of ^{125}I-labeled rat immunoglobulin, and a review of 11 privately sponsored QWBA studies using ^{125}I-labeled large molecule drugs for which biodehalogenation was monitored (Solon et al. 2013), it has been shown that anywhere from 10 to 60 % of the total ^{125}I measured was bound to protein in plasma of animals given a ^{125}I-labeled large molecule between 5 and 60 min post-dose. Although it was not clear from the description in the FDA report, the results suggested that the investigators did not account for biodehalogenation as they reported high concentrations of ^{125}I derived radioactivity in blood, kidneys, and especially thyroid. This is an expected result

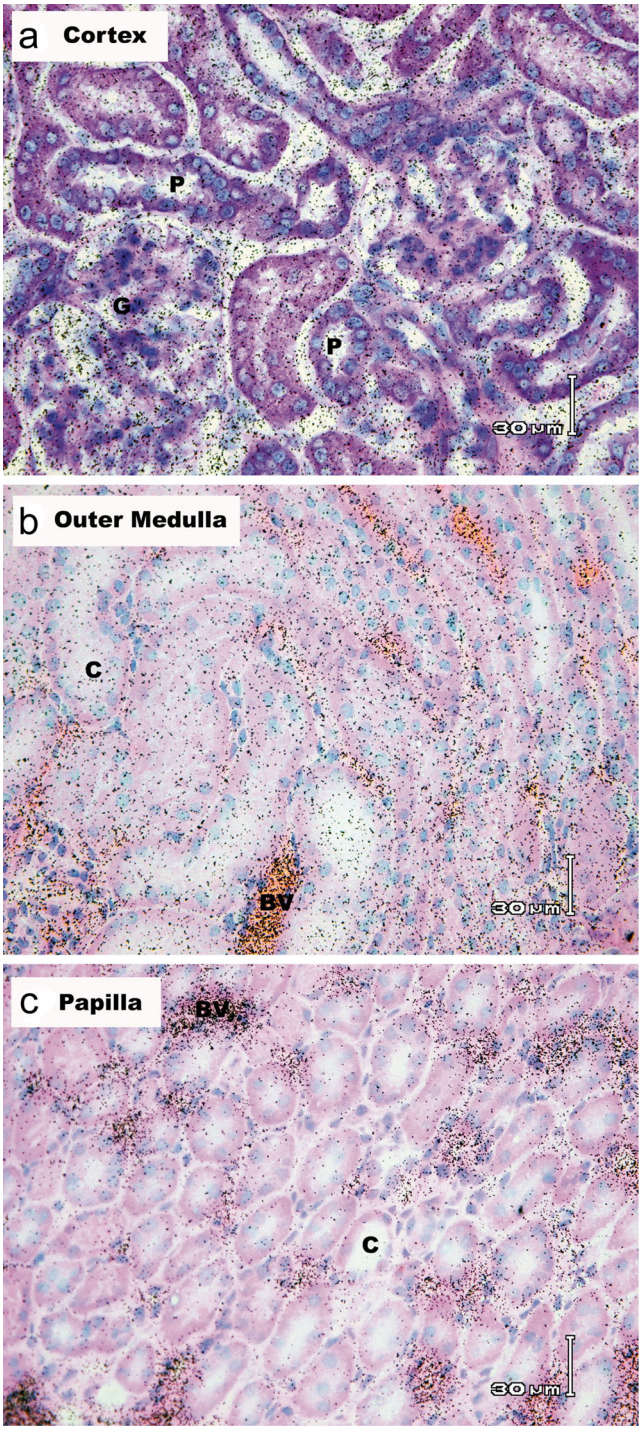

Fig. 10 Microautoradiographs of kidney sections from a male Cynomolgus monkey 48 h post-i.v. dosed with [^{14}C]-peginesatide at 2.1 mg/kg. *G* glomerulus; *P* proximal convoluted tubule; *D* distal convoluted tubule; *C* collecting duct; *BV* blood vessel. [^{14}C]peginesatide associated biodistribution is represented by the dark brown granules overlaid on H&E staining. Reproduced with permission from Woodburn et al. (2013)

for distribution of free ^{125}I and it was probably not reflective of the N8 distribution. In the experience of the author, this is a common misinterpretation and one that can lead to faulty conclusions about the distribution of biological drugs, which underscores the importance of characterizing biodehalogention when using ^{125}I-labeled test articles for any in vivo examination of drug disposition.

As previously discussed, tritium is another isotopic label that can become unstable in vivo and requires monitoring when used for quantitative analysis of animal plasma, tissue, and excreta samples. Such was the case in a study performed on the metabolism and distribution of unformulated internally ^{3}H-labeled siRNAs in mice (Christensen et al. 2013). The objective of their study was to assess the ADME properties of two ^{3}H-labeled siRNAs in mice after a single IV administration. These investigators, who performed a thorough review of the published literature on the use of radiolabeled siRNAs to determine ADME properties, observed potential drawbacks in how siRNAs were being radiolabeled in unstable positions and used for research. This motivated them to develop a novel ^{3}H-siRNA radiolabeling procedure, which they tested and reported. These researchers carefully monitored the amount of possible in vivo ^{3}H exchange with endogenous water in blood and plasma obtained from mice used for QWBA analysis. QWBA showed that the overall highest concentrations of ^{3}H-siRNa-derived radioactivity were present in the kidney, salivary gland, spleen and liver. Their investigation into the metabolic stability of the ^{3}H radiolabel revealed that approximately 9 % of the label had exchanged with endogenous water at 2 h post-dose and that it increased to 26 % at 48 h after dosing, which "indicated that the formation of tritiated water was an ongoing process". This report underscores the importance of monitoring the in vivo stability of radiolabels when performing quantitative analyses. However, despite this, many investigators are either unaware of the in vivo instability of ^{3}H labels and/or do not realize the extent of the potential loss and thus the negative impact it could have on their interpretation of results and conclusions. One such example is in a study comparing the tissue distribution of ^{3}H-labeled recombinant fusion protein linking factor VIIa with albumin (^{3}H-rVIIa-FP), and ^{3}H-labeled recombinant fusion protein linking factor VIIa (^{3}H-rVIIa) in rats (Herzog et al. 2014). This paper reported quantitative tissue distribution, mass balance excretion, and plasma PK results obtained using QWBA, LSC, and radio-HPLC techniques. The paper did not describe any methods to evaluate the in vivo metabolic stability of the ^{3}H on either test article. Although it reported that the identity of the ^{3}H-related radioactivity in plasma and urine, which was determined by radio-HPLC, corresponded to the intact test articles, the chromatographs show large wide peaks related to "[^{3}H]-aggregates" and very wide multi-peaked chromatographs that the investigators claimed to be related to ^{3}H-rVIIa-FP. Their report claims the data to be quantitative evidence of plasma concentration data based on these chromatograms; however, wide multi-peaked chromatograms are highly suspect and are at best semi-quantitative. This fact, coupled

with the fact that these investigators did not evaluate the presence of ^{3}H-water which would reveal the extent of in vivo instability of the ^{3}H on either test article, renders any interpretation and conclusions related to quantitative data as flawed. At best, all the data presented in this article are semi-quantitative for the positive quantitation of the test articles being studied and most likely underestimate concentrations of rVIIa-FP, rVIIa, and the control albumin used. Interestingly, these investigators compare their QWBA data to QWBA data reported in an earlier publication that used ^{125}I-labeled human plasma-derived FV11a and –FX with MC710 in rats (Nakatomi et al. 2012), and in that report the investigators also did not evaluate the extent of biodehalogenation. Those authors actually reported relatively high "radioactivity levels in the thyroid, … kidney, … stomach, … and intracystic urine…" and that "at 168 h , radioactivity persisted in the thyroid gland…". These observations are clear indications of biodehalogenation, but that fact was apparently unknown to the authors, and the assumption that the presence of ^{125}I was related to the presence of their test article was obviously misleading. It is unfortunate, but there are many such studies being reported in the literature that do not examine the in vivo stability of the radiolabeled compound being used for quantitation of drug concentrations in tissue, plasma, and/or excreta.

Receptor autoradiography studies

Although the in vivo stability of the radiolabel may present problems for the reliable quantitation of drug concentrations in tissues of animals during in vivo studies, it is less of an issue for their use in vitro where metabolic processes are more or less eliminated and/or much reduced. Thus, in vitro quantitative tissue autoradiography using ^{125}I- and ^{3}H-labeled test drugs offer investigators reliable results without needing to worry as much about the instability of the radiolabel due to metabolic effects. Although autoradiography techniques have been used to study in vitro drug localization for years, the advent of high resolution phosphor imaging has provided for the ability to obtain quantitative results, and there is an increase in published reports of its use. A novel application of in vitro quantitative autoradiography for drug concentration is in the area of tissue receptor research. Mizoguchi et al. (2014) performed such a study while investigating specific binding characteristics of 18β-glycyrrhetinic acid (GA) in rat brain. GA is a component of glycyrrhiza, which is derived from a traditional Japanese medicine called yokukansan, and has known pharmacological action in the central nervous system. In these experiments, thin sections of rat brain, which were obtained by cryosectioning, were incubated in solutions of ^{3}H-GA at varying concentrations with or without unlabeled GA. Sections were then dried and exposed to phosphor imaging plates, and specific binding of GA was determined in various regions of the brain using quantitative image analysis. These investigators also performed MARG on sections used for phosphor imaging, and merged the MARG images with photomicrographic images of serial sections used for immunohistochemical images for glial fibrillary acidic protein (GFAP). This approach enabled the confirmation of co-localization of ^{3}H-GA to small cells in the hippocampal region, which were stained by the GFAP immunostain. Together, their experiments showed evidence of specific binding sites in specific regions of the brain, and it suggested that the astrocyte was a major target cell for GA. This work is important in understanding the pharmacology of GA in the brain after administration of glycyrrhiza and/or the traditional Japanese medicine, yokukansan.

Autoradiography has also been used to determine receptor localization and binding to characterize disease states, and there are many examples in the current literature. A review of several of these type studies that were conducted over the past few years revealed that several different methods for autoradiography have been used. In all articles reviewed, the authors claim quantitative tissue analysis to determine tissue concentrations of drug. Some of the reports cited outdated methods, such as the use of x-ray film or photographic emulsion autoradiography and the use of commercially available radioactive calibration standards. It has been shown repeatedly that quantitation using x-ray film or emulsion, which have a very limited linear quantitative range, requires special considerations for reliable quantitation (Cross et al. 1974; Longshaw and Fowler 1978; Coe 1982; Franklin 1985). It has also been known by professional autoradiographers since the mid-1990s that commercial standards are not accurate in their reporting of the associated concentration values, and that recalibration is required as previously discussed. Investigators who are not trained in quantitative autoradiography to determine tissue concentrations of drugs and/or who did not fully research the pitfalls of the various techniques are prone to generating questionable tissue concentration data using autoradiography.

One such study reported the "quantitative autoradiography" of hippocampal glutamate NMDA receptor (NMDAR) loss in postmortem human brain samples obtained from an Alzheimer's patient (Kravitz et al. 2013). In that study, the authors exposed human brain sections to the surrogate markers ^{3}H-MK-801 (to label NMDARs), and ^{3}H-PK11195 [to label a translocator protein (TSPO)] in vitro. Then, they analyzed scanned images of x-ray film that had been exposed to the brain sections along with commercially available image calibration standards (a single 3-week exposure was used) using densitometry analysis software. These authors did not cite or discuss any other methods that would indicate that they followed the special considerations required for the use of x-ray films for quantitative image analysis. Despite those pitfalls, they concluded that there was a "highly significant negative correlation between NMDAR and TSPO in the hippocampal CA1 field, but that TSPO density in the basal

ganglia was positively correlated with NMDAR density". They suggested that "the response neuroinflammation is inherently different in hippocampus and basal ganglia and that hippocampal NMDAR expressing cells are preferentially vulnerable to neuroinflammation". Another study using these semi-quantitative autoradiographic methods reported "quantitative autoradiography of N-methyl-D-aspartate receptors (NMDARs) in the adult rat brain after repeated exposure to phencyclidine (PCP) in adolescence" by monitoring ^{3}H-MK-801 (Metaxas et al. 2014). In this paper, the authors also reported the use of x-ray and film and commercially available autoradiographic standards to characterize NMDAR density as it was related to PCP administration and behavior. It was clear from the description of the methods and lack of cited methods that they were probably not aware of the pitfalls and limitations of the methods they were using. Nevertheless, they relied on their "quantitative autoradiographic data" on NMDAR localization to conclude that the "differential effects of adolescent PCP administration on social and exploratory behaviors are not driven by alterations in NMDAR density, at least at the time of measurement". Another paper presented a comparison of in vivo magnetic resonance imaging (MRI) using a gadolininium-labeled bovine serum albumin (Gd-BAS) and a "terminal quantitative autoradiography" analysis, which used ^{125}I-serum albumin to measure the forward transfer constant (K_a^{trans}) of albumin in cerebral tumor in rats to characterize vascular permeability (Paudyal et al. 2011). This study more clearly stated the methods used, and clearly stated that commercially available "^{125}I microscales" were "used as received", and that x-ray film was also used for the "quantitative autoradiography" analyses. They also reported that "Upon inspection of the films, it was clear that the calibration standards, placed in adjacent parallel bars on a strip, exhibited substantial overlap on the radiographic film" and that "strongly elevated background due to adjacent and distant calibration standards of higher intensity" had an effect on image calibration. Furthermore, these investigators did not characterize the biodehalogenation of the ^{125}I-albumin used in their quantitative tissue concentration study, which has a big impact on quantitative autoradiography, as previously discussed. It appeared that these investigators did not have the necessary experience and/or knowledge of proper quantitative autoradiography techniques to realize that the data they were attempting to obtain were flawed in several critical ways (i.e., using an x-ray film detection media with limited quantitative linearity; using non-validated ^{125}I calibration standards for quantitation; and by not characterizing the instability of the ^{125}I radiolabel used for quantitation). These researchers observed several other problems they had in obtaining and comparing the quantitative autoradiography data and MRI data. Nevertheless, they went on to make quantitative comparisons, and concluded that "Nevertheless, such comparisons are useful in the ability to supply validation for MRI techniques". In the opinion of this reviewer, the interpretation and conclusions from this study are compromised due to these oversights. It is not possible to validate one technique against another if the baseline technique has supplied faulty data for comparison. In contrast, a well-executed QWBA study that revealed liver enrichment of Faldaprevir, which is a drug being developed by Boehringer-Ingelheim to treat hepatitis C virus, served as a comparator for an in vitro model called "HepatoPac" to predict liver enrichment and metabolism (especially regarding glucuronidation), and the authors concluded that Hepatopac was a superior model over the use of suspended hepatocytes for those types of studies (Ramsden et al. 2013).

Another recent study coupled the use of histochemical staining and an attempt at quantitative MARG to study the micro-distribution and kinetics of ^{131}I-huA33 antibody in patients with colorectal cancer (Ciprotti et al. 2014). These investigators infused patients with ^{131}I-huA33 and obtained tumor samples for analysis by gamma counting, x-ray autoradiography, and histochemical staining for GPA33 expression and CD31-positive blood vessels. The tumor samples for MARG were processed using paraffin-embedded tissue sections on glass slides that were co-exposed with ^{131}I and ^{125}I calibration standards to x-ray film, and then analyzed to determine tissue concentrations using densitometry software. These investigators did not monitor the in vivo stability of the ^{131}I- huA33, nor did they account for the possible loss of test article after tissue fixation and processing, which can dislocate and wash out soluble test articles. However, they did use a variety of exposure times which would enable the determination of a wider range of tissue concentrations than that provided by a single exposure. The co-registration of the histological images and autoradiographs provided a good means of comparison of semi-quantitative tissue concentration of the huA33 antibody versus morphological characteristics of the histological techniques, despite the neglected evaluation of the in vivo stability of the ^{131}I label. By targeting GPA33 and huA33, the researchers were able to show how well huA33 was taken up by cancer cells and provided support for the development of huA33 for targeted treatment of colorectal cancer.

History and experience teaches a lesson here. In the regulated setting of the pharmaceutical industry, corporate researchers rejected the use of x-ray film and the use of non-validated commercial standards for their routine tissue distribution studies because the resulting "quantitative" data was unreliable, required a long time to acquire data, and the quantitative methods would not pass the scrutiny of fellow analytical researchers. X-ray film whole-body autoradiography images were only used to support the organ homogenate concentration data obtained by organ dissection and assay by LSC. Thus, it was not until the advent of phosphor imaging, with its superior linear range for quantitation, high resolution, and well-validated methods, that quantitative autoradiography was validated and accepted across the pharmaceutical industry

as a suitable replacement for regulated tissue distribution studies which were previously conducted using organ dissection, homogenization, and LSC techniques. The use of x-ray film for the determination of quantitative tissue concentration determinations is a technological step back, and investigators using those outdated techniques must realize that their entire study may be compromised if they do not have the experience to perform all the technical steps to assure data quality.

In contrast, several papers have reported the use of quantitative autoradiography to determine targeted receptor binding to characterize disease states. In some cases, investigators coupled histological images with serially obtained phosphor images to provide high resolution localizations at near-cellular levels in attempts to offer a view of quantitative microautoradiography. One such study performed in vitro autoradiography of carcinoembryonic antigen (CEA) in tissues of colorectal cancer patients and rats using ^{67}Ga-Ga-IMP461 and ^{68}Ga-Ga-IMP461 (Hall et al. 2012). The study provided visual high-resolution evidence that autoradiography and histochemical techniques offered highly specific complementary techniques to diagnosis biopsies for colorectal cancer, and went on to state that this pre-targeting technique using ^{67}Ga-labeled IMP and SPECT, or ^{68}Ga-labeled IMP and PET, might be useful for diagnosis of patients with colorectal cancer.

In a different study, investigators used phosphor imaging and re-calibrated commercial standards to determine the concentration of tritium-labeled Cl-994 (^{3}H-Cl-994), which is a benzamide that inhibits class I histone deacetylase (HDAC) (Wang et al. 2013). The goal of this study was to use ^{3}H-Cl-994 as a probe for the ex vivo binding to, and thus localization of, HDAC in the rat brain. These researchers also used western blot analysis of rat brain samples to examine and compare HDAC expression in various regions of the brain to the data they obtained by quantitative autoradiography. They showed compelling quantitative autoradiographic data to support the use of ^{3}H-Cl-994 as a probe for examining the regional variations in HDAC density in preserved postmortem human brain samples, which would be important in choosing compounds under development as in vivo PET imaging agents to diagnose various epigenetic brain defects, such as contextual memory defects, and certain neurotoxicities, among other applications.

Conclusions

This review of the recent literature has shown that the use of quantitative autoradiography remains one of the most powerful and highest resolution techniques to quantitatively determine the concentration of drug-derived radioactivity in all tissues of laboratory animals. These studies are relied upon by drug discovery and development companies, and by regulators, due to the robust and reliable results provided when the studies are performed properly. However, this reviewer has observed an increase in the number of studies that reported the use of typically unstable radiolabeled compounds (e.g., ^{125}I-proteins, ^{3}H-compounds) without characterizing the in vivo stability of label, and the use of x-ray film and non-validated autoradiographic image calibration standards for determining tissue concentrations of radiolabeled compounds in vivo. This may be an unfortunate result of the decreasing number of professional autoradiographers in recent years due to the consolidation of those areas in the pharmaceutical industry. Quantitative autoradiography relies on the careful control of each step in the seemingly straightforward process, and small oversights can result in large errors in quantitation.

Molecular imaging has grown dramatically over the last 50 years, and most recently in vivo imaging modalities, such as PET (Morris et al. 2005) and fluorescence (Gross and Piwnica-Worms 2006) imaging, have captured the attention of scientists in drug discovery and development owing to the ability to visualize organ in vivo distribution in preclinical species. However, these new in vivo modalities have their limitations, such as relying on the use of short-lived isotopes as in PET imaging, which limits the ability to study tissue pharmacokinetics over days or weeks, or encountering issues with reliable quantitation due to background fluorescence and poor resolution, which occurs when imaging fluorescent tags in vivo. Additionally, the positron-emitting isotopes for PET and the fluorescent labels used can alter the structure and/or binding capabilities of molecules, thus rendering them ineffective at providing the needed information. The inability to distinguish between labeled drug, metabolite(s), and/or impurities is also a limitation of these modalities. However, with the recent developments in MALDI MSI, DESI, SIMS technologies (Solon et al. 2010), which rely on the use of whole-body sections, the ability to distinguish between the parent molecule and metabolites is possible. Unfortunately, these technologies have not reached the point of being quantitative, which means that QWBA is expected to remain the technique of choice for the reliable quantitation of drug concentrations in tissues. The resurgent interest in the use of MARG and the future development of reliable quantitation of drug concentrations at the cellular level remains to be realized, but new technology and research has begun to make this a possibility in the future. To these ends, ex vivo, in situ imaging modalities, such as QWBA and MARG, will undoubtedly be relied upon for many years to come to provide the kind of detailed tissue distribution information required for drug discovery and development programs.

References

Appleton TC (1964) Autoradiography of soluble labeled compounds. J R Microsc Soc 83:277–281

Baker JRJ (1989) Autoradiography: A Comprehensive Review. Royal Microscopical Society, Microscopy Handbooks 18. Oxford University Press, p 30–32

Blackett NM, Parry DM (1973) A new methodfor analyzing electron microscope autoradiographs using hypothetical grain distributions. J Cell Biol 57:9–15

Brown LA (2013) Pharmacology/Toxicology Primary Discipline Review; Subject: STN 125466/0 - Novo Nordisk;s Original Biological License Application (BLA) NovoEight®, Antihemophilic Factor (Recombinant) Plasma/albumin Free beta (β)-domain deleted (BDD); FDA File (original BLA STN 125466/0

Charpak G, Imrie D, Jeanjean J, Miné P, Nguyen H, Scigocki D, Tavernier SPK, Wells K (1989) A new approach to positron emission tomography. Eur J Nucl Med 15:690–693

Christensen J, Litherland K, Faller T, van de Kerkhof NF, Hunziker J, Krauser J, Swart P (2013) Metabolism studies of unformulated internally [^{3}H]-labeled short interfering RNAs in mice. Drug Metab Dispos 41(6):1211–1219

Ciprotti M, Chong G, Gan HK, Chan A, Murone C, MacGregor D, Lee F-T, Johns TG, Heath JK, Ernst M, Burgess AW, Scott AM (2014) Quantitative intratumoural microdistribution and kinetics of ^{131}I-huA33 antibody in patients with colorectal carcinoma. EJNMMI Res 4:22

Coe RAJ (1982) An evaluation of X-ray films suitable for autoradiographs using ß^{14}C radiation. Int J Appl Radiat Isot 36:93–96

Cross SAM, Groves AD, Hesselbo T (1974) A quantitative method for measuring radioactivity in tissues sectioned for whole body radiography. Int J Appl Radiat Isot 25:381–386

Dain JD, Collins JM, Robinson WT (1994) A regulatory and industrial perspective of the use of carbon-14 and tritium isotopes in human ADME studies. Pharm Res 11(6):925–928

Davenport L (2013) Teriflunomide: no effects on sperm. Poster at 29th Congress of European Committee for Treatment and Research in Multiple Sclerosis October 2–3, 2013, Copenhagen, Denmark

Downs AM, Williams MA (1984) An improved approach to the analysis of autoradiographs containing isolated sources of simple shape: method, theoretical basis and reference data. J Microsc 114:143–156

Flitney FW (1969) Tritium-labelled Araldite as an internal standard for quantitative autoradiography using the electron microscope. J Microsc 90(2):151–156

Franklin ER (1985) The use of measurements of radiographic film response of X-ray film in quantitative and semi-quantitative autoradiography. Int J Appl Radiat Isot 36:193–196

Gross S, Piwnica-Worms D (2006) Molecular imaging strategies for drug discovery and development. Curr Opinions Chem Biol 10(4):33442

Haglund J, Borg N (2013) ADME characterization in rtas revealed immediate secretion of AZD7903 into the stomach after IV dosing. Xenobiotica 43(9):823–835

Hall H, Velikyan I, Blom E, Ulin J, Monazzam A, Påhlman L, Micke P, Wanders A, McBride W, Goldenberg DM, Långström B (2012) In vitro autoradiography of carcinoembryonic antigen in tissue from patients with colorectal cancer using multifunctional antibody TF2 and $^{67/68}$Ga-labeled haptens by pretargeting. Am J Nucl Med Mol Imaging 2(2):141–150

Héroult M, Steinke W, Frisk A-L, Borkowski S, Meyer K, Petrul H, Heisler I, Quanz M, Neuhaus, Buchmann B, Miller T, Bauser M, Hägenbarth A, Brands M, Ziegelbauer K (2014) Effects of selective and broad glucose transporter inhibition on glucose distribution in tumor-bearing mice. Poster at Annual Meeting of the American Association for Cancer Research, San Diego

Herzog E, Harris S, McEwen A, Henson C, Pragst I, Dickneite SS, Zollner S (2014) Recombinant fusion protein linking factor VIIa with albumin (rVII-FP): tissue distribution in rats. Thromb Res 134(2):495–502. doi:10.1016/j.thrombres.2014.05.031

Hesk D, Koharski D, Saluja S (1997) In: Synthesis and applications of isotopically labeled compounds. Wiley, New York

Jeavons AP, Hood K, Herlin O (1983) The high density avalanche chamber for positron emission tomography. IEEE Transcripts Nucl Sci 30:640–645

Kim H, Prelusky D, Wang L, Hesk D, Palamanda J, Nomeir A (2002) The importance of radiochemical analysis of biological fluids before and after lyophilization from animals dosed with [^{3}H]-labeled compounds in drug discovery. Am Pharm Rev 7:44–48

Kolbe H, Dietzel G (2000) Technical validation of radioluminography systems. J Regul Toxicol Pharmacol 31(2):S5–S14

Kolkhof P, Delbeck M, Kretschmer A, Steinke W, Hartmann E, Bärfacker L, Eitner F, Albrecht-Küpper B, Schäfer S (2014) Finerenone, a novel selective nonsteriodal mineralocortoid receptor antagonist protects from rat cardiorenal injury. J Cardiovasc Pharmacol 64(1): 69–78

Kravitz E, Gaisler-Salomon I, Biegon A (2013) Hippocampal glutamate NMDA receptor loss tracks progression in Alzheimer's disease: quantitative autoradiography in postmortem human brain. PLoS ONE 8(11):e81244

Lacassagne A, Lattes J (1924) R'éparitiondu polonium (injecté sous la peau) dans l'organisme de rats porteurs de griffes cancereuses. C R Séance Soc Biol 90:352–353

LeBlanc B, Jezequel S, Davies T, Hanton G, Taradach C (1998) Binding of drugs to eye melanin is not predictive of ocular toxicity. Regul Toxicol Pharmacol 28:124–132

Liquier-Milward J (1956) Electron microscopy and radioautography as coupled techniques in tracer experiments. Nature 177:619

Longshaw S, Fowler JSL (1978) A poly (methy ^{l4}C) methacrylate source for use in whole-body autoradiography and beta-radiography. Xenobiotica 8:289–295

Luckey G (1975) US Patent 3:859,527

Märs U, d'Argy R, Hallbeck K, Miller-Larsson A, Edsbäcker S (2013) Tissue accumulation kinetics of ciclesonide-active metabolite and budesonide in mice. Basic Clin Pharmacol Toxicol 112(6):401–411

Metaxas A, Willems R, Kooijman EJM, Renjaän VA, Klein PJ, Windhorst AD, Ver Donck L, Leysen JE, van Berckel BNM (2014) Subchronic treatment with phencyclidine in adolescence leads to impaired exploratory behavior in adult rats without altering social interaction or N-Methyl-D-aspartate receptor binding levels. J Neurosci Res. doi:10.1002/jnr.23433

Mizoguchi K, Kanno H, Ikarashi Y, Kase Y (2014) Specific binding and characteristics of 18b-Glycyrrhetinic. Acid in rat brain. PLoS ONE 9(4):e95760

Morris ED, Yoder KK, Wang C, Normandin MD, Zheng QH, Mock B, Muzic RF Jr, Froehlich JC (2005) ntPET: a new application of PET imaging for characterizing the kinetics of endogenous neurotransmitter release. Mol Imaging 4(4):473–489

Motie M, Schaul KW, Potempa LA (1998) Biodistribution and clearance of 125I-labeled C-reactive protein and ^{125}I-labeled modified C-reactive protein in CD-1 mice. Drug Metab Dispos 26(10):977–981

Nakatomi Y, Tsuji M, Nakashima T, Gokudan S, Miyazaki H, Tomokiyo K, Ogata Y, Harano S, Matsui H, Shigaki T, Nakamura T, Mogi M (2012) Pharmacokinetics, distribution, and excretion of ^{125}I-labeled human plasma-derived-FVIIa and -FX with MC710 (FVIIa/FX mixture) in rats. Thromb Res 129:62–67

Paudyal R, Ewing JR, Nagaraja TN, Bagher-Ebadian H, Knight RA, Panda S, Lu M, Ledbetter K, Fenstermacher JD (2011) The concerdance of MRI and quantitative autoradiography estimates of the trasnvascular transfer rate constant of albumin in a rat brain tumor model. Magn Reson Med 66(5):1422–1431

Ramsden D, Tweedie DJ, St George R, Chen L-Z, Li Y (2013) Generating an in-vitro-in vivo correlation for metabolism and liver enrishment of a hepatitis C Virus drug, Faldaprevir, using a rat hepatocyte mode (HepatoPac). Drug Metab Dispos 42(3):407–414

Rind HB, Butowt R, von Bartheld CS (2005) Synpatic targeting of retrogradely transported trophic factors in motorneurons: comaprison of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and cardiotrohon-1 with tetanus toxin. J Neurosci 25(3):539–549

Robbie SJ, Lundh von Leithner P, Ju M, Lange C, King AG, Adamson P, Lee D, Sychterz C, Coffey P, Ng Y-S, Bainbridge JW, Skima DT (2013) Assessing a novel depot delivery strategy for non-invasive administrationof VEGF/PDGF RTK inhibitors for ocular neovascular disease. Investig Ophthalmol Vis Sci 54(2):1490–1500. doi:10.1167/iovs. 12-10169

Salpeter MM, Bachmann L, Salpeter EE (1969) Resolution in electron microscope radioautography. J Cell Biol 41:1–40

Solon E (2007) Autoradiography: high-resolution molecular imaging in pharmaceutical discovery and development. Expert Opin Drug Discov 2(4):503–514

Solon EG (2012) Use of radioactive compounds and autoradiography to determine drug tissue distribution. Chem Res Toxicol. doi:10.1021/tx200509f

Solon EG, Kraus L (2002) Quantitative whole-body autoradiography in the pharmaceutical industry. Survey results on study design, methods and regulatory compliance. J Pharmacol Toxicol Methods 43:73–81

Solon E, Lee F (2002) Methods determining phosphor imaging limits of quantitation in whole-body autoradiography rodent tissue distribution studies affect predictions of ^{14}C human dosimetry. J Pharmacol Toxicol Methods 46:83–91

Solon EG, Schweitzer A, Stoeckli M, Prideaux B (2010) Autoradiography, MALDI-MS, and SIMS-MS imaging in pharmaceutical discovery and development. AAPS J 12(1):11–26

Solon E, Lordi A, Lander J, Shen H, (2013) Important considerations for the use of ^{125}I-labeled proteins to examine ADME characteristics of iodinated compounds. 2013 AAPS National Biotechnology Conference. San Diego, CA

Stumpf WE (2003) Drug localization in tissues and cells. Library of Congress Control Number 2003105179. IDDC Press

Stumpf WE, Roth LJ (1964) Vacuum freeze drying of frozen sections for dry-mounting high resolution autoradiography. Stain Technol 39:219–223

Ullberg S (1954) Studies on the distribution and fate of ^{35}S-Labelled benzylpenicillin in the body. Acta Radiol Suppl 118:1–110

Ullberg S (1977) The technique of whole-body autoradiography: cryosectioning of large specimens. In: Elvefeldt O (Ed.) Special issue on whole-body autoradiography. Sweden LKB Instr. J. Science Tools, Bromma

Venturi S, Venturi M (1999) Iodide, thyroid and stomach carcinogenesis: evolutionary story of a primitive antioxidant? Eur J Endocrinol 140:371–372

von Bartheld CS (2001) Tracing with radiolabeled neurotrophins. Methods Mol Biol 169:195–216

Walker MD, Goorden MC, Dinelle K, Ramakers RM, Blinder S, Shirmohammad M, van der Have F, Beekman FJ, Sossi V (2014) Performance assessment of a preclinical PET scanner with pinhole collimation by comparison to a coincidence-based small-animal PET scanner. J Nucl Med 55(8):1368–1374. doi:10.2967/jnumed.113.136663

Wang Y, Zhang Y-L, Hennig K, Gale JP, Hong Y, Cha A, Riley M, Wagner F, Haggarty SJ, Holson E, Hooker J (2013) Class I HDAC imaging using [^{3}H]CI-994 autoradiography. Epigenetics 8(7):756–764

Ward PD, La D (2014) Testicular distribution and toxicity of a novel LTA4H inhibitor in rats. Toxicol Appl Pharmacol 278(1):26–30

Williams MA (1969) In: Advances in optical and electron microscopy. Barer R, Cosslett VE (eds) Advances in optical and electron microscopy, vol 3. Academic, New York, pp 219–272

Woodburn KW, Fong KL, Wilson SD, Sloneker S, Strzmeinski P, SolonE MY, Tagawa Y (2013) Peginesatide clearance, distribution, metabolism, and excretion in monkeys following intravenous administration. Drug Metab Dispos 41(4):774–784

Yue Q, Mulder T, Rudewicz PJ, Solon E, Budha N, Ware JA, Lyssikatos J, Hop CE, Wong H, Khojasteh SC (2013) Evaluation of metabolism and disposition of GDC-0152 in rats using ^{14}C labeling strategy at two different positions: a novel formation of hippuric acid from 4-phenyl-5-amino-1,2,3-thiadiazole. Drug Metab Dispos 41(2):508–517

Zane PA, Brindle SD, Gause DO, O'Buck AJ, Raghavan PR, Tripp SL (1990) Physicochemical factors associated with binding and retention of compounds in ocular melanin of rats: correlations using data from whole-body autoradiography and molecular modeling for multiple linear regression analyses. Pharmacol Res 7(9):935–941