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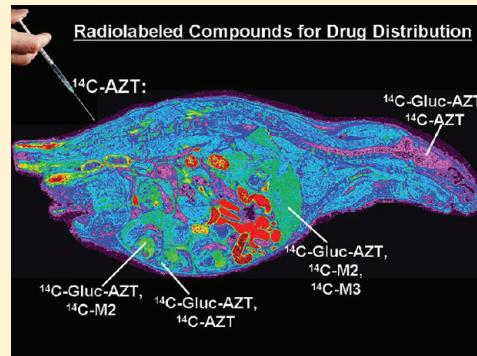
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Use of Radioactive Compounds and Autoradiography to Determine Drug Tissue Distribution

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ABSTRACT: Radioactivity has been used in drug discovery and development for several decades because it offers researchers a highly sensitive way to quantitatively assess the absorption, distribution, metabolism, and/or excretion (ADME) of chemical entities by incorporating a radioactive isotope into the structure of the drug molecule. Regulatory agencies around the world require drug makers to characterize the ADME properties of prospective new drugs as one way to help ensure that patients are not exposed to dangerous drug and/or drug metabolite levels before they can be approved for human use. Radiolabeled compounds have consistently proved to be the most efficient tool for determining that information, even though attempts have been made to use nonradioactive techniques. The techniques of quantitative whole-body autoradiography (QWBA) and microautoradiography (MARG), which rely on the use of radiolabeled drugs, are two techniques that are routinely used to examine tissue distribution of drugs in discovery and development. These techniques provide drug researchers with quantitative tissue concentration data and a visual location of those concentrations in intact organs, tissues, and cells of laboratory animals. It is important for readers to realize that these techniques visualize total radioactivity, which can include the parent molecule along with its metabolites, and/or degradation products or impurities. This requires investigators to treat the quantitative data with caution unless the identity of the radioactivity is determined using some type of other bioanalytical techniques, such as mass spectroscopy and/or radio-HPLC, which can be easily performed on the tissue obtained from the animals used for QWBA and/or MARG. Nevertheless, these data are used in drug discovery and development to answer questions related to tissue penetration, fetal/placental transfer, tissue retention, routes of elimination, drug–drug interactions, enzyme induction/inhibition, formulation comparisons, *in vivo* compound solubility, differential metabolite distribution, interspecies comparisons, and to predict human exposure to parent drugs, metabolites, and radiation during clinical studies. This review will consider the strategic use of WBA, QWBA, and MARG in the pharmaceutical industry. Case studies and anecdotal information will also be presented; however, readers should realize that these are general examples and that some details have been omitted for brevity and/or because the data is proprietary and could not be presented at this time. Nevertheless, the images and discussions are provided to demonstrate how the techniques can and have been used to examine *in situ* tissue distribution of therapeutic compounds.



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incorporating a radioactive isotope into the structure of the drug molecule. Regulatory agencies around the world require drug makers to characterize the ADME properties of prospective new drugs as one way to help ensure that patients are not exposed to dangerous drug and/or drug metabolite levels before they can be approved for human use. Drug makers have found that they can increase their odds of developing a new drug by improving/optimizing the ADME profile of their drugs, which had been the cause of approximately 40% of test drug failure in the past.¹ International cooperation and harmonization regarding the development and registration of new drugs began in earnest in the early to mid 1980s, and today, organizations such as the World Health Organization (WHO) and the International Commission on Radiological Protection (ICRP)

INTRODUCTION

Radioactivity has been used in drug discovery and development for several decades because it offers researchers a highly sensitive way to quantitatively assess the absorption, distribution, metabolism, and/or excretion (ADME) of chemical entities by

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have been coordinating international efforts to establish some international guidelines for the safe development of new drugs.² Roffey et al.³ outlined the following 4 critical questions that should be addressed by radiolabeled studies: "(1) Is the proposed clearance mechanism sufficiently supported by the identities of the drug related materials in excreta, so as to provide a complete understanding of clearance and potential contributors to inter-patient variability and drug–drug interactions? (2) What are the drug-related entities present in circulation that are the active principals contributing to primary and secondary pharmacology? (3) Are there findings (low extraction recovery of radiolabel from plasma, metabolite structures indicative of chemically reactive intermediates) that suggest potential safety issues requiring further risk assessment? (4) Do questions 2 and 3 have appropriate preclinical support in terms of pharmacology, safety pharmacology and toxicology?" Radiolabeled compounds have consistently proved to be an efficient tool for helping to answer these questions; however, researchers are attempting to utilize newer techniques, such as compounds labeled with fluorescent tags,⁴ and mass spectroscopy (MS) techniques to study ADME characteristics, but these techniques have limitations. Currently, fluorescent tags cannot provide reliable quantitation owing to variable tissue background and a lack of image resolution. However, today fluorescent compounds are widely used as an *in vivo* imaging tool in drug discovery to screen such things as the localization of compounds in target organs and tumors of xenograft animal models. Another limitation of fluorescently labeled compounds is that sometimes the fluorescent tag can alter the activity and/or binding of the compound due to the size and possible chemical modifications. Consequently, the pharmaceutical activity of each fluorescently labeled compound should be verified before it is used to study ADME characteristics to ensure that accurate data will be obtained. Mass spectroscopy is also used to gather ADME information related to quantitation and molecular identification, but its use is also limited.⁵ Although reliable quantitation can be obtained using MS, it requires much more work to get that data because appropriate standards are required, and more resources are needed to process individual organ homogenates to determine individual tissue concentrations. Furthermore, the extraction efficiency and characterization of different matrix effects on quantitation need to be evaluated for each organ, and the process does not offer true tissue level quantitative data, such as that obtained using radiolabeled compounds and autoradiography imaging techniques. However, in the last 10–15 years matrix-assisted laser desorption imaging MS (MALDI-MS) has been gaining attention as a potential technique to identify both parent compounds and/or their metabolites in whole-body and individual tissue sections.⁶ Currently, this technology provides the ability to identify the location of different chemical entities and high resolution images of biological samples, but reliable and routine quantitation has not been possible. To this end, the use of radiolabeled compounds to determine ADME characteristics has remained the best option because it provides the most efficient way to visually localize and quantify the amount of a chemical entity throughout the body and in discrete organs, tissues, and cells of animals used for drug research. Of course, radiolabeled studies have their limitations, among which is the fact that the data provided reflect the distribution of both the parent compound as well as any radiolabeled metabolites. To that end, quantitative data must be viewed with some caution and that understanding. Nevertheless, the techniques of quantitative whole-body

autoradiography (QWBA) and microautoradiography (MARG) have become widely used in drug discovery and development to provide unmatched quality for determining the concentration and localization of test compounds in biological samples.

Autoradiography is an eloquent collection of high resolution imaging techniques that enable the qualitative and quantitative assessments of drug localization in lab animals. Applications of the techniques have been used to support drug discovery and development for over 60 years in both academia and industry. Qualitative and quantitative whole-body autoradiography (WBA and QWBA, respectively) and microautoradiography (MARG) are widely used techniques to study the distribution of radiolabeled compounds in organs, tissues, and cells of lab animals.

WBA typically refers to the original technique, which relied on a film detection system/imaging system and was developed by Sven Ullberg, who published the first WBA study on the *in situ* biodistribution of ³⁵S-penicillin in 1954.⁷ The basic technique of WBA entails the administration of a radiolabeled test compound [most often hydrogen-3 (³H) or carbon-14 (¹⁴C) isotopes] to laboratory animals (e.g., rodents and nonhuman primates) followed by euthanasia and freezing of the carcasses of animals at different time points after drug administration. The frozen intact carcasses are cryosectioned (at 30–50 μm thickness) at different levels through the carcass to capture all tissues, and the sections are imaged using X-ray film. The technique remained qualitative for about 25 years because film is not a linear detection system that could be easily used for quantitation. It was not until after 1979 when phosphor imaging technology⁸ enabled the quantitative imaging of beta radiation in solid samples. The technology was applied to imaging radiolabeled drugs in whole-body sections, and results were obtained in a much shorter period of time than that for film (e.g., days versus weeks or months). QWBA, which was possible with digital phosphor imaging, was quickly adopted and validated by the pharmaceutical industry, and it has all but replaced the classical analysis of organ homogenates by liquid scintillation counting (LSC).^{9,10} QWBA now provides quantitative distribution information about drugs at the tissue level in intact whole-body specimens. QWBA has been widely validated and accepted as a robust quantitative technique to determine the tissue distribution of new drug entities, and it is now accepted and currently often requested by regulatory agencies around the world.

MARG is an autoradiographic histology technique that utilizes photographic detection media (e.g., nuclear/photographic emulsion) to spatially locate drug-derived radioactivity at the cellular level in organ/tissue samples that have been removed from lab animals given radiolabeled test compounds. Fresh tissue or organ samples are snap-frozen, cryosectioned (at 4–10 μm thickness), and exposed to nuclear imaging emulsion that has been applied to glass microscope slides.¹¹ The sections are allowed to expose the emulsion-coated slides, and then the slides are developed like film, stained, and examined under a light microscope to visually identify the localization of the drug-derived radioactivity at the microscopic level.

This review will consider the strategic use of WBA, QWBA, and MARG in the pharmaceutical industry. Case studies and anecdotal information will also be presented. Although many of the details of each case study are not presented (to keep this review at a reasonable length), the examples presented demonstrate how the techniques have been used by many pharmaceutical companies to help answer questions during their discovery

and preclinical development programs as defined by those companies.

AUTORADIOGRAPHY STUDIES IN DRUG DISCOVERY

The pharmaceutical industry has been using WBA (and to a much lesser extent MARG) since it was first introduced in the mid 1950s; however, it was not used extensively because it usually took months to obtain the images, and the images could not be adequately analyzed to provide quantitative data. WBA images were often presented to support the organ homogenate LSC data and to provide clues about the relative distribution to tissues, which could not be provided by LSC of organ homogenates. Large pharmaceutical companies (e.g., Eli Lilly, Burroughs-Wellcome, Glaxo, Bristol-Myers Squibb, Parke-Davis, and Sandoz) and a limited number of large contract research organizations (e.g., Oread BioSafety Center, Corning Hazelton, and Institute of Whole-body Metabolism [Japan]) began using QWBA in drug discovery to help answer pivotal questions since the late 1980s. However, it was not widely used by the industry because the instrumentation was very expensive, and only the largest of companies could afford it. These large companies often also had the resources to radiolabel their own compounds, and the results were not often published. Therefore, the utility and value remained largely unknown by many researchers outside of large pharmaceutical companies. However, in the early to mid-1990s the Society for Whole-Body Autoradiography (SWBA) and the European Autoradiography Club (now called the European Society for Autoradiography [ESA]) raised awareness about the value of WBA and QWBA in pharmaceutical research, and regulators began asking for these studies to support drug registration. At the 1994 meeting of the SWBA, the utility of QWBA was obvious, and presentations ranged from the disposition of ^{14}C - β -guanidinopropionic acid in rats¹² to the determination of ^{14}C -risedronate in rat bone.¹³ The use of MARG was also discussed at these early meetings, and several examples were presented by scientists from Monsanto¹⁴ and laboratories at SUNY Stony Brook and Brookhaven National Laboratory,¹⁵ the latter of which discussed the use of MARG to study the prevention of renal ischemia using ^{99}Tc -arginine-glycine-aspartic acid peptide. Although many of these presentations were rarely published, abstracts were circulated, and the use of QWBA and MARG quickly spread by word of mouth to scientists in the field. Since then the use of QWBA and MARG to support drug discovery efforts has increased, and it is now commonly used to screen lead compounds for progression to development and to answer specific questions related to tumors, brain, and skin penetration, fetal transfer, tissue retention, unusual routes of elimination (ocular, intestinal, saliva, sweat, and hair), hormone/enzyme redistribution due to drug effects, effects of enzyme induction/inhibition, drug-drug interactions, formulation comparisons, *in vivo* compound solubility, differential metabolite distribution, and interspecies comparisons to name a few.

In the 1970s and 1980s, the pharmaceutical industry began recognizing that most drugs failed in the development stage due to poor ADME characteristics.¹ To solve this problem, most large pharmaceutical companies increased their radiochemistry capabilities to provide radiolabeled compounds to support early preclinical drug development studies. Researchers quickly realized the benefits of identifying potential ADME issues at the discovery phase before much time and money were spent in the development phase. Furthermore, in-house radiochemistry

expertise enabled researchers to specifically label their compounds, and radiochemistry methods were improved to provide more stable radiolabeled compounds more quickly. This approach has saved companies millions of dollars that may have been spent performing expensive drug development studies by eliminating compounds with poor ADME characteristics early on. Today, it is a common strategy for companies to radiolabel lead discovery compounds with ^3H within 2 weeks and then run small but useful ADME studies, which now include QWBA. The use of ^3H (over ^{14}C) for discovery support is beneficial because it often takes less time to synthesize a ^3H -labeled compound. The image resolution obtained using ^3H is also better than that obtained using ^{14}C or other higher energy isotopes because the low energy β particle of ^3H does not travel as far from its source. However, much more ^3H must be administered (10-fold higher than ^{14}C) to help reduce the exposure time and to enable reliable image quantitation. A typical strategy has been to radiolabel the most promising discovery drug candidates and then to nominate the one with the best ADME characteristics for further development. Although the initial cost for QWBA instrumentation is high, the resources to conduct QWBA analysis is relatively low, but expertise in the technique is crucial. While sectioning and the other processing steps may only take a few months of regular practice, it can take many more months to develop a thorough knowledge of anatomy and a complete knowledge of the technique to fully interpret data and to address issues when they arise.

Discovery ADME studies are often designed to include two groups of animals. One group ($n = 3$) is used to determine mass balance and excretion patterns and another group to determine tissues distribution. The samples obtained from these experiments can also be used to examine metabolism profiles and identification. A typical group for QWBA analysis, which may utilize only 4–6 rats, may include both oral (po) and intravenous (iv) dose routes. Usually there is only 1 animal per time point and a few time points postdose (typically at the expected time of expected maximal plasma concentration and 24 h postdose), but this can provide a wealth of information. These studies may also include a third group of animals to provide individual tissues for possible MARG analysis, which could be conducted at anytime during discovery and/or development. A separate group of animals to supply tissue samples for MARG is needed because the freezing step used for QWBA is relatively slow and leads to poor microscopic morphology.

Discovery ADME studies are valuable because they not only help to answer specific questions but also provide a snapshot of overall tissue distribution that can be used to better design a more definitive tissue distribution study and can alert toxicologists and pathologists to potential safety issues. The image data can also be used later on during drug candidate selection to choose the discovery candidate with the best tissue distribution profile and/or to follow-up on new questions that often arise during discovery and development.

There has been a resurgent interest in the use of MARG in drug discovery, especially in the areas of ocular,¹⁶ brain,¹⁷ and skin¹⁸ distribution, where low but efficacious tissue concentrations can be localized at the cellular level to help scientists understand the action of their test compounds. This resurgence has been partially attributed to the availability of MARG as a contract service because the technique requires highly skilled scientists who are able to maintain their expertise by performing the procedures on a routine basis. The use of MARG requires a technical and scientific understanding of histology, drug disposition,

and the use of radioactivity. Histology expertise normally falls into the toxicology/pathology departments of pharmaceutical companies, and the use of radioactivity is unfamiliar and often not allowed. As a result, these studies are usually conducted in a drug metabolism/pharmacokinetic (DMPK) department where the scientists are familiar with using radioactivity, but unfortunately, most do not have a good understanding of histology and/or anatomy and physiology. This is another reason that MARG has been underutilized in the pharmaceutical industry.

QWBA and MARG have been used to study a variety of compounds that include antibodies, liposomal compounds, nanoparticles, oligonucleotides, peptides, proteins, radiopharmaceuticals, and small organic molecules.¹⁹ The need for radio-labeled compounds can affect the role of medicinal chemistry/process chemistry departments by drawing resources from the discovery of new compounds. Thus, large pharmaceutical companies often have dedicated radiochemistry laboratories that mainly serve the needs of the DMPK department. These departments may or may not be part of a larger medicinal chemistry department, but it is probably safe to say that medicinal chemistry departments and radiochemistry departments could benefit from regular interactions on projects to leverage data about their lead compounds and about bioanalytical method development and method transfers to gain corporate efficiency. Pharmaceutical companies that enjoy a positive and close relationship between the principal scientists in the two groups often coordinate their knowledge to enable the tracking of the parent drug plus its metabolites. They do this by working together to position the radioactive tag(s) at the optimal location(s) on the molecule to track expected or known metabolites as well as the parent drug. Pharmaceutical companies that do not have their own radiochemistry and/or DMPK laboratories would be well-served to choose a contract research organization (CRO) with DMPK staff experienced in working with radiochemistry laboratories so that the test article is labeled in the most useful position to examine ADME characteristics in the most economical but scientifically valid fashion.

The molecular structure and matching isotope must be suitable to provide both optimal image resolution and reliable quantification, which relies heavily on the radiopurity and stability of the radiolabel on the molecular entity. Radiopurity may be defined as the percentage of the radiolabeled test molecule versus the percentage of radioactive degradation products and/or other radioactive contaminants that can be present in a given batch of a radiolabeled compound. 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. Investigators must remember that all WBA, QWBA, and MARG results are a direct reflection of total radioactivity, and the images and quantitative results do not necessarily equate to the administered parent test compound. Radiopurity can be affected by the stability of the isotope label in the test molecule, and this should be considered when choosing which isotope should be used for labeling. If the radiolabel does not remain on the chemical entity *in vivo*, then the quantitative results will include radioactive counts from the labeled test article as well as unknown radioactive impurities. This can result in gross over or underestimations of the tissue concentrations of the drug and/or metabolites. Labeling small molecules with ¹⁴C at known positions usually results in the most stably labeled compounds, which is most preferable for autoradiographic studies, especially when quantitative tissue concentration data are needed.

In contrast, labeling of small molecules with ³H (and large molecules with ¹²⁵I) is often less stable, and it is crucial to monitor this *in vivo* when quantitative results are needed.²⁰

Small organic molecules are generally labeled with ¹⁴C or ³H,²¹ and larger molecules (e.g., proteins and peptides) are typically labeled using ¹²⁵I (and sometimes ³⁵S). Tritium-labeled compounds have the potential to undergo hydrogen exchange with water, and this possibility increases *in vivo*. However, talented radiochemists are often able to place the ³H in stable locations on the test molecules, which decreases the possibility of H-exchange. Nevertheless, it is necessary to verify and monitor the stability of the ³H label when used *in vivo* by obtaining urine and/or plasma from the animals being used for QWBA (and to a lesser degree for MARG and WBA).²² The extent of *in vivo* stability can be monitored by determining the concentration of radioactivity in fresh (wet) and evaporated samples of plasma and/or urine obtained from the animals used on the study. When the amount of ³H radioactivity in the wet samples is higher than that observed in dried samples, researchers must presume that ³H on the test compound has exchanged with H of endogenous water and that QWBA results must be corrected and/or interpreted with caution. Despite this limitation, the use of tritium has proved to be a valuable tool to support discovery programs because compounds can be tritiated more quickly than labeling with ¹⁴C (often within 2 weeks), and experienced radiochemistry laboratories often synthesize tritiated compounds with good stability. Furthermore, in some cases it is too difficult and/or costly to label a compound with ¹⁴C, and in these cases, the researchers have little choice but to use a tritiated compound for their definitive studies to support regulatory submissions.

The stability of ¹²⁵I on large molecules is also an issue that requires attention, especially with the growing need to understand the disposition of biological drugs. Similar to ³H, but even more common, is the instability of ¹²⁵I labeling on large molecules where cleavage *in vivo* is inevitable, and the production of free ¹²⁵I is practically guaranteed. This means that the radioactivity imaged will undoubtedly reflect some free ¹²⁵I, which needs to be accounted for accurate interpretation of the results. To this end, ¹²⁵I autoradiography of labeled biopharmaceuticals is semiquantitative at best, and it requires careful consideration, especially if quantitative data are sought. Additionally, care must be taken when interpreting tissue concentrations of ¹²⁵I-labeled compounds in the epidermis, choriod plexus, kidneys, mammary gland, salivary gland, stomach, thymus, and thyroid, which contain a sodium-iodide symporter that is involved in the organization and/or elimination of free ¹²⁵I.^{23,24} These tissues often have relatively high concentrations of free and/or organized ¹²⁵I, which are not drug-related and thus provide misleading results. The administration of nonradiolabeled sodium-iodide to test animals prior to dosing with the ¹²⁵I test article is one way to reduce the uptake of free ¹²⁵I by these issues and thus reduces the confounding effects of measuring free ¹²⁵I. This also helps to shunt free ¹²⁵I to the kidneys, thus facilitating elimination, which also reduces the influence of free ¹²⁵I on tissue quantitation. Researchers often use trichloroacetic acid (TCA) protein precipitation of plasma collected from animals used for QWBA to determine the ratio of protein-bound ¹²⁵I versus free ¹²⁵I to characterize their ¹²⁵I-labeled test article *in vivo*.²⁵ That procedure presumes that all protein-bound ¹²⁵I is bound to the test article, but to positively identify the protein-bound ¹²⁵I, a binding assay, gel analysis, specialized mass spectroscopy technique, or some other analysis should be performed. The ratio of unbound to

free ^{125}I can be used to normalize tissue concentrations obtained using QWBA, but it does not account for the further possibility of *in vivo* binding of free ^{125}I to animal proteins nor does it distinguish between parent drug, metabolites, and degradation products. Tissue quantitation of drug-derived radioactivity using ^{125}I and QWBA or gamma counting techniques can, at best, be considered as a semiquantitative technique, although it is one of the only practical techniques currently available for examining tissue distribution of large molecules.

■ DRUG DISCOVERY QWBA/MARG CASE STUDIES

This section presents examples of how autoradiography has been used to support drug discovery programs, as defined by the respective pharmaceutical companies, and/or nonregulated preclinical development studies used to support company claims. QWBA is an excellent tool to understand how chemical entities are absorbed and eliminated from not only the body but also, most importantly, into and out of discrete tissue compartments, which include target organs/tissues. Many researchers are familiar with the analysis and utility of plasma PK as an indicator of drug exposure; however, target tissue PK profiles are often very different from plasma PK. Dynamic and interacting factors such as the physicochemical properties of the compound, plasma protein binding, tissue-specific drug transporters, varying tissue and biological fluid concentrations, tissue specific metabolism, and various biological and physiological processes make the prediction of specific *in vivo* tissue distribution and tissue pharmacokinetics impossible. Furthermore, individual tissue PK parameters vary widely, and modeled generalizations about tissue distribution based on the physicochemical properties²⁶ cannot be relied upon to describe what actually occurs *in vivo*. Discovery QWBA and MARG studies can help scientists by providing *in situ* drug concentration data specifically for target tissues. In addition to gaining information about tissue PK, QWBA not only provides information about renal and biliary excretion, but it has the ability to show other routes of elimination that may or may not be expected during early discovery programs. These routes may include intestinal secretion, hair, tears, saliva, seminal fluid, lymphatic fluid, cerebrospinal fluid, and/or sweat. Although intestinal secretion and the role of transporters are now well-understood and are routinely studied using *in vitro* techniques such as Caco-2 cell preparations, QWBA has the advantage of using an *in vivo* model, which provides researchers with quantitative and spatial data related to the actual *in vivo* situation, which can vary greatly among discovery drug candidates. Furthermore, the *in vivo* dynamics of the secretion (and possible reuptake) can be visualized over time using QWBA, which cannot be done using *in vitro* models. Figure 1 shows an example of a drug that was observed to be secreted into the intestinal lumen after an iv dose. This small study was conducted to investigate why radioactivity was observed in feces of bile-duct cannulated rats given a single iv dose of a radiolabeled drug in a discovery mass balance study²⁷ before any *in vitro* assay was conducted. QWBA showed drug-derived radioactivity in the intestinal tract at early time points, which indicated that a small but significant portion of the drug-derived radioactivity was being secreted directly into the lumen of the gastrointestinal tract as well as in bile and urine.

QWBA samples can also provide tissue samples for analysis of metabolites that are present in various tissues over time to provide detailed information about the entire ADME profile of a test article. The use of whole-body sections and/or the

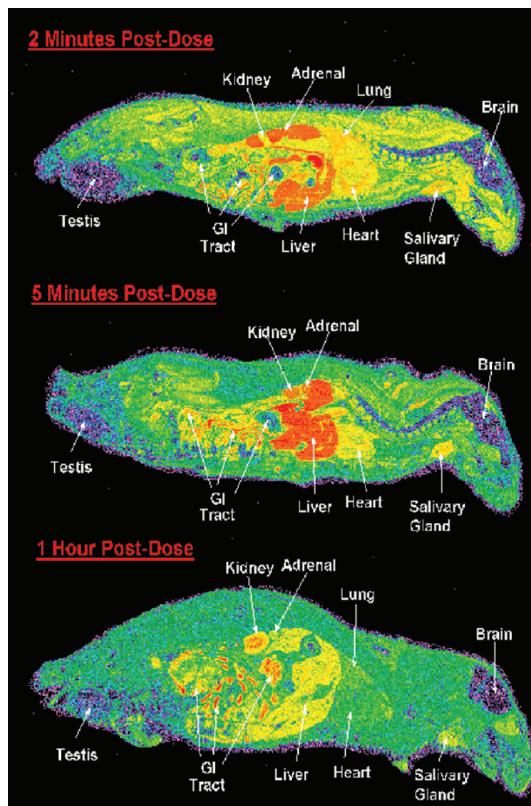


Figure 1. Intestinal secretion as shown by WBA. Autoradiograph of bile-duct cannulated rats given an iv dose of a ^{14}C -labeled drug. QWBA demonstrated intestinal secretion as a route of elimination for this compound early on in the discovery process before *in vitro* assays (e.g., Caco-2 cell model) were conducted. The red color in the gastrointestinal (GI) tract is a high amount of drug-derived radioactivity.

residual frozen tissue available from partially sectioned carcasses to supply tissue samples for bioanalysis using radio-HPLC and/or mass spectroscopy is nothing new but is often overlooked as an important and simple method to positively identify the chemical identity of the radioactivity imaged and quantified by QWBA. For example, a method development study was conducted at QPS, LLC to demonstrate how a small integrated study could provide preliminary ADME data related to paclitaxel (taxol) and its metabolites by combining QWBA, liquid chromatography and flow through scintillation radiodetection (LC/FSD), and liquid chromatography and dual mass spectroscopy (LC/MS/MS).²⁹ ^{14}C -taxol was given to rats, and blood, plasma, excreta and carcasses were collected for analysis of excretion and tissue distribution patterns. Samples of liver, kidney, spleen, heart, lung, skeletal muscle, and bone marrow were collected from the carcasses during sectioning for QWBA and were extracted and analyzed by LC/MS/MS to identify metabolites in tissues and plasma at each time point postdose. Figure 2 shows the QWBA results, which showed differential distribution of drug-related radioactivity in the kidney, liver, and brain at the later time points, which was helpful in further elucidating the metabolism.

QWBA also showed relatively high concentrations in the central nervous system and testis, which prompted further analysis that identified potentially neurotoxic metabolites. LC/FSD/MS/MS data showed that low specific activity hindered LC/FSD profiling; however, the absolute amounts of drug-derived

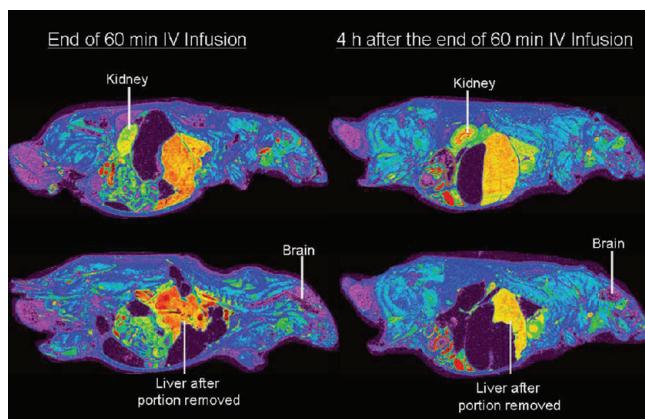


Figure 2. Autoradiographs of ^{14}C -taxol in rats after a 60 min infusion. Portions of liver, kidney, spleen, heart, lung, skeletal muscle, and bone marrow were removed from the residual partially sectioned carcasses and analyzed by LC/MS/MS and LC/radioflow detection to profile and identify metabolites.

compounds were adequate for LC/MS/MS identification. Two hydroxylated metabolites were found in low amounts in the liver, lung, kidney, and blood and an unknown metabolite was found in urine. The developmental toxicity of taxol has been known for over a decade before this study was conducted; however, this example demonstrates how a small study that integrates the use of QWBA and LC/FSD/MS/MS can provide a lot of useful information to help guide further studies and to help point out areas of possible concern during further development.

Understanding the clearance of compounds is paramount to developing a new drug, and QWBA is an excellent technique that shows clearance and/or retention from every compartment of the body, and as such, it is useful in identifying tissue(s) that may pose potential safety risks and/or be beneficial for therapy. Figure 3 shows an example of how QWBA analysis of 2 rats

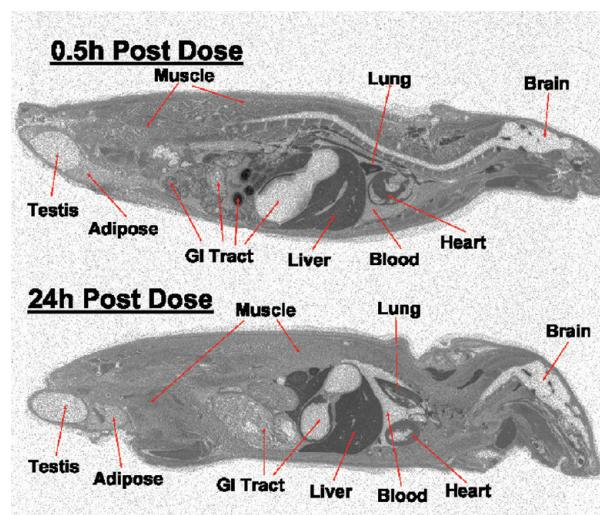


Figure 3. Retention of drug-derived radioactivity in rat tissues. Whole-body autoradiographs of two rats given similar iv doses of a ^{14}C -labeled compound sacrificed at 0.5 (a) and 48 h (b) postdose. Autoradiographs showed little or no decline in the amount of drug-derived radioactivity in tissues at 48 h postdose, while blood concentrations were below quantifiable limits.

clearly demonstrated the retention of a discovery drug candidate that showed poor recovery in a discovery mass balance

study. In this example, plasma data alone showed clearance from that compartment within 24 h, but QWBA images and tissue concentration data showed retention of drug-derived radioactivity in practically all tissues. In this case, the project team stopped further discovery efforts on this compound and focused efforts on another compound in the same class that did not display this characteristic.²⁸ It is important to mention that tissue retention may also be a benefit when prolonged exposure is necessary, although the dosing regimen and patient dosing compliance must also be carefully considered when developing a drug with such a prolonged exposure profile.

Conversely, QWBA can also reveal the tissue distribution characteristics of high clearance drugs and/or the effects of enzyme induction, which may end or prolong further development of the drug. Figure 4 shows the resulting distribution

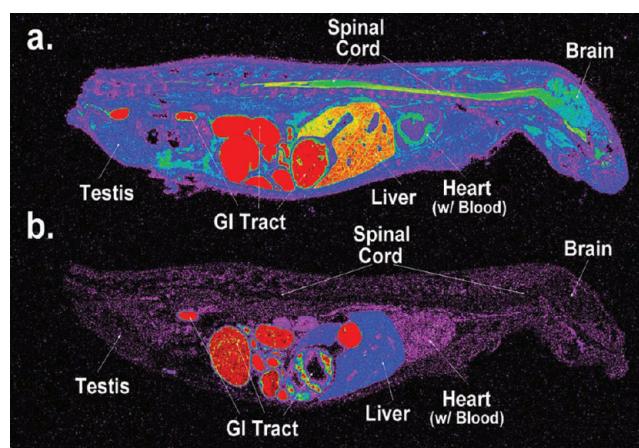


Figure 4. Autoradiographs of a ^{14}C -drug in rat before and after enzyme autoinduction and tissue distribution. (a) Whole-body autoradiograph of drug-derived radioactivity in a rat at 24 h after a single oral dose of a ^{14}C -labeled drug. (b) Whole-body autoradiograph of drug-derived radioactivity in a rat at 24 h after the last of five daily oral doses of the same drug (days 1–4, unlabeled drug administered; day 5, ^{14}C -labeled drug administered).

pattern of a drug that induced its own metabolism and clearance.²⁸ The data obtained at 24 h after the last of five daily oral doses of the same drug showed that the elimination after multiple dosing was more rapid than after a single dose, and thus, tissue exposure was notably lower. In most cases, this might end further development of the compound; however, concentrations in the heart, liver, and intestinal tract were present, which may be high enough to be efficacious for certain therapeutic treatments.

QWBA is often used in drug discovery to answer a variety of questions and/or to understand issues related to drug–drug interactions, inhibition, and induction of metabolism, and the effects of drug transporters. These studies may utilize genetically modified animals such as *p*-glycoprotein knockout models or the intentional use of specific drugs that are known to induce or inhibit specific metabolic enzymes. Figure 5 shows the effect of aminobenzotriazole (ABT), which is an indiscriminate inhibitor of P450 enzymes, on the tissue distribution of an aromatase inhibitor designed to treat cancer.³² This example showed researchers that a metabolite of the drug was able to penetrate the brain, which was an intended target, while the intact parent compound did not. This could be a benefit or liability depending on the nature of the metabolite, but this

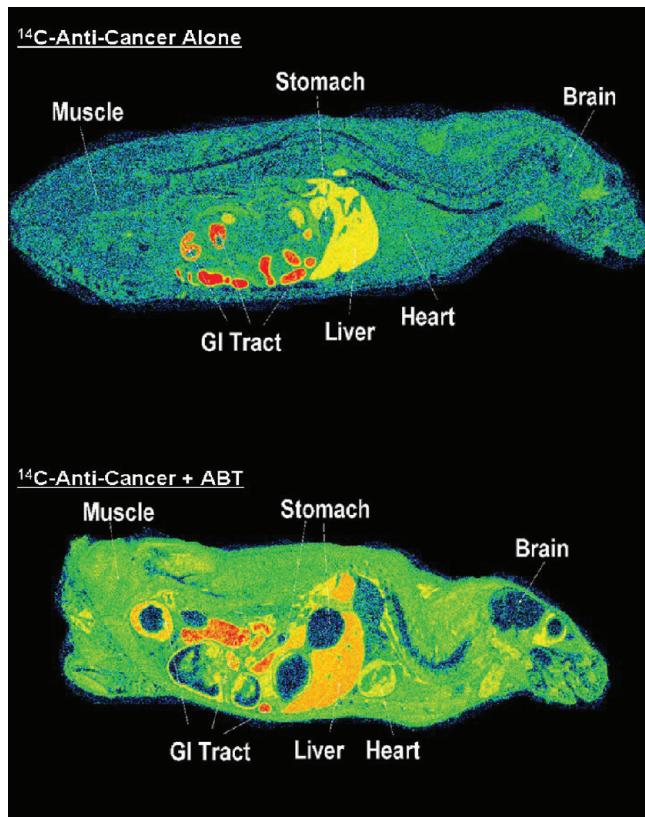


Figure 5. Rat whole-body autoradiographs of ^{14}C -anticancer drug before and after metabolism inhibition by ABT. ABT decreased tissue/blood ratios from 0.96 to 0.11, showed that brain radioactivity was probably due to the metabolite instead of the parent drug, and suggested that more intestinal receptors were occupied by the parent molecule instead of the metabolite.

simple 2 animal study pointed out a potential toxicity issue that might need to be considered while moving the drug candidate through discovery.

It is crucial to determine the penetration and exposure of antibiotics into target tissues as early as possible to ensure that a given compound will be efficacious. This is especially true when the tissue to be treated is difficult to penetrate (e.g., brain, testis, and bone). Figure 6 shows an autoradiograph of the distribution of ^{14}C -dalbavancin into bone and related tissues that can be infected by methicillin-resistant staphylococcus after related surgical procedures and/or other trauma.³⁰ In this study, rabbits were given a single IV dose of ^{14}C -dalbavancin, and one rabbit each was euthanized at various time points up to 336 h postdose. The leg of each rabbit was frozen, sectioned, and examined using quantitative autoradiography, which showed that high concentrations of dalbavancin rapidly penetrated bone marrow, epiphyseal plate, periostium, and articular cartilage up to 336 h after the single dose. These data suggested that dalbavancin would be clinically useful in treating bone and joint infections. This example shows how quantitative autoradiography can be used to examine the pharmacokinetics of compounds in deep tissues where the concentrations and kinetics, and thus, exposure can be very different from that seen in plasma. This may be a positive or negative finding depending on the drug and the desired therapy to be achieved.

The utilization of quantitative phosphorimaging and MARG to study drug distribution in individual complex organs such as the eye, skin, and blood vessels has helped many investigators

gain a better understanding of their drug candidates and has become a strategy to select lead compounds. The use of the two technologies offers a powerful combination to obtain a high resolution image of the distribution into very discrete tissue compartments while providing reliable quantitative data on tissue concentrations. Figure 7 shows a phosphorimage of a dog eye after treatment with a radiolabeled test article and MARG images that show detailed qualitative distribution at the cellular level. In this example, the test article was applied topically, and tissue concentrations in the sclera, cornea, iris, conjunctiva, uveal tract, aqueous and vitreous humors, and lens were determined using the phosphorimages obtained from sections adjacent to the sections used for MARG.³¹

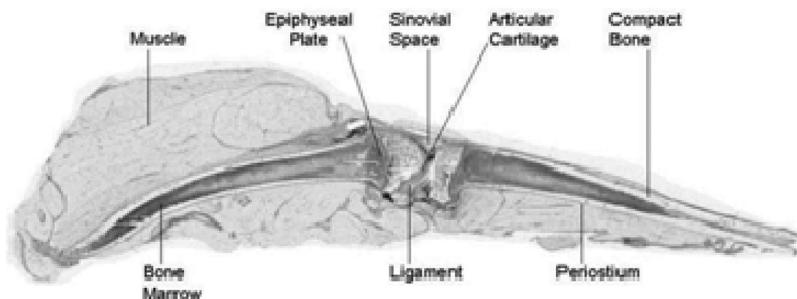
This technique was recently used by Miyaji et al. to examine the distribution of a novel δ -protein kinase C inhibitor, which was reversibly conjugated to a cell penetrating peptide (KAI-9803) in rats.³² In this study, the authors used both QWBA and MARG to show that KAI-9803 was delivered to target cells in the liver, heart, and kidney.

■ RADIOACTIVITY STUDIES IN DEVELOPMENT

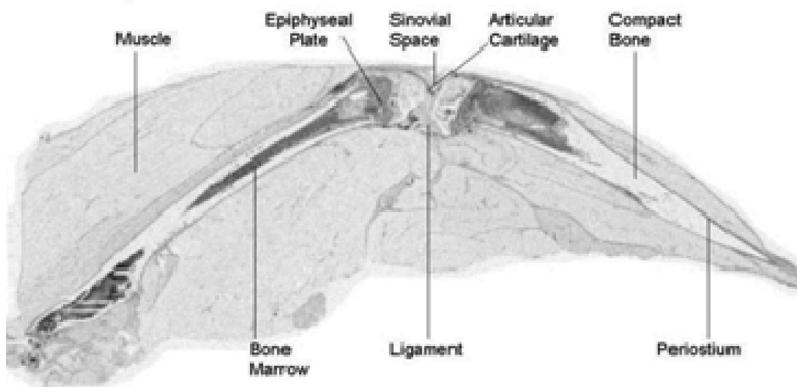
The determination of tissue distribution of new drugs is often required by regulatory agencies for new drug registration and for their development. In the mid-1990s regulatory agencies, such as the Japanese Ministry of Health and the US FDA, made formal statements¹⁰ about the acceptability of QWBA data for new drug registration, and currently, they often ask sponsors to perform tissue distribution studies using QWBA because the data provides much more detailed information than the former organ homogenate techniques.

Tissue PK information not only provides researchers with a way to examine detailed compartmentalized drug concentration information but also enables the determination of specific target tissue PK/pharmacodynamic (PK/PD) modeling and the estimation of human tissue radiation exposure during human radiolabeled studies that are needed to examine human drug metabolism. Prior to the implementation of QWBA, organ dissection and LSC assays provided only a rough estimate of organ exposure to radiation and unfortunately less accurate tissue exposure information. This becomes important when a radiolabeled drug is retained in a discrete tissue within an organ with heterogeneous tissue composition, such as the eye, kidney, and adrenal glands. In these cases, the actual tissue concentration would be underestimated by the LSC assay of organ homogenates because high concentrations in a discrete tissue would be diluted by low concentrations in surrounding tissues in an organ homogenate. This could lead to incorrect decisions about drug and/or radiation exposure. Furthermore, classical dissection/LSC organ distribution studies often used an insufficient number of time points to reliably determine PK parameters for many organs. This was primarily a resource decision because organ homogenate studies require the use of 3 animals per time point due to the inherent variability introduced by organ collection (i.e., variable trimming and exsanguination at necropsy) and the extensive sample processing required for that technique. QWBA, which utilizes intact animals that have not been processed to any great extent, has shown much less variability, and so these studies are designed to include the analysis of more time points, which lead to more reliable PK data for more tissues.^{9,10} A typical, well designed QWBA tissue distribution study includes the use of 1 animal per time and 8–12 time points. The study may also include extra animals that are maintained in the study after the QWBA data from the initial time

a. 24 h post-dose



b. 120 h post-dose



c. 336 h post-dose

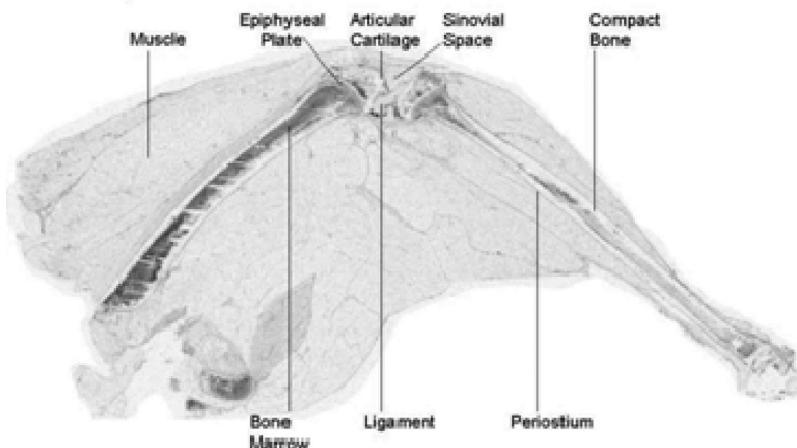


Figure 6. Autoradiographs of ^{14}C -dalbavancin distribution in the hindleg of a male rabbit after a single iv dose at 20 mg/kg rabbit. Relatively high concentrations of drug-related radioactivity remained in deep target tissues for up to 336 h after a single dose.

points are examined. Most studies are designed such that the last animal carcass is collected at 5–7 days after a single dose administration. Another general guideline is to set the last time point to be at least 10 times the drug half-life in plasma, but researchers must consider that tissues will probably have very different half-lives, and often it is many times longer than the half-life observed in plasma. To ensure that elimination can be verified from all tissues, an extra animal may be added to the study such that it could be analyzed at a time point after the main study data have been examined. This provides researchers with an opportunity to determine the elimination of drug-related radioactivity from tissues that may retain it much longer

than other tissues. This is especially useful if a drug and/or its metabolites associate with melanin in pigmented tissues, such as the retina of the eye, meninges of the central nervous system, and pigmented skin, which could pose a radiation safety concern to humans during a clinical radiolabeled study.³ In these situations, it is not unusual to see very slow elimination from pigmented tissues that can last for several weeks.

Researchers are often faced with a decision regarding where to place a radioactive label for their development studies. One important consideration is whether the isotope placement will enable the tracking of a particular metabolite and/or if a particular metabolite will be retained for a longer period of time



Figure 7. Phosphor image of a dog eye (A) and microautoradiographs of the anterior eye structures (B) and posterior eye structures (C) after a topical administration of a ^{14}C -labeled test drug showing the localization of drug-derived radioactivity in the cornea (C), iris (I), conjunctiva (Conj), posterior chamber (PC), anterior chamber (AC), uveal tract (U), lens (L), nuclear layer (NL), rods and cones (RC), and pigmented layer (PL). Radioactivity in A appears as a gray scale image where black is high radioactivity, and white is background. Radioactivity in B and C appears as black grains on the hematoxylin and eosin stained cryosections.

Table 1. Distribution of a Dual Radiolabeled Compound in Rat Kidney

time (h)	concentrations (μg equiv of drug/g of tissue)							
	brain (cerebellum)		brain (cerebrum)		kidney (cortex)		kidney (medulla)	
	label A	label B	label A	label B	label A	label B	label A	label B
1	0.088	0.229	0.070	0.201	7.820	6.036	4.917	8.296
2	0.089	0.407	0.076	0.353	6.368	7.962	5.391	10.446
4	0.072	0.600	0.074	0.526	5.477	7.596	4.512	12.804
8	0.050	0.604	0.037	0.606	2.810	4.708	2.848	4.809
24	BQL ^a	0.257	BQL	0.298	0.435	0.715	0.166	0.191
48	BQL	0.134	BQL	0.145	0.141	0.268	0.065	0.138
72	BQL	0.094	BQL	0.048	BQL	0.222	BQL	0.050
168	BQL	0.046	BQL	0.028	BQL	0.093	BQL	0.037
240	BQL	0.030	BQL	BQL	BQL	0.056	BQL	BQL
336	BQL	BQL	BQL	BQL	BQL	0.040	BQL	BQL

^aBQL = below the lower limit of quantitation.

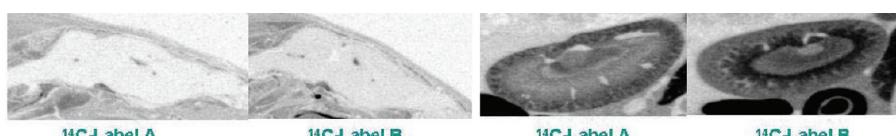


Figure 8. Distribution of a dual radiolabeled compound in rat brain and kidney. Two batches of the parent molecule were prepared, and each was labeled with ^{14}C at two different positions on the molecules so that the parent molecule and metabolites could be imaged and tissue concentrations determined using QWBA. The goal was to determine specific tissue exposure to each metabolite to address safety concerns.

than another. To help address this, researchers sometimes perform a QWBA tissue distribution study using a test article that has been labeled in two different places and then dosing 2 separate groups of animals to compare the tissue distribution patterns and PK of the drug-derived radioactivity from the two versions of the radiolabeled test article.

■ DRUG DEVELOPMENT QWBA CASE STUDIES

Table 1 and Figure 8 show some of the results of such an experiment where a tissue distribution study in rats was conducted for Compound X.³³ Two batches of Compound X were prepared, and each was labeled with ^{14}C at different positions on the molecule because Compound X had two known metabolites that were from two different parts of the molecule.

The two versions of the ^{14}C -drug were administered to two groups of rats and tissue concentrations determined using QWBA analysis. The goal was to track the tissue distribution of the radioactivity associated with each metabolite and parent drug and to correlate specific tissue exposure to each metabolite

in an attempt to address safety concerns. It also helped investigators to see if the radioactivity from one of the radiolabeled versions would be eliminated faster than the other, which would provide decreased radiation exposure to humans during a clinical radiolabeled study. In this example, the concentration of drug-derived radioactivity from Label A in brain tissue was approximately half that observed after the administration of Label B. Differential distribution was also observed in the kidney cortex and medulla. The resulting human estimations of radiation exposure to the brain and the different tissues of the kidney obtained using the two versions of the radiolabeled compound were substantially different and helped to guide the choice of radiolabel for the human study as well as providing clues to the localization of a metabolite responsible for potential safety concerns.

Male pigmented rats are used most often for tissue distribution studies; however, the animal model that best represents the expected pharmacokinetics in humans should be used whenever possible. This includes the examination of placental

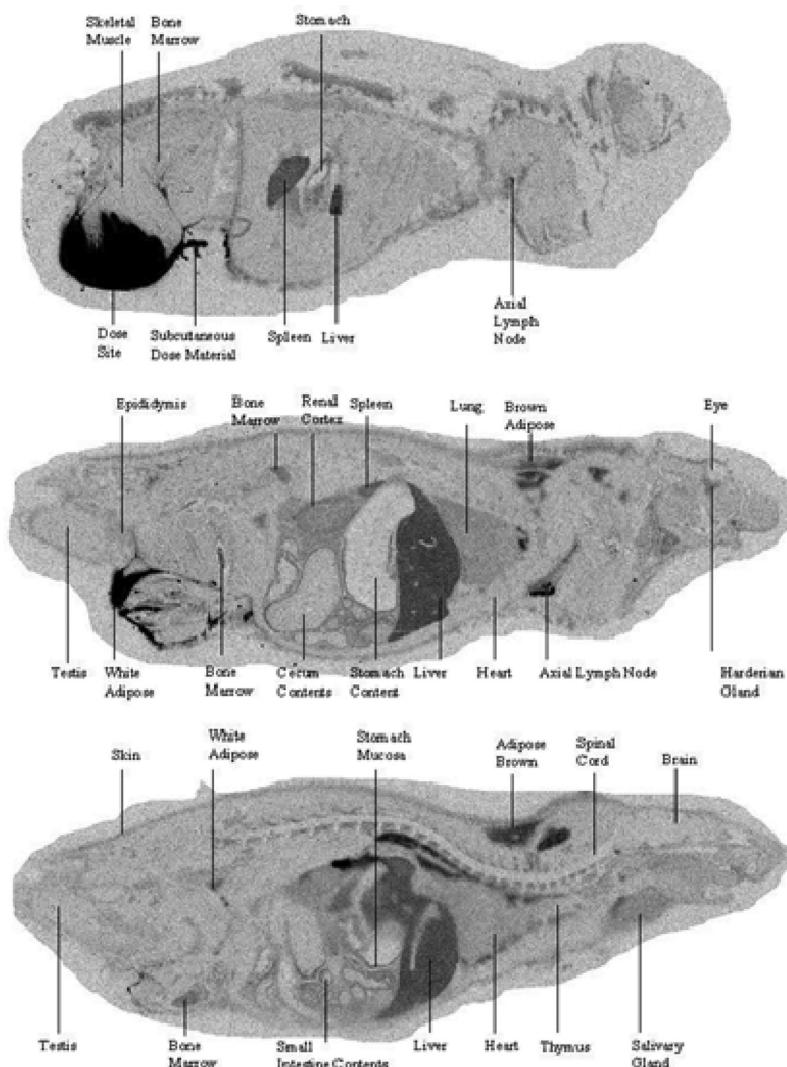


Figure 9. ^{14}C -DepoFoam in rats after a subcutaneous injection. Whole-body autoradiographs at 2 days after single subcutaneous administration of ^{14}C -DEPC in DepoFoam formulation.

transfer of radiolabeled drugs in lab animals, such as that conducted at the Walter Reed Army Institute of Research, which used QWBA to further clarify embryotoxicity of artesunate and its metabolite dihydroartemisinin in pregnant rats.³⁴ Their work highlighted the importance of obtaining knowledge about partitioning of xenobiotics after introduction into the body and how that data can be used in the assessment of physiology-based pharmacokinetic modeling.

QWBA is routinely performed using all types of laboratory animals, which includes mice, hamsters, rats, guinea pigs, rabbits, ferrets, cats, dogs, and nonhuman primates. The large format cryomicrotome used for QWBA can adapt to any carcass or portion of a carcass that can fit into a frozen block that is approximately 40 cm long by 15 cm wide by 15 cm high. This added versatility provides for interspecies comparisons, which, for example, can be useful when researchers need to compare tissue PK parameters of the toxicology model (e.g., rat, mouse, or dog) to the pharmacology/efficacy model (e.g., guinea pig, rabbit, or cat). This can be very valuable in explaining different results across species and was used by Pharmacia and Upjohn to study the pharmacokinetics of latanoprost in the cynomolgus monkey.³⁵ In this study, radiolabeled latanoprost was

administered topically to the eye of monkeys, and QWBA was performed. The results revealed that drug-derived radioactivity had traveled through the lachrymal ducts and that high concentrations were present in the gastrointestinal tract, kidney, liver, blood, bile, and urine.

The best time to conduct radiolabeled studies, which includes mass balance and metabolite profiling and identification, is often a strategic decision for pharmaceutical companies because they are expensive to conduct but necessary before a human radiolabeled study can be performed. Many times a preclinical mass balance study will be conducted before a tissue distribution study because it can help guide the choice of time points by evaluating the elimination pattern. However, by the time the decision is made to determine definitive ADME characteristics, it is often more efficient and economical to combine the mass balance, tissue distribution, and metabolite profiling and identification under a single protocol and/or by using the same samples. A simple but complete study design would include groups of animals for determining: biliary excretion (using bile-duct cannulated animals for bile collection), plasma PK (intact animals), mass balance, and excretion patterns (intact animals); tissue distribution for human

radiation dosimetry (intact pigmented animals to examine possible association of the drug-derived radioactivity with melanin); and tissue distribution to bridge toxicology data (intact albino rats). This saves resources by performing only 1 study setup and adds more consistency because all animals are treated under the same conditions. A recent example of such a complete ADME radiolabel that utilized QWBA to determine tissue distribution was conducted at Genentech where ^{14}C -GDC-0449 (vismodegib) was studied in rats and dogs.³⁶ The radiolabeled compound was used to determine the complete ADME profile of a novel orally active Hedgehog Pathway inhibitor, which displayed a unique metabolic pathway through pyridine ring-opening. Most often these studies were conducted during phase 2 and just before the human radiolabeled study would be performed, which helped reduce the chance of wasting resources if a decision was made not to develop a compound. However, investigators should not wait too long before requesting the radiolabeled material and scheduling the preclinical ADME studies. There can be unforeseen delays in obtaining the radiolabeled compound and/or in conducting the QWBA study, which could ultimately delay and/or prevent a previously scheduled human radiolabeled study, and there may be little time to make a good decision regarding the conduct of the human study. Another strategy that has been used by drug development teams is to conduct the tissue distribution study earlier, for example, during phase 1. The benefit of doing this is that the resulting tissue distribution can be used to aid in the understanding of toxicology, pharmacology, and efficacy studies, and/or to identify potential issues that may affect the human study well ahead of time. A potential negative side of this approach is that resources would be wasted if the decision was made not to develop a drug; however, the information gained from one study can often help to guide decisions about the development of other new drugs in the same molecular/therapeutic class.

Regulatory agencies are also concerned with the safety of substances used as drug carriers to facilitate the sustained release of drugs, and QWBA has been used to track the released drug as well the carriers, such as liposomes. Willis et al. examined the distribution of the ($[1-^{14}\text{C}]$ -2-erucoyl)-DEPC DepoFoam formulation (Pacira Pharmaceuticals, Inc., San Diego, CA) in rats and showed that it was widely distributed and metabolized via normal pathways.³⁷ This multivesicular liposome product was widely distributed, after a subcutaneous injection, and relatively high concentrations were found in lymph nodes and brown adipose tissue (see Figure 9).

■ REGULATORY CONSIDERATIONS

Regulatory agencies in developed countries have played pivotal roles in the development of new drugs, and these agencies have made great progress in harmonizing their requirements to approve new drugs. Communication among members of the US FDA, European Union, World Health Organization, Japanese Ministry of Health, and other regulatory agencies around the world has vastly improved since the early 1980s, and now many of the requirements to register new drugs are very similar. Furthermore, developing countries can now more easily model their new drug approval processes after those in the developed nations due to technologies such as the Internet and outreach organizations such as the World Health Organization.

Although most regulatory agencies do not require tissue distribution information during the drug discovery phase, it is often helpful for researchers to have some radiolabeled ADME

data prior to elevating a drug into the development phase. Early ADME data can alert researchers to potential issues such as those discussed earlier. For companies that focus on out-licensing of their new drugs, a limited ADME package is also a good sales tool that gives prospective buyers and/or collaborators information to make better decisions on how to proceed to a deal. The decision to perform and include pre-clinical radiolabeled ADME study data in an innovative new drug (IND) application is usually up to the pharmaceutical company, and most companies will include that data if it is available. However, it is not required unless a human radiolabeled study will be run prior to or during phase I studies, and in that case, a human radiation dosimetry estimation, which relies on animal tissue distribution data, must be performed; the FDA has issued a guidance for that situation.³⁸

Today most regulatory agencies require that pharmaceutical companies include preclinical radiolabeled ADME studies as part of new drug applications (NDA). This is because they provide the best data for choosing the toxicology models and doses, and they provide insight into human metabolism for safety evaluation. Thus, better decisions can be made about tissue exposures of the parent compound and its metabolites. Furthermore, it is absolutely necessary to perform preclinical radiolabeled tissue distribution studies in nonclinical models prior to human radiolabel studies so that human radiation dosimetry estimates can be made.

Of course, there are exceptions to the rule that radiolabeled studies need to be conducted for NDAs. The most common exception is when the new drug is to treat a life-threatening disease such as terminal cancer or orphan diseases. In these cases, limited tissue distribution studies may be performed, but they are usually to confirm that targeted tumor and/or other possible therapeutic targets will be adequately exposed to the test drug. Otherwise, efficacy and toxicology studies are usually adequate to enable further clinical studies.

■ CONCLUSIONS AND FUTURE PERSPECTIVES

The impact of the use of radiolabeled compounds for studying tissue distribution has been enormous over the history of drug discovery and development. The use of autoradiography image analysis has all but replaced the former techniques of organ homogenate LSC analysis in less than 2 decades. Autoradiography has been proven time and again to extend the knowledge of drug disposition by providing true tissue-level concentration data that details tissue exposure, helps to guide the decisions about drug safety, pharmacology, efficacy, and can help solve the challenges that can turn a chemical into a useful drug that saves lives. Until technology can offer efficient ways to quantify chemical entities in complex matrices, such as the multitude of tissues in the human body, radiolabeled compounds will need to be used as the most efficient way to do this job. Whole-body and microautoradiography techniques will continue to be utilized to uncover the mysteries surrounding tissue distribution of new drugs. This is because newer *in vivo* radio-imaging modalities, such as positron emission topography or fluorescence imaging,³⁹ cannot offer the same high resolution and length of tracking time provided by stable, low energy ^{14}C and tritium labeled compounds. Furthermore, the new applications of MALDI-MS imaging and secondary ion mass spectrometric imaging (SIMS) technologies, which can identify nonradiolabeled parent drug molecules and metabolites in tissue samples, cannot yet be used to provide quantitative data. This is due to the various matrix effects imposed by each tissue type, which need to be accounted

for in order to provide reliable quantification, and this is not thought to be possible for many years to come.⁶

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ADME, absorption distribution metabolism excretion; WHO, World Health Organization; ICRP, International Commission on Radiological Protection; MS, mass spectroscopy; LC, liquid chromatography; WBA, whole-body autoradiography; QWBA, quantitative whole-body autoradiography; MARG, microautoradiography; ³⁵S, sulfur-35 isotope; ³H, hydrogen-3 isotope; ¹⁴C, carbon-14 isotope; ¹²⁵I, iodine-125 isotope; LSC, liquid scintillation counting; SWBA, Society for Whole-Body Autoradiography; ESA, European Society for Autoradiography; RGD, Arg-Gly-Asp Peptide; po, *per os*; iv, intravenous; DMPK, drug metabolism and pharmacokinetics; CRO, contract research organization; TCA, trichloroacetic acid; PK, pharmacokinetic; GI, gastrointestinal; FSD, flow through scintillation detection; ABT, aminobenzotriazole; C, cornea; I, iris; Conj, conjunctiva; PC, posterior chamber; AC, anterior chamber; U, uveal tract; L, lens; NL, nuclear layer; RC, rods and cones; PL, pigmented layer; KAI-9803, selective δ -protein kinase inhibitor derived from δ V1–1 portion of δ -protein kinase; PD, pharmacodynamic; BQL, below quantifiable limits; GDC-0449, vismodegib; IND, innovative new drug; NDA, new drug application; MALDI-MS, matrix-assisted laser desorption imaging mass spectroscopy; SIMS, secondary ion mass spectrometric imaging.

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