13 Whole-Body Autoradiography and Microautoradiography in Drug Discovery and Development

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13.1 SUMMARY

Tissue distribution studies, which are part of a suite of experiments known as *absorption, distribution, metabolism, and excretion* (*ADME*) studies, are performed to understand the disposition of xenobiotics and biotherapeutic drugs after administration to animals and humans. This chapter describes the following three approaches used to study the tissue distribution of chemical and biological compounds in laboratory animals: (i) organ collection, homogenization/combustion, and liquid scintillation counting (LSC) analysis; (ii) whole-body autoradiography (WBA), which includes quantitative whole-body autoradiography (QWBA); and (iii) microautoradiography (MARG), which qualitatively resolves the localization of compounds to the cellular level in a histological preparation. LSC, WBA, QWBA, and MARG studies require the use of a radiolabeled chemical or biological test compound, which provides for the tracking and quantification of both the parent molecule and potential metabolites that might be formed. A radiolabeled test drug is administered to laboratory animals, which are later euthanized at different time points. Samples of the animal's entire body, organs, tissues, and excreta are then analyzed to determine the concentrations of the test-drug-derived radioactivity in the various biological matrices. Tissue concentration versus time profiles can then be constructed to provide data for organ- or tissue-specific pharmacokinetic (PK) compartmental analysis. The animal tissue distribution data are then

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used to help evaluate pharmacology and toxicology findings and to predict human exposure to drugs and metabolites.

Dissection and LSC techniques were the prevalent methods for evaluating drug distribution up until about 1995, when QWBA began to be used. This technique entails the removal of organs followed by further processing and analysis of solubilized organ homogenates and/or the combustion of the organ and/or homogenates. The resulting drug-derived radioactivity is then quantified using LSC to determine the radioactivity concentration in the organ.

QWBA is an imaging technique that provides both spatial and quantitative radioactivity concentrations in tissues of intact laboratory animals. The main advantages are minimal sample processing/alteration and provision of true tissue concentrations. This technique begins when each animal is euthanized at their respective time point post dose, 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 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. Sven Ullberg pioneered the WBA technique in 1954, and the technique was made quantifiable by Luckey in 1975 when he developed phosphor imaging technology or radioluminography. QWBA limitations include (i) tissue concentrations determined by QWBA (or LSC) can include parent drug, plus its metabolites, and/or degradation products and (ii) it is difficult to evaluate short-lived isotopes.

MARG provides pharmaceutical scientists with a high resolution tool to investigate spatial localization of radiolabeled drugs at a tissue and cellular levels. The basic procedure requires dosing of animals with a radiolabeled substance, followed by the removal and freezing of organs/tissues, cryosectioning and section mounting, and exposure to nuclear photographic emulsion. The tissue sections are then developed, stained, and evaluated microscopically to evaluate distribution at the cellular level. 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 calibration standards are not applied, which makes true quantitation problematic. This chapter discusses the history, advantages, limitations, and examples of the application of each technique.

13.2 INTRODUCTION TO TISSUE DISTRIBUTION ASSAYS

Tissue distribution studies, which are part of a suite of experiments known as *absorption, distribution, metabolism, and excretion (ADME)* studies, are performed to understand the disposition of xenobiotics and biological drugs after administration to animals and humans. The goal of these studies is to determine how the body absorbs drugs and then how the drug is distributed throughout the body, metabolized, and then eliminated from the body. Regulatory agencies around the world require that this information be determined for the registration of new drugs. This chapter focuses on three approaches used to study the tissue distribution of chemical and biological compounds (small and large molecular weight entities) in laboratory animals and how they are used in preclinical drug research. The three approaches discussed are (i) organ collection,

homogenization/combustion, and LSC analysis, which is commonly referred to as the *cut and count* procedure; (ii) WBA, which includes QWBA; and (iii) MARG, which qualitatively resolves the localization of compounds to the cellular level in histological preparations.

LSC, WBA, QWBA, and MARG studies require the use of a radiolabeled chemical or biological test compound. Small molecular entities (chemical compounds that have molecular weights of <600) Dalton 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 quantified in various tissues, fluids, and excreta. The radiolabeled test drug is synthesized in a radiochemistry laboratory, and the position of the radiolabel on/in the molecule is usually known for small molecules (but may not be for large molecules). The radiolabel provides a method for tracking both the parent molecule and metabolites that retain the labeled portion of the molecule. The radiolabel also enables the specific identification and quantification of metabolites that contain the radiolabel, using radio-high performance liquid chromatography (HPLC) techniques and mass spectroscopy.

In general, small organic molecules are labeled with ^{14}C, ^{35}S, or ^{3}H [1], and larger molecules, such as proteins and peptides, are commonly labeled using ^{125}I (and sometimes ^{35}S). ^{3}H is often used in drug discovery because it can usually be done relatively quickly, and it is easier to label compounds with ^{3}H than with ^{14}C. However, it is important to verify the stability of the ^{3}H label since ^{3}H-labeled xenobiotics are known to sometimes undergo hydrogen exchange with water [2]. 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 to be used for the study. The radioactive counts obtained from the wet and dried samples may be compared to see if there is a difference. A similar situation exists for large molecule peptide and protein drugs that are radiolabeled with ^{125}I. In this case, the protein-bound ^{125}I often becomes dissociated from the test article *in vivo*, and this must be characterized to interpret tissue concentration data. This is discussed further in Section 14.4.2.10.

The study designs for examining tissue distribution are varied, but a few basic designs are currently being used in the pharmaceutical industry. In the most basic design, a radiolabeled test drug is administered via a relevant dose route [e.g., orally, intravenously (IV), topically, inhalation, ocular] to laboratory animals. At various time points after dosing, subjects are euthanized, and samples of the animal's organs, tissues, and excreta are then analyzed to determine the concentrations of the test-drug-derived radioactivity [e.g., organs, tissues, plasma, bile, intestinal contents, urine, feces, cerebrospinal fluid (CSF)]. Tissue concentration versus time profiles can then be constructed to provide data for tissue-specific PK compartmental analysis.

Drug tissue distribution studies are routinely conducted in rodents, rabbits, canines, and nonhuman primates. The choice of the animal test model depends on the purpose of the study. In most cases, the animal model will mimic the expected PK in humans, but it may answer specific questions (e.g., toxicology, pharmacology, oncology, genetic modifications). The number of time points used and the overall duration of the study are also important variables to be considered. Time points should be designed to obtain useful and reliable tissue concentration–time profile data. The following tissue PK parameters can then be determined: maximal drug concentration reached (C_{max}); time

of maximal concentration (T_{max}); area under the time–concentration curve (AUC), which describes tissue exposure to a drug; and terminal elimination half-life ($T_{1/2}$), which describes how long it takes for a tissue to eliminate a drug. The choice of time points to be used for the study will determine how reliable the PK data will be, and it requires at least 6, but 10 usually provide enough to enable the determination of reliable PK parameters for many tissues. To obtain reliable tissue PK parameters, there must be enough time points to capture the T_{max} and establish a reliable $T_{1/2}$. Statistically speaking, the portion of the time–concentration curve used to determine the $T_{1/2}$ for each tissue must have an r^2 value of 0.8 or higher [3], 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 using enough time points then predictions of human tissue PK exposure must be treated with caution.

Tissue distribution study designs have changed over the years in response to new technology and ethical animal treatment concerns. Most notable has been the change from using three animals per time point and six to eight time points to using one animal per time point and more time points. Before the implementation of QWBA, when organ homogenization and LSC was used to examine tissue distribution, studies were designed to use three animals per time point and six to eight time points because of high variability observed in the organ concentrations. This variability was presumably due to inconsistent effects of organ exsanguination, cross-contamination of organs by body fluids, and inconsistencies associated with organ collection, homogenization, and further sample processing (sample combustion and/or solubilization and LSC detection efficiency). However, as QWBA techniques began replacing the LSC method, many investigators found relatively lower variability in tissue concentrations among animals at the same time point [4]. This was believed to be due to analysis of intact animal carcasses that were snap frozen on termination, thus preserving a "near-*in vivo* " snapshot of drug distribution over time. This also eliminated the variable effects of exsanguination, organ homogenization/processing, and/or the possibility of organ cross-contamination during organ removal and sample handling. Early investigators who developed QWBA techniques also recognized that the use of more time points resulted in more reliable tissue PK parameters [5]. In this way, QWBA has provided the ability to produce the highest quality, and true, "tissue" concentration data (as opposed to organ homogenate data), as well as the most conservative and ethical use of laboratory animals. Nevertheless, organ dissection/homogenization and LSC are still used today by some pharmaceutical companies that have not yet realized the benefits of and/or accepted QWBA as the method for conducting drug distribution studies. To those ends, this chapter describes the techniques of "cut and count" LSC organ analysis and autoradiography (ARG) tissue analysis and how they are used in drug discovery and development.

13.3 ORGAN DISSECTION, HOMOGENIZATION, AND LIQUID SCINTILLATION ASSAYS

Until the early 1990s, organ dissection, homogenization, and LSC techniques were the prevalent methods for evaluating drug distribution. The technique [6] begins with the process of euthanasia. Typically, animals are deeply anesthetized, and blood is

removed via cardiac puncture, followed immediately by the dissection of the animal, where organs of interest are removed, trimmed, rinsed, weighed, and samples are obtained for homogenization, solubilization, and/or oxidation. Investigators should be aware that exsanguination effects many physiological processes [7] that can alter drug distribution, and so tissue concentration data obtained from tissues removed from a carcass need to keep that in mind when interpreting data obtained in this manner. Technicians performing the necropsy are normally required to wash their instruments between individual organ collection to reduce the possibility of cross-contamination of organs during removal. Technicians must assure that all connective tissue, fat, and other fluids are consistently removed from each organ. The number of organs removed can vary considerably, but normally, $\sim$25–30 organs are removed, which include adipose (abdominal and brown) tissue, adrenal gland, blood, bone, bone marrow, brain, cecum (and contents), epididymis, esophagus, eyeball, Harderian gland, kidney, large intestine (and contents), liver, lung, lymph nodes, myocardium, ovary, pancreas, pituitary gland, prostate, salivary gland, seminal vesicles, skeletal muscle, skin (nonpigmented and pigmented), small intestine (and contents), spinal cord, spleen, stomach (and contents), testis, thymus, thyroid, urinary bladder (and contents), and uterus. Organs are then prepared for homogenization and/or combustion (or oxidation). Organs with a heterogeneous tissue composition, such as bone/bone marrow, pituitary gland, spleen, liver, kidney, brain, and muscle, are usually homogenized using a probe homogenizer and/or manual mincing/crushing before sample aliquots are obtained for drying and combustion. Small organs (usually weighing <1 g) such as adrenal glands, pituitary gland, and small samples of tissues for combustion may be placed directly on combustion cones. All samples are allowed to dry to improve complete and uniform combustion. Often triplicate aliquots (0.100 g) of the homogenized sample or an entire organ will be analyzed to monitor/control variability. Each sample aliquot for analysis must be weighed before combustion in the sample oxidizer to the determination of the concentration of drug-derived radioactivity in the sample. Each sample is individually placed into a combustion instrument and then burned completely. The liberated $^{14}CO_2$ or ^{3}H gas is then trapped in a solution, and the radioactivity is determined by LSC. Blank samples that contain a known amount of radioactivity are often oxidized and compared to noncombusted standards to determine the percentage of recovery and efficiency of the combustion process, although it must be realized that each tissue may have different oxidation efficiencies and this is not usually determined due to the significant amount of extra work involved. Nevertheless, the efficiency of sample oxidizers must be verified before and during sample oxidation because the performance of these instruments can vary as samples are being processed. Furthermore, the potential exists for carryover of radioactivity from one sample to another, which, of course, could alter the concentration data. Most sample oxidizers require loading and unloading and careful monitoring by a technician.

An alternative to sample combustion is sample solubilization, where organ homogenates and/or entire small organs are solubilized using a strong caustic reagent. Aliquots of the solubilized sample are then bleached to remove any color that can impede the efficiency of the LSC instrument, and this is typically done by adding peroxide to the sample before scintillation fluid is added and the samples are counted by LSC.

LSC of the resulting samples relies on the performance of the scintillation analyzer. The LSC analyzer detects photons of light that are produced as the radioactive β

particles emitted from decaying isotopes, such as ^{14}C or ^{3}H, excite the scintillant. The photons emitted are detected by photomultipliers and the signal is translated into a "count" of the radioactivity. The radioactivity [i.e., counts per minute (cpm)] in each sample is then converted to disintegrations of radioactivity per minute (dpm) by means of an external standardization and a quench curve that are part of LSC analyzer protocols. Quenched standards are typically used to calibrate the liquid scintillation counter on each day of analysis, and samples are typically counted for at least 5–10 min each.

The LSC data (dpm) is then used to determine the concentration of drug-derived radioactivity in the sample by dividing the decay per minute by the weight of the sample, which if diluted during homogenization, must be corrected using a dilution factor. It is important for researchers to recognize that the concentration data obtained this way is from organ homogenates and is not true tissue concentration. All organs are composed of several different tissue types, and each tissue of an organ can have vastly different concentrations of the test drug. The ability to determine organ concentration enables researchers to determine the percentage of the radioactive dose that has been taken up in an organ because the total organ weight was obtained at necropsy. This percentage of radioactive dose per organ per time point is usually determined and reported.

In conclusion, exsanguination, organ dissection, homogenization, oxidation or solubilization, and LSC techniques offer pharmaceutical researchers a method to determine organ concentrations; however, true tissue level concentration determinations cannot be obtained. Nevertheless, LSC techniques have been a valuable tool to study the ADME properties of drugs, and it will undoubtedly be used in the future as an alternative method to WBA.

13.4 WHOLE-BODY AUTORADIOGRAPHY

Today, most tissue distribution studies used to support new drug registrations are performed using QWBA techniques [8], which is the imaging and quantitation of drug-derived radioactivity concentrations in tissues of preclinical species. QWBA has numerous advantages over "cut and count" LSC analyses. The key advantages are tissue, rather than organ, concentrations are obtained; there is no cross-contamination from tissues; and the variability due to exsanguniation is eliminated. As previously mentioned, QWBA typically uses one animal per time point, and each animal is euthanized and then the carcass is rapidly snap frozen typically in a dry ice/hexane bath (Fig. 13.1a). This process eliminates redistribution of compounds post death. A small blood sample is normally obtained before freezing and can be collected via cardiac puncture or alternative methods. Plasma may then be prepared from a portion of the blood sample. The frozen carcass is then embedded in a frozen block of supporting media (Fig. 13.1b) and precision cryosectioned (Fig. 13.1c) in a sagittal orientation at 40 μm thickness using a special macrotome to obtain representative *in situ* samples of all organs, tissues, biological fluids, ingesta, and excreta. Approximately 5–10 whole-body sections at various depths through the animal are necessary to collect the typical set of 40–50 tissues analyzed by this method. These samples, collected on adhesive tape, are then dehydrated (approximately two days in a cryomacrotome or more rapidly using a lyophilizer). Phosphor imaging has replaced traditional film ARG

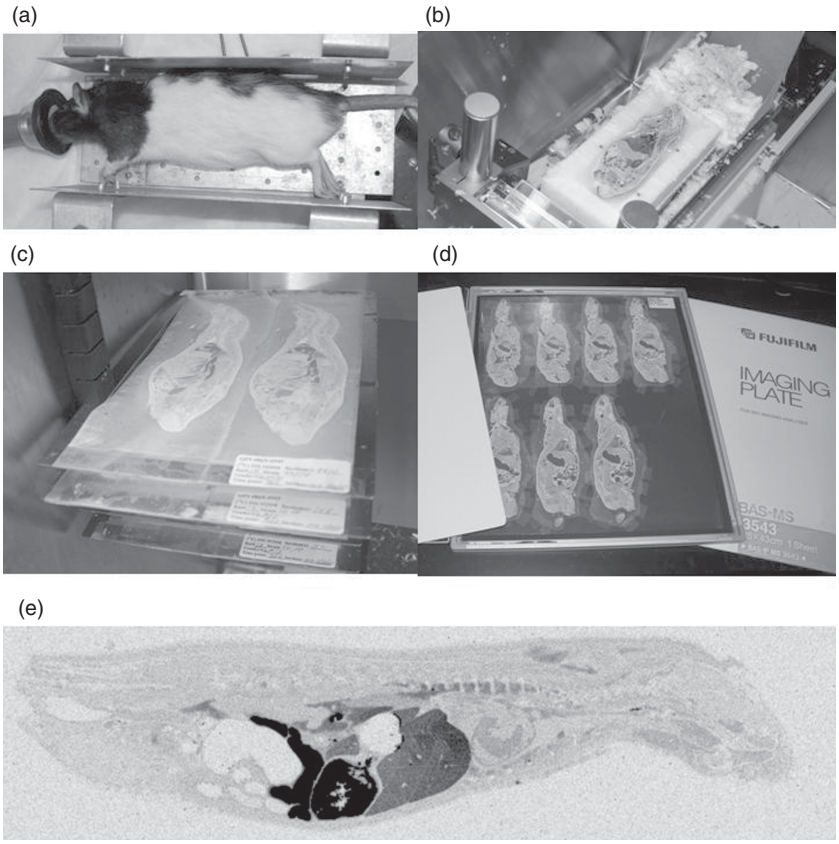

Figure 13.1 Whole-body autoradiography methods. (a) The carcass is frozen in a hexane-dry ice bath, (b) whole-body sections are dehydrated, (c) sections are exposed to phosphor imaging plates along with radioactive image calibration standards, (d) the imaging plates are scanned by a phosphor imaging plate scanner, and (e) grayscaled digital images of the radioactivity in the sections are obtained for image analysis to determine tissue concentrations. (See color insert.)

because of its larger linear range and shorter exposure times. Samples are opposed to phosphor imaging plates along with radioactive calibration standards (Fig. 13.1d). 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 plate scanner, and grayscaled digital images of the radioactivity in the sections are obtained (Fig. 13.1e). The digital images are then calibrated using image analysis software so that different grayscale levels, which are assigned by the image analysis software, are interpolated to provide a quantitative value of radioactivity in all tissues imaged. Data can be reported as nanocuries (nCi) or microcuries (μCi) of radioactivity per gram of the tissue or in terms of drug equivalents per gram of tissue (or matrix). Digital QWBA images typically have pixel resolutions of $100\,\mu m^2$; 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 $25\,\mu m^2$, which are among the highest resolving powers available.

13.4.1 History, Strengths, and Limitations of Whole-Body Autoradiography

The eloquent method of QWBA came from crude beginnings in the 1800s. In 1867, Niepse de Saint Victor first described the phenomenon of ARG as the "persistent activity due to an unknown chemical radiation" [9,10]. This observation lead another scientist named London to perform an experiment in which an autoradiographic image of a frog treated with radium was first produced in 1904 [11]. To that end, ARG is truly the first molecular imaging technique used for the localization of radioactivity in biological specimens. Fifty years later, Dziewaitkovski used β radiation to investigate the localization of compounds in biological samples [12]. This was followed by the development of the WBA technique by Ullberg in 1954 [13], who pioneered the technique by administering ^{35}S-penicillin to mice followed by freeze embedding them in water-soaked cotton using dry ice. He then sectioned their entire body bodies using a microtome in a walk-in freezer. The whole-body sections he produced were then exposed to X-ray film, which produced the autoradiographs that revealed the tissue distribution of drug-derived radioactivity. Methods for sectioning the whole bodies of laboratory animals were also developed and included the use of dry-ice-cooled microtomes [14], grinding down frozen carcasses [15], abrasion of resin-embedded carcasses [16], thick sectioning using a circular saw [17], and, finally, the development of a large microtome held inside a chest freezer [18]. Leica Microsystems, Inc. (Nussloch, Germany) began manufacturing commercially available large format cryomicrotomes and remains the leading provider of large cryomicrotomes used for WBA.

Quantitation of autoradiographs was the next challenge for pioneering macroautoradiographers. Berlin and Ullberg [19] and Kutzim [20] made the first attempts to quantify tissue concentrations in autoradiographs using early image analysis techniques. Unfortunately, the results achieved were only semiquantitative, but in the following years (1974–1987), several investigators [21–26] researched methods to better determine quantitative data from autoradiographs with limited success because of the inherent nonlinearity of film. Also during this time, Schweitzer [27] developed an image calibration method using ^{14}C-spiked blood standards at concentrations that bracketed the sensitivity of phosphor imaging plates. This robust technique continues to be used by many investigators today. Luckey revolutionized WBA in 1975 by developing and patenting phosphor imaging technology or radioluminography [28], which provided digital images from whole-body sections within days. Most importantly, these images enabled the direct determination of quantitative tissue concentrations over four to five orders of magnitude. Technical validation of the phosphor imaging instrument and QWBA methods, which more completely described the principals, specifications, and limitations of the instrumentation and QWBA, was published in 2000 [29].

In 1994, a group of autoradiographers in the pharmaceutical industry formed the Society for Whole-Body Autoradiography (SWBA) whose mission was to promote the use of QWBA over traditional organ dissection homogenate methods to determine tissue distribution of new drugs. Further work parameterized quantitative aspects to meet stringent bioanalytical expectations [30–35]. In 1990, a Japanese collaboration of $>$20 companies proposed that QWBA should replace the use of traditional dissection and LSC assay to determine true tissue distribution during the drug development [36]. Dr. Yasuo Ohno of the National Institute of Health Science (Tokyo, Japan) concluded his presentation at the 1997 meeting of the SWBA by stating that the Japanese Ministry of Health and Welfare would accept QWBA data in lieu of traditional organ

dissection distribution studies for the approval of new drugs as long as the procedures were appropriately validated. Today, pharmaceutical companies have almost entirely eliminated the use of dissection studies to determine the definitive tissue distribution of new pharmaceuticals, and these studies are now routinely submitted to regulatory agencies.

In addition to ARG and autoradioluminography, direct nuclear imaging technologies were also developed. These instruments utilize ionization chambers and different imaging technologies [e.g., scintillating sheet, charge coupled device (CCD) camera] and were first developed by Jeavons in 1983 [37]. Today's instrument consists of a parallel plaque avalanche chamber, which is based on the invention by Charpak in 1989 [38]. These instruments (Beta Imager and Micro Imager), 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 provide data relatively quickly. However, relatively few sections can be analyzed at one time, and the instruments require regular and careful maintenance.

The key strength of the QWBA technique is its ability to provide true tissue distribution of radiolabeled test articles in a relatively unadulterated, *in situ* sample. Phosphor imaging has also been shown to be a very robust yet sensitive technology for the quantification of radioactivity in whole-body sections. Its wide linear detection range (four to five orders of magnitude) and sensitivity that can reliably quantify ~45 dpm distributed over an area of 0.5 cm^2 far exceed those achieved by LSC for similar sized samples. 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 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 special isotopes with half-lives generally much less than one day. In general, QWBA provides a much higher quality data set than that yielded by organ dissection and homogenate assay by providing concentration data on all tissues, not just those chosen for typical dissection studies, which eliminates the possibility of missing organs that may have high concentrations. The images can also be reviewed and analyzed at any time so that if an issue arises after the tissue distribution has been completed then investigators can go back, review, and quantify the nonroutine tissue(s) of interest.

QWBAs does, however, have several limitations that need to be considered. One of the major limitations of QWBA is its inherent nonselectivity, which is true for all radiolabeled studies. The actual molecular identity of the radioactivite signal detected may be due to unchanged parent drug, one of many metabolites that have retained the radiolabeled portion of the molecule, and/or degradation products. 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 is difficult to evaluate short-lived isotopes, such as those used for radiopharmaceuticals, due to the processing time required, although it is possible to alter the processes to obtain data more quickly, but it requires special attention. 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, Kaim *et al*. [39] were able to demonstrate that the uptake of ^{18}F-fluoroethyl-L-tyrosine (^{18}F-FET) in nonneoplastic inflammatory cells in an experimental soft tissue infection model was lower than that of ^{18}F-fluorodeoxyglucose (^{18}F-FDG), and they predicted a higher specificity for the detection of tumor cells using ^{18}F-FET.

Cost of instrumentation is another limitation. The instrumentation for QWBA is relatively expensive, and highly trained technicians are needed to perform the processing and analysis. QWBA is also limited, in that it cannot adequately provide data at the microscopic level because of the freezing technique. Despite the "snap freezing" techniques used, the freezing of all tissues is too slow to prevent cellular damage due to ice crystal formation. This makes histological receptor localization/staining and cellular identification difficult. Therefore, alternative methods are needed to evaluate cellular distribution and receptor occupancy.

Other general considerations for both dissection and QWBA tissue distribution studies include the radiochemical purity of the test compound, the radioactive dose, and methods of euthanasia. Generally, impurities should account for less than 5% of the material, and so the radiopurity of test articles should be greater than 95%. The use of anesthesia and the method of euthanasia can also affect the tissue distribution of any test article and should be carefully considered. For example, it is commonly thought that CO_2 alters the permeability of the blood–brain barrier (acidosis) and therefore alters normal brain penetration. Thus, euthanasia by CO_2 inhalation may not be a good technique to use if the objective is to study brain penetration. Likewise, euthanasia by exsanguination will undoubtedly have an effect on tissue distribution due to the massive fluid changes that take place in the body during this process [7].

13.4.2 Whole-Body Autoradiography in Drug Discovery

New initiatives in research, such as combinatorial chemistry, expanded compound libraries, parallel syntheses, and high throughput biological screening, have resulted in the generation of large numbers of compounds as putative drug candidates at pharmaceutical companies. The net result has been and will continue to be an increase in the number of drug candidates recommended for successful development. However, to increase the likelihood of developing a new drug, adequate screening for metabolic and PK liabilities during the early discovery process have become mandatory and efforts and cost to evaluate relevant metabolic characteristics of each new chemical entity (NCE) have increased substantially. It is, therefore, critical to reevaluate the current utilization of new technologies and the role of drug metabolism studies to support the discovery of NCEs. More sensitive and novel detection systems have made this process less cumbersome than in previous years. PK screening, drug stability studies, evaluation of metabolites, cytochrome P450 (CYP) involvement, enzyme induction and inhibition, and excretion studies play a major role in the drug discovery process. However, notably absent were tissue distribution studies, especially those conducted using WBA, which before the early 1980s demanded many resources and required a long time to achieve results (4–10 weeks). Quantitation of the autoradiographic images was also difficult because of the limited linear response of the films used to capture the autoradiographic images. However, within the last 15 years, the established methods of WBA have been combined with phosphor imaging technologies, which now provide reliable, quantitative tissue distribution information for

radiolabeled drug discovery compounds. This information can often be obtained within two weeks of dosing. WBA has also been used to answer pivotal questions regarding tissue PKs, routes of elimination, drug–drug interactions, drug localization, clearance, tissue targeting, solubility and formulation issues, routes of administration, tumor and brain penetration, interspecies comparisons, and tissue retention.

13.4.2.1 Brain and Cerebrospinal Fluid Penetration. A common clinical surrogate marker for central nervous system (CNS) penetration of drugs is the plasma to CSF concentration ratio [40]. However, unless this model is validated for each compound, it can be misleading when predicting the extent of CNS penetration of a particular compound. Discrepancies between CSF and CNS penetration can result from functional and structural differences between the blood–CSF and blood–brain barriers (i.e., permeability and surface area differences), variable diffusion of compounds from CSF into CNS tissue, and bulk CSF flow kinetics [41]. Numerous studies performed by preclinical PK laboratories have shown that many drugs get distributed differently into CSF and the brain [42,43]. Despite the evidence, which has demonstrated clear differences between CSF and CNS drug levels, the HIV literature contains many comparisons of CNS permeability of various therapeutic agents based on their relative CSF concentrations. Figure 13.2 shows the WBA results obtained using two different compounds that were being developed to treat HIV-1 [40]. The images clearly show different tissue distribution patterns between brain and CFS levels of drug-derived radioactivity. Thus, drug-A-derived radioactivity distributed preferentially in the CNS, whereas drug B distributed into the CSF but not in the brain tissue. This comparison revealed that there was no correlation between the distribution of drug-derived radioactivity in CSF and brain levels. Although the radioactivity observed in both compartments does

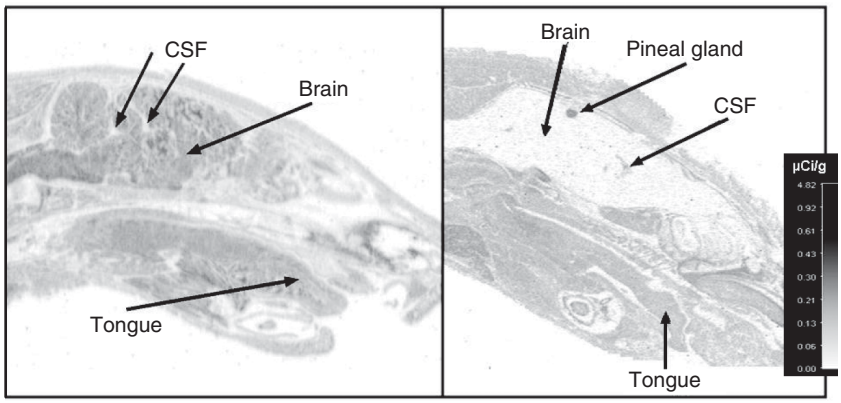

Figure 13.2 Cerebrospinal fluid (CSF) and brain penetration comparison in rats. Whole-body autoradiographs reveal differential distribution of drug-derived ^{14}C between brain and CSF of rats treated with different compounds. The comparison shows that drug levels measured in the CSF do not necessarily predict brain penetration and such models should be appropriately validated before making this assumption. (a) A brain with high concentrations of drug-derived radioactivity in the CSF but absent in the brain tissue. (b) An opposite situation to that in (a) Both are small molecule drugs that were expected to penetrate the brain because of high lipophilicity.

not discriminate between parent drug and/or its metabolites, the unequal distribution observed in all cases strongly suggested that each compound needed to be individually evaluated before making the assumption that levels of parent compound are distributed equally between CSF and brain.

13.4.2.2 Drug–Drug Interactions. Drug–drug interactions are a concern to drug developers whenever the potential exists for the coadministration of two drugs. It is, therefore, often valuable to know whether or not the drug(s) in question will change the disposition of the coadministered drug. An example is presented in Fig. 13.3, where a ^{14}C-labeled compound being developed to treat cancer was examined using WBA [40]. In this study, a single dose of the compound was administered alone (Fig. 13.3a) or coadministered with either 1-aminobenzotriazole (ABT) (Fig. 13.3b) or LY335979 (Fig. 13.3c). ABT is a known inhibitor of the cytochrome P450 metabolizing enzymes [44], the latter of which is responsible for the metabolism of the cancer drug being studied. LY335979 is a potent inhibitor of the transporter protein P-glycoprotein (Pgp) [45] for which the cancer drug was a substrate. Figure 13.3b shows that the pretreatment of animals with ABT resulted in reduced levels of radioactivity in the brain, whereas the use of LY335979 resulted in an enhancement of the radioactivity (Fig. 13.3c) in the brain as compared to the control (Fig. 13.3a). The results of this study suggested that the metabolites, but not the parent compound, of the drug contributed most to the amount of drug-derived radioactivity observed in the brain. This type of finding could have significant impact on the interpretation of *ex vivo* receptor autoradiographic binding assays performed to investigate the pharmacology of the compound in the CNS. Additionally, the study revealed that brain, and theoretically tumor penetration, could be improved by using LY335979. Similar studies with amprenavir [46] revealed an enhancement of concentration of the radioactivity in the brain of mice pretreated with GF120918, which is another potent Pgp inhibitor [47]. These examples show the potential of using known drug–drug interactions to study all types of toxicological, physiological, and pharmacological mechanisms of action, as well as PKs.

13.4.2.3 Melanin Binding. Melanin is a compound responsible for coloration in pigmented animals but is absent in albino animals, such as the Sprague Dawley (SD) rat, which is widely used in toxicology studies. Melanin is found in pigmented skin; choroid plexus and meninges of the CNS; the pigmented epithelial layer of the retina of the eye, such as in Long-Evans (LE) rats [48]; and in nerve cells of primates [49] and amphibians [50]. Many lipophilic drugs with pK_a values above 7 bind to melanin; however, the toxicological consequence of this binding has been debated for many compounds in the literature [51]. Structure–activity relationship studies using 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 [52]. Chloroquine, ofloxacin, norephedrine, and diazepam bind to synthetic melanin with varying affinity [53]. Diazepam, flunitrazepam, and Ro-5-4864 are known to induce melanogenesis [54]. Covalent binding of drugs to melanin is rare; however, its polyanionic nature and high content of carboxyl and semiquinone subunits facilitates noncovalent binding with many drugs. Although melanin binding of NCEs is not predictive of toxicity, it may prohibit the conduct of human radiolabeled studies where prolonged exposure of

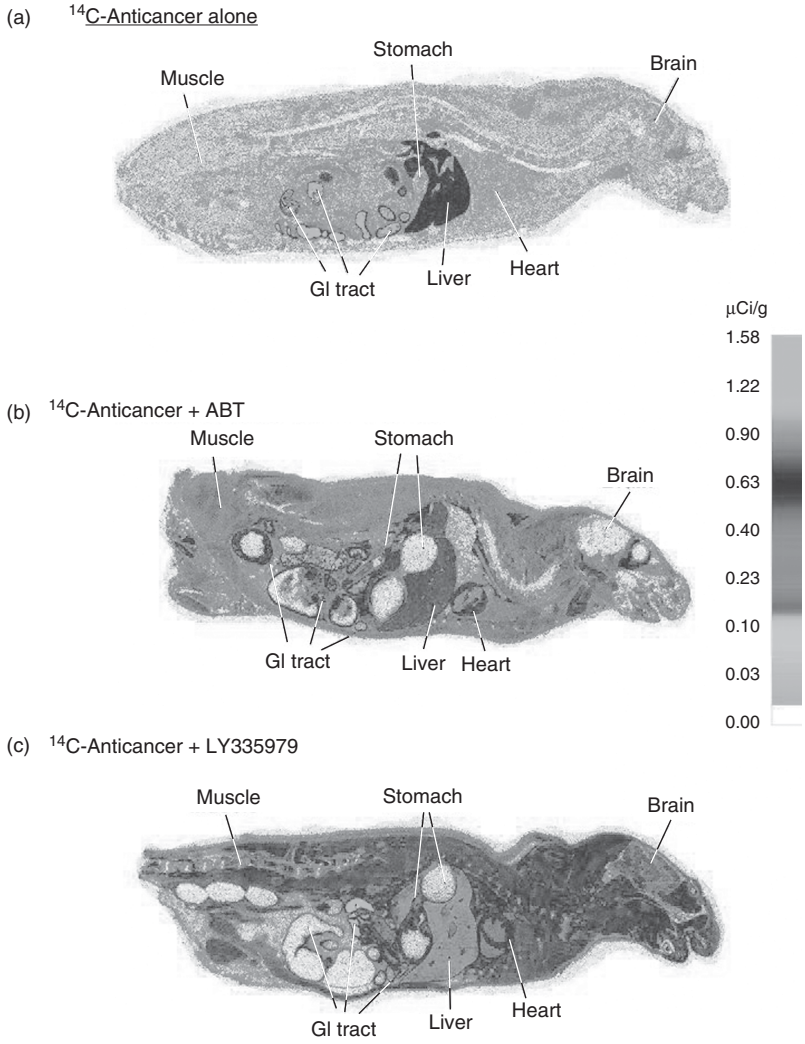

Figure 13.3 Tissue distribution of an anticancer drug after the application of known drug–drug interactions. (a) The tissue distribution of drug-derived radioactivity after a single oral dose of an anticancer drug. (b) The effect of the coadministration of 1-aminobenzotriazole, which is a potent inhibitor of the CYP450 class of metabolizing enzymes, on the tissue distribution of the drug-derived radioactivity after a single oral dose of an anticancer drug. (c) The effect of the coadministration of LY335979, which is a potent P-glycoprotein inhibitor, on the tissue distribution of the drug-derived radioactivity after a single oral dose of an anticancer drug. (See color insert.)

pigmented tissues, especially the eye, to radioactivity could pose a health risk [55]. 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 in drug discovery and development. Figure 13.4a shows a SD 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 13.4b, which is an autoradiograph of an albino rat that

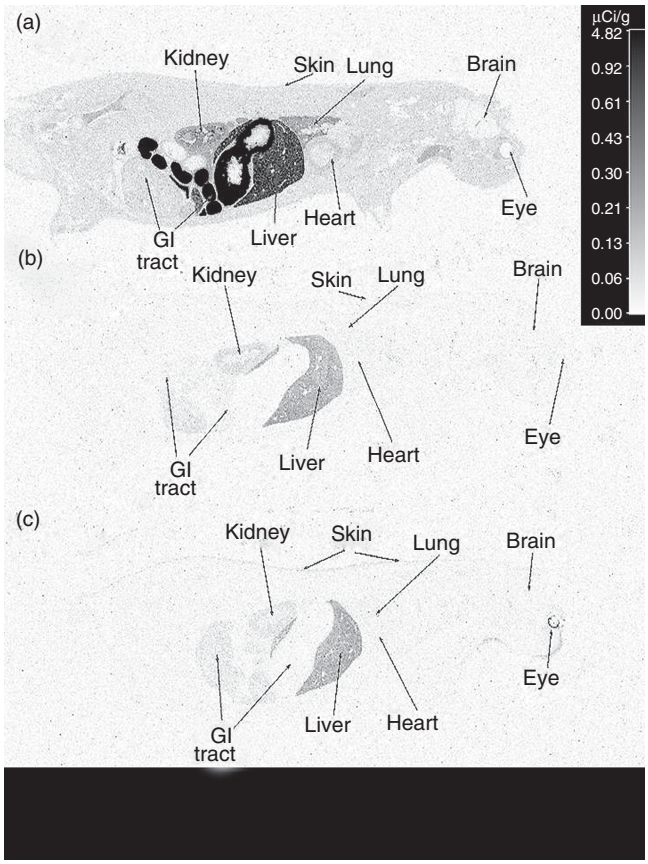

Figure 13.4 Melanin binding. Whole-body autoradiographs reveal tissue distribution of a ^{14}C-labeled drug in albino and pigmented rats. (a) A Sprague Dawley rat at 2 h post dose shows wide distribution of drug-derived radioactivity. (b) A Sprague Dawley rat at 96 h post dose shows limited distribution of drug-derived radioactivity and, notably, no radioactivity in the eye. (c) A pigmented Long-Evans rat at 72 h post dose shows limited tissue distribution of drug-derived radioactivity and, however, a high concentration of radioactivity in the eye, which is due to melanin binding.

was prepared for WBA at 96 h after a single dose, shows the absence of drug-derived radioactivity in the ocular tissues. Figure 13.4c shows the binding of drug-derived radioactivity in the pigmented skin and uveal tract of the eye of a pigmented LE 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 drug-derived radioactivity in humans during a proposed human ^{14}C metabolite identification study was shown to be a cause for concern.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective destruction of melanin-containing monoaminergic neurons of the brains in primates [49] and amphibians [50]. MPTP is metabolized by monoamine oxidase type B to 1-methyl-4-phenylpyridine (MPP^+), which is known to bind to neuromelanin with high affinity and causes cell death and neurochemical features of Parkinson's disease.

The antimalarial drug chloroquine, which also has high affinity for melanin, competitively inhibits the binding of MPP^+ to neuromelanin in monkeys and provides a protective effect against motor abnormalities [56]. Such distribution studies could be monitored easily by WBA. MPTP-related toxicities were not seen in rodents that lack neuromelanin. The pigmented cells acted as depots for drug and/or metabolites, which results in toxic cytoplasmic concentrations causing nerve cell death. Similar melanin-binding studies have also been reported for ^{14}C-*N*-*N*′-dicyclopropyl-methyl-piperazine-dichlorohydrate in cynomolgus monkeys [49] and ^{3}H-aflatoxin in pigs [57].

13.4.2.4 Enzyme Induction/Inhibition. The induction and/or inhibition of drug-metabolizing enzymes by NCEs is a concern in drug development because it can effect the disposition and thus activity of the drug being developed and/or coadministered drugs [58]. The enzyme induction or inhibition caused by a compound may be a benefit, liability, or nonissue to the clinical use of the drug. For this reason, the drug's ability to induce or inhibit metabolism must be considered. WBA has been used to investigate the effects of enzyme induction by the compound on the tissue distribution and PKs of drugs [40]. Figure 13.5 demonstrates the effect of enzyme induction on the tissue distribution of a ^{14}C-labeled drug 24 h after a single oral administration (Fig. 13.5a) and 24 h after the last day of five days of single daily oral administration (Fig. 13.5b). In this instance, enzyme induction was known to occur *in vitro* and *in vivo*, but the actual effect on targeted tissues was unknown. The levels of drug-derived radioactivity in all tissues were lower in the induced animals (multiply dosed) than those of single-dosed animals. Apparently, induction of CYPs led to increased metabolism and excretion of metabolites via bile. This type of study can provide investigators with

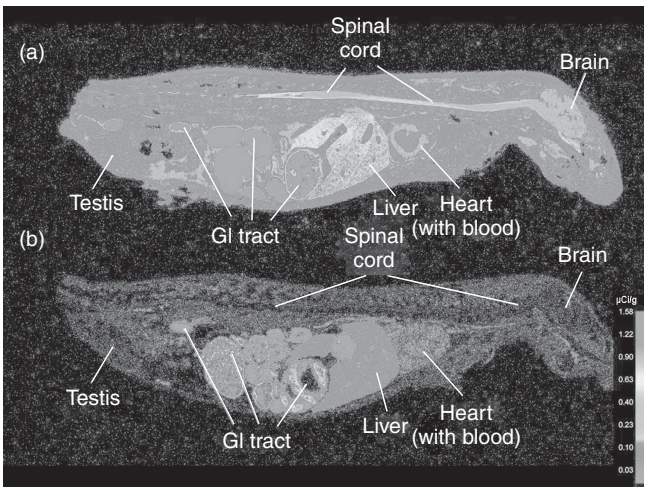

Figure 13.5 Enzyme induction/inhibition and tissue distribution and PK. (a) Whole-body autoradiograph of drug-derived radioactivity in a Sprague Dawley rat at 24 h after a single oral dose of a ^{14}C-labeled drug. (b) Whole-body autoradiograph of drug-derived radioactivity in a Sprague Dawley rat at 24 h after the last of five daily oral doses of the same drug (days 1–4 administered unlabeled drug, day 5 administered ^{14}C-labeled drug). The data obtained at 24 h post dose showed that the elimination after multiple dosing was more rapid than that after a single dose and thus tissue exposure was notably lower. (See color insert.)

important information regarding exposure to target organs/tissues after multiple daily dosing and, in some cases, may require alteration in the dosing regimen in the clinic. The effect of enzyme inhibition on the tissue disposition of NCEs can also be studied by observing the effects that coadministered drugs have on the distribution of NCEs.

13.4.2.5 Tumor Penetration. WBA has been an invaluable tool in cancer research for its ability to provide a visual depiction of tumor penetration, as well as providing a snapshot of general tissue distribution in both tumor and toxicological animal models. Figure 13.6 shows the distribution of a ^{90}Y-labeled anticancer agent, which was administered orally to transgenic mice that expressed human mammary tumors. This study, which was performed very quickly (within three days) because of the short half-life of the radioisotope, showed excellent tumor penetration as well as retention in the tumor versus normal tissues [40]. Importantly, since this technique involves examining all

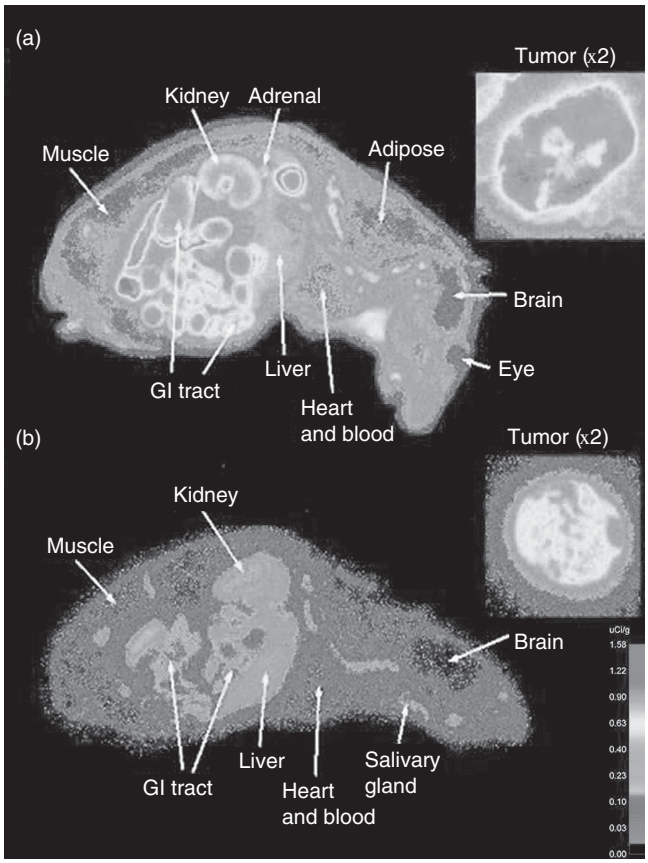

Figure 13.6 Tumor penetration model. Whole-body autoradiographs of tumor-bearing mice that were administered a single IV dose of a ^{90}Y-labeled anticancer drug and sacrificed at (a) 2 h or (b) 24 h post dose. Insets show close-ups (enlarged $\times 4$) of the tumor in each mouse. WBA revealed that high levels of drug-derived radioactivity remained in the tumors for up to 24 h post dose, while radioactivity in normal tissues declined to lower levels. (See color insert.)

other major tissues, distribution of the compound to other potential target organs, such as pancreas, liver, and stomach, can be monitored.

In other oncology studies conducted, mice implanted with ovarian Oca-1 tumors and dosed with ^{3}H-paclitaxel (TXL) in Cremophor EL and alcohol vehicle or ^{3}H-poly(glutamic acid)-paclitaxel (PG-TXL) showed that the better efficacy of PG-TXL over TXL in inhibiting tumor growth due to the broader distribution of TXL and/or its metabolites in tumors when dosed as the macromolecular conjugate [59]. The concentration of free and conjugated TXL was determined by excision and processing of tumors in mice in a parallel study. By WBA, the distribution of radioactivity in the tumor was determined to be initially located in the periphery of tumors on day 1 and was homogeneously distributed into the center of the tumor by day 6. Similarly, a comparison of intratumoral injection of mitomycin C and its dextran conjugate in rats bearing Walker 256 carcinosarcoma, by WBA, showed that while mitomycin C cleared the tumor rapidly, the intratumor clearance was greatly retarded when it was conjugated with dextran [60].

13.4.2.6 Fetal Penetration. WBA can also be used to address questions about placental transfer and tissue retention because clear pictures of the distribution to various organs within the fetus can be investigated. For example fetal penetration of [^{3}H]deramciclane, an anxiolytic agent, after IV and oral administration in rats was not observed [61], which was a useful finding in animals and was used in the package labeling to support safe administration in pregnant women. On the other hand, [^{14}C]olanzapine [62], *Fusarium* mycotoxin zearalenone [63], and chloroacetonitrile [64] have been shown to cross the placental barrier in rats by WBA, which posed a potential safety liability for development of these drugs.

13.4.2.7 Formulation Selection. Formulation changes are sought to bring about desired alterations in the absorption and tissue distribution and also to avoid certain side effects. QWBA was used to determine that a submicron lipid emulsion of the drug tirilazad over an aqueous solution helped to avoid venous irritation without sacrificing PKs and tissue distribution in rats [65]. WBA of holmium-166 (^{166}Ho) chitosan complex, a radiopharmaceutical for cancer therapy, after intrahepatic administration to rats and mice, showed that the drug was not distributed to tissues but was retained at the site of administration [66].

13.4.2.8 Metabolism/Covalent Binding. The use of WBA coupled with other procedures can be adopted for studying metabolism and covalent binding of compounds to specific tissues that show retention of radioactivity. Notable examples include the use of (i) whole-body sections where specific areas of the sections were punched to obtain tissue samples that were subsequently extracted and analyzed for metabolites [67] using mass spectroscopy analysis and (ii) freeze-dried tissue sections from a lamb, which was dosed with ^{3}H-aflatoxin, were extracted successively with 5% trichloroacetic acid (TCA), 50% ethanol, 99.5% ethanol, heptane, and water and then dried and imaged to study the distribution of nonextractable radioactivity [68]. These studies enabled the differentiation of metabolites and tightly bound compounds in tissues. New technologies such as the Advion Nanomate® instrument that facilitates the extraction and collection of compounds from whole-body sections can make this process easier. There are other examples of similar analysis that used the residual frozen animal carcasses after WBA samples were collected [64,69,70].

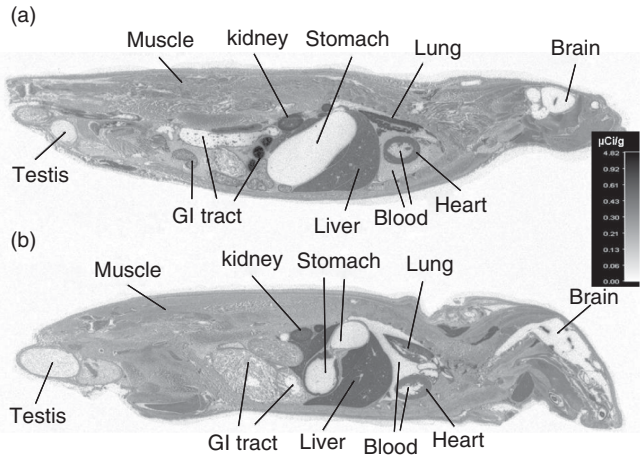

Figure 13.7 Retention in rat tissues. Whole-body autoradiographs of two rats given similar IV doses of a ^{14}C-labeled compound and were sacrificed at (a) 0.5 and (b) 48 h post dose. Autoradiographs showed little or no decline in the amount of drug-derived radioactivity at 48 h post dose, which suggested that the compound was retained in the tissues of the rat.

13.4.2.9 General Tissue Retention. WBA can also been used to determine sites of retention of drug-related material when the mass balance of administered material in excreta samples is low and/or when unusual plasma PK is observed. In an early mass balance study in our laboratories, the total radioactivity of a new compound was not recovered from rats 24 h after IV administration of the compound, with only small amounts of radioactivity recovered in urine, feces, and bile [40]. QWBA was used to identify the unknown sites of possible tissue retention, and it showed that the compound was widely distributed at both 0.5 and 48 h post dose (Fig. 13.7) and tissue levels of radioactivity were essentially unchanged from 0.5 to 48 h post dose. Data suggested that drug-derived radioactivity was retained in the tissues of the rat, while being rapidly cleared from the systemic circulation. The observed tissue retention raised concerns of possible toxicological problems, especially in liver, kidney, heart, and lungs where concentrations were highest. In another study, IV administration of [^{14}C]-D- or -L-serine to rats led to the detection of accumulation of radioactivity in the corticomedullary areas of kidneys, which suggested a possible link between the observed acute necrosis of the renal proximal tubules and administration of large doses of D-serine [71].

13.4.2.10 Application of QWBA to Therapeutic Peptides and Proteins. ^{125}I-labeled proteins and peptides are being used for QWBA analyses to study tissue distribution and PKs 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. 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. Semiquantitative data can be obtained with confidence when the *in vivo* stability and amount of free ^{125}I circulating in the body is characterized.

Currently, ^{125}I ARG is most often used to provide a quantitative method for determining tissue distribution of large molecules because it is either not possible or extremely expensive to radiolabel these molecules with ^{14}C or ^{3}H. Although ^{35}S can

be used in some instances to label large molecules, the synthesis and location of the radiolabel can be difficult, but the resulting stability of the radiolabel, image resolution, and quantification are an improvement over ^{125}I labeling, which is much more instable. The stability of ^{125}I labeling on large molecules is subject to cleavage *in vivo*, which often results in relatively high concentrations of free ^{125}I. The determination of drug concentrations in tissues using ^{125}I labeled molecules must be considered as semiquantitative because of the inevitability of measuring free ^{125}I along with the test article. Thus, care must be taken when interpreting tissue concentrations of ^{125}I-labeled compounds for the thyroid, stomach, kidneys, mammary gland, salivary gland, thymus, epidermis, and choriod plexus [36]. These organs contain a sodium-iodide symporter that are involved in the organification and/or elimination of free ^{125}I [72,73] and often have very high concentrations of free and/or organified ^{125}I, which is not drug related and thus provide misleading results. Most iodopeptides are absorbed intact through the intestinal tract, but once in the blood stream, deiodination can occur to varying degrees depending on the labeling [72,74]. Iodide has a volume of distribution that is about 38% of body weight and is mostly extracellular. In humans, total plasma iodide clearance is about 12% per hour (rate = 45–60 mL/min) and thyroid clearance varies widely depending on dietary intake and can be as low as 3–4 mL/min [73,74]. These data must be considered when considering tissue quantification and imaging with ^{125}I-labeled biotherapeutics. Finally, regulatory agencies have begun requesting that biotechnology companies show the ADME characteristics of large molecules. This can be a challenge in some cases because large molecules are usually not easily labeled with conventional more stable isotopes (e.g., ^{14}C) and their metabolism is often similar to that of endogenous proteins and peptides.

Administration of nonradiolabeled sodium iodide to test animals before giving the ^{125}I test is one way to reduce the uptake of free ^{125}I by shunting free ^{125}I to the kidneys to increase the rate of clearance and reduce the effect on tissue quantitation. Furthermore, the concentrations of free ^{125}I in the body must be characterized to better understand the true drug-derived concentrations in tissue and this can be done by using protein precipitation with TCA protein precipitation of plasma and determining the ratio of protein-precipitable ^{125}I versus free ^{125}I [73]. This ratio can then be used to correct tissue concentrations obtained using QWBA. Further analysis to determine the radiostability of ^{125}I-labeled compounds in tissues may be done using gel electrophoresis, thin-layer chromatography, ELISA, and/or specialized mass spectroscopy techniques. To these ends, tissue quantitation of drug-derived radioactivity using ^{125}I and QWBA or gamma counting techniques can, at best, be considered as a semiquantitative technique, albeit one of the only practical techniques currently available.

Skofitsch *et al*. [75] used ^{125}I-galatin to identify specific binding sites in rat brain using ARG, which is one of the earlier examples of the use to study large molecule distribution. As many types of antibodies are being developed to treat cancer and other diseases, ^{125}I-labeled antibodies have been used increasingly to evaluate tumor penetration and localization. Figure 13.8 shows such an example where QWBA demonstrated localization in the highly vascularized portions of the tumor. Targeting of drugs to necrotic portions of a tumor can be studied by QWBA since differences in uptake between different regions of tumors can be observed, whereas this information could not be obtained through a typical excision/homogenization experiment. The increased interest in delivering oligonucleotide therapeutics including small interfering RNAs [small nuclear ribonucleic acid (snRNA)] to their desired site of action has prompted

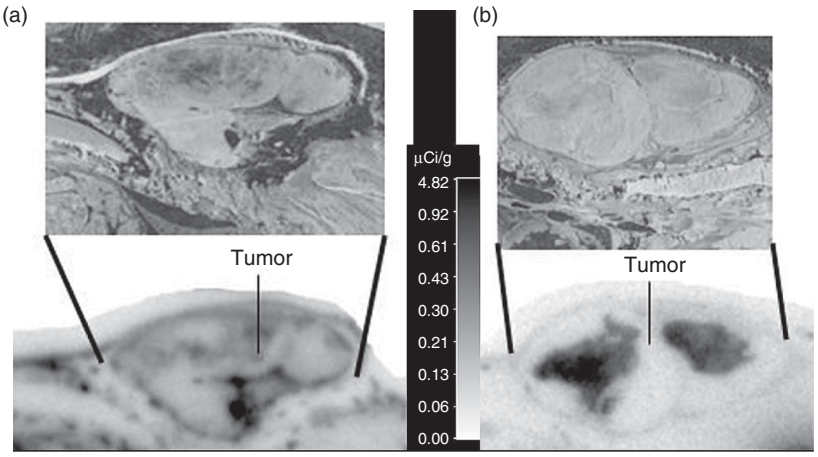

Figure 13.8 Tumor penetration and distribution. ^{125}I-Peptide distribution in different tissue regions of tumors are shown. Note the higher concentrations (darker regions of autoradioluminograph) in the more vascularized areas of the tumor (a) as compared to the regions that show a high concentration in the solid tissue regions (b). (See color insert.)

distribution studies of these entities. ^{3}H or ^{14}C labels can be incorporated for QWBA investigations.

13.4.3 Whole-Body Autoradiography in Drug Development

Most often, QWBA is used during the drug development stages and to support regulatory submissions. The data are used to show tissue PKs and to predict human radiation dosimetry for the human radiolabeled ADME studies. A routine tissue distribution performed using QWBA typically evaluates 35–40 tissues using 6 or more time points designed around known plasma or tissue PKs.

13.4.3.1 ADME Regulatory Studies. The design of tissue distribution studies for drug development and regulatory submission has changed since QWBA has been implemented [36]. In the past, the number of time points used was rarely sufficient to determine reliable tissue PK parameters. Most notably was the determination of the $T_{1/2}$ of drugs in individual tissues. This has implications for predicting the tissue PK and pharmacodynamics of drugs as well as the exposure of human tissue to radiation during human radiolabeled studies conducted to determine human excretion patterns and identify metabolites. Albino strains are most commonly used for whole-body autoradioluminography (WBAL) because they are used most often as the toxicology model; however, pigmented animals are often included in studies conducted to support human radiodosimetry predictions in order to gain exposure information in pigmented tissues such as the skin and eye. When pigmented and albino rats are used, investigators must be aware of strain differences, especially to physiological differences related to drug metabolism and brain physiology [76,77]. Large animals such as rabbits, dogs, and monkeys are also frequently performed for drug development purposes, especially when the species demonstrates plasma kinetics similar to humans. Furthermore, samples of blood, plasma, bile, urine, and feces may be collected from the animals on the QWBA

studies and analyzed by other means such as liquid chromatography/mass spectrometry (LC/MS) or radio-HPLC to provide information regarding metabolism. Investigators and regulatory agencies have embraced these approaches and currently recommend that tissue distribution studies be conducted using validated QWBA methods [78].

Another important consideration is the choice of using a single dose or multiple doses, and a uniform guidance regarding this has been issued by worldwide regulatory agencies [78,79]. They state repeated dose distribution studies may be appropriate when

1. A single-dose distribution study suggests that the apparent half-life of the test article and/or metabolites in organs/tissues significantly exceeds the elimination half-life in plasma and is also more than twice the dosing interval of toxicology studies.
2. Steady state concentrations of the compound/metabolite in circulation of PK or toxicology studies are higher than those predicted by a single-dose kinetic study.
3. Critical histopathological findings of safety studies are observed that would not be predicted by a short-term toxicology and/or single-dose distribution or pharmacology study. The focus of the distribution study should be on those target organs.
4. The pharmaceutical is being developed for site-specific targeted delivery.

The regulatory guidelines and requirements for the designs are less specific but state that the study design should utilize radiolabeled compounds or alternative methods with sufficient sensitivity and specificity to provide quantitative data. They also suggest that metabolites be surveyed as well as the parent molecule [80].

13.4.3.2 Studies for Human Radiation Dosimetry Predictions. Human radiolabeled drug studies are often performed as part of phase II clinical trials to determine human metabolism and PKs of new drug entities. ^{14}C- and ^{3}H-labeled compounds are routinely used in human beings to determine ADME characteristics of NCEs in human subjects. In order to perform these studies, a sponsor needs to provide a prediction of radiation exposure to tissues and/or organs in the body that might result from the administration of a radiolabeled drug. Clinical investigators must assure that the dose of radioactivity associated with the radiolabeled drug will not be harmful to volunteers and falls within acceptable exposure ranges before radiolabeled drug studies can be performed in humans. The Food and Drug Administration (FDA) also requires that pharmaceutical companies perform a prediction of human exposure to radioactivity as a result of the administration of radiolabeled test compounds.

Dosimetry predictions rely on mathematical models and known radioactive tissue/organ concentration profiles and/or excretion patterns, which are obtained from QWBA or "cut and count" tissue distribution studies. The FDA and the International Commission on Radiological Protection (ICRP) have set allowable limits for exposure of radiation involving the administration of single doses of radionuclides to human volunteers. The FDA has limits of 3 rem, which is a measurement of equivalent radiation exposure to whole body and critical organs, and 5 rem to other organs. The ICRP Category IIa effective dose upper limit has been set at 1 mSv, which is another unit of radiation dose equivalent. Recommendations for the methods to determine human and dosimetry predictions have been published by the FDA and ICRP. However, the calculations and data obtained from the various methods can produce different predictions

of radiation exposure and can present problems for clinical review boards considering the approval of proposed human radiolabeled drug studies [81].

Several mathematical prediction methods and calculations have been proposed over the years, but most pharmaceutical companies rely on guidance from the ICRP and those proposed by the Medical Internal Radiation Dose (MIRD) Committee of the Society for Nuclear Medicine [82]. Representatives of the FDA have also published a guidance on the prediction [56]; however, the FDA initiated an updated guidance in 2009 [78]. Additionally, several textbooks have been written over the years, and the basic calculations have not changed much since the early 1960s [83]. However, the equations suggested by the MIRD Committee have been revised over the years and two software packages called MIRDOSE and OLINDA, which were based on the MIRD equations and developed by the Oak Ridge National Laboratory, were created to aid in dosimetry predictions. These softwares utilize mean residence times (MRTs) obtained from the animal tissue/organ distribution information obtained from earlier studies to predict the radioactive exposure to humans given a radiolabeled NCE or other radioactive substances. MIRDOSE provides predictions for whole-body exposure and major organs only. MIRDOSE does not calculate the exposure for many other tissues, and for this reason, its utility for dosimetry is limited.

The equation referenced by Dain *et al*. [56] was originally developed by Marinelli [84] and then refined, to account for animal data, by Hendee [85]. The calculations and methods for predicting human dosimetry presented by the ICRP appear to be related more to industrial radiation workers and the general population and not necessarily focused on clinical radiolabeled drug studies.

The literature on radiation exposure predictions often refer to and consider the $T_{1/2}$ and/or MRT of the isotope used to label the test compound but not necessarily the biological $T_{1/2}$ or MRT of the test article, which may vary considerably from the isotope. This has presented a challenge for pharmaceutical researchers who need to determine what calculations and methods can be used along with the detailed tissue distribution data offered by QWBA.

Anecdotal evidence suggested that there are misunderstandings about dosimetry and different dosimetry methods provide different estimates. A rodent tissue distribution study, which was designed to provide reliable tissue PK parameters by Solon [81], tested this hypothesis by choosing the following three sets of recommended calculations: (i) "Hendee–Marinelli" equation [56]; (ii) MIRD Committee, MIRD equation [86]; and (iii) ICRP recommendations (weighting factors for whole-body exposure [87]). The results showed that each method produced a different prediction of tissue and whole-body exposure. It was also observed that some equations (e.g., MIRD) were best designed for organ homogenate data, which are obtained from organ dissection/homogenate analyses, and do not readily account for the use of actual tissue concentrations. The equations also did not adequately consider allometric scaling from rodents to humans. Furthermore, the use of weighting was not appropriate for use on individual tissues but for determining the contribution of tissues to whole-body exposure only. A common practice among laboratories is also to use PK parameters that have been obtained from studies where reliable parameters cannot be obtained because of study designs that do not adequately capture time points. This results in unrealistic $T_{1/2}$ and AUC values that can grossly over or underestimate exposures. QWBA results have repeatedly shown that tissue concentrations often vary considerably within organs, thus predictions obtained by using organ homogenate data can greatly

under- or overestimate the actual tissue exposure to radioactivity (e.g., concentration found in the cortex and medulla of the kidney). This finding suggests that the current dosimetry methods may need to be revised to better utilize true "tissue" concentration data from QWBA studies for determining the tissue level exposure. This study showed the need to reexamine in detail the guidelines and methods being used to determine human ^{14}C dosimetry from administered radiolabeled drugs for human studies.

13.4.3.3 Postapproval Studies. QWBA has also been used to examine drugs after they have been approved for use to support marketing efforts and/or to address postapproval issues. Schweitzer *et al*. [88] investigated the distribution of [^{14}C] diclofenac sodium (Voltaren®), which is a drug that has been marketed for years, after a single oral administration in rats. These investigators showed that diclofenac preferentially distributed into the inflamed tissues and achieved exposures that were 26- and 53-fold higher in the inflamed neck and inflamed paws of treated animals than in control rats, while all other tissues in treated animals and control animals showed similar distribution and exposure. In this case, the study was done in response to a request from a project team and it also supported marketing efforts for the compound. It also provided for comparing the dermal penetration and distribution of future formulations that could improve exposure and efficacy.

13.5 MICROAUTORADIOGRAPHY

MARG provides pharmaceutical scientists with a high resolution tool to investigate spatial localization of radiolabeled drugs at a tissue and cellular level. MARG is especially good at providing insight regarding *in vivo* receptor binding in various cell types and has predictive value for specific drug targeting. In this respect, it has been used widely in academic settings to answer basic research questions and/or to support pharmacologic studies where it can provide important information on cellular mechanisms. MARG has applications in all areas of science, but this report discusses examples in drug metabolism, pharmacology, toxicology, and molecular biology.

The methods used by the author, which are described below, are based on the methods of Appleton [89] and Stumpf [90], but it is important to realize that there are many variations of the method presented in the literature. 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 in liquid nitrogen. The tissue is then cryosectioned at $-20\,^{\circ}C$ (or the optimal cutting temperature for a given tissue/organ), to obtain 4- to 5-μm thick sections. Then, under darkroom conditions, sections are thaw mounted onto dry glass microscope slides that have been precoated with nuclear photographic emulsion. The slides are placed into a light-tight box with desiccant and allowed to expose for an appropriate amount of time. The collection of sections onto dry slides is a key step developed by Appleton [91] and eliminates the possibility of diffusion of soluble compounds, which can happen during slide and section dipping into an aqueous emulsion. In contrast, the original Stumpf and Roth method [90] involved collection of the section into vials for freeze-drying, which required very careful section handling, was very time consuming, and prone to sample destruction. Following exposure, the slides are developed in a manner similar to developing photographic film before being stained using conventional histological

staining protocols. This may include immunostaining techniques that can provide positive colocalization of drug-derived radioactivity to known cell types, receptors, and/or other structures/markers for which antibody staining protocols exist [90].

There are several examples in the literature of investigators attempting to obtain quantitative data from MARG preparations. A few methods have been proposed to determine subcellular concentrations of drugs. These methods, which date back to the late 1960s, have been presented in books by Stumpf [90] and Baker [92]. One of the earliest methods, which was known as *restricted method*, was based on the analysis of structures of similar shape and size [93] and 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 [94]. This method established a "universal curve" and required that the two-dimensional structures associated with radioactivity were of similar shape. An "unrestricted method" or "circle method" was developed by Williams in 1969 [95]. 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 *hypothetical grain* method was developed by Blackett and Parry in 1973 [96]. This method has five stages that include (i) overlay screen preparation, (ii) collection of "hypothetical grains" and construction of a "crossfire" matrix, (iii) collection of real grain data, (iv) fitting of hypothetical and real grain data using chi-square, and (v) modification of the matrix until an "acceptable fit" is obtained. Each of these methods claimed to be able to estimate the number of molecules in subcellular regions if the specific silver grain yield (average number of disintegrations to produce one silver grain) was determined. Then, it was "theoretically" possible to determine the number of molecules in a cell or nucleus. The formulas must consider exposure time, specific activity, silver grain yield (dependant on uniform method conditions), section thickness, volume of the compartment, and "other parameters," which are very difficult to control because of the various artifacts inherent in an inherently 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., emulsion-coated slide) is unknown and not characterized in almost all examples in the literature. Tissue absorption of radioactivity is also rarely considered or characterized. The number of cells counted/quantified must be sufficient for robust statistical analysis. Background subtraction and variable background can invalidate results. There are no tissue section thickness quality control standards and/or internal calibration standards used in MARG. Changes in daily cosmic and background radiation can adversely affect an entire, several months long study. In short, most pharmaceutical researchers do not have the resources available to develop routine MARG procedures to enable and or prove that MARG is a quantitative technique, and thus its limited application to date.

13.5.1 History

The first microautoradiographic data were produced by Lacassagne in 1924 [97], which lead to further work by Bélanger and Leblond [98], who poured liquid photographic emulsion onto histological sections to reveal the location of radioactive substances in the tissues. Joftes and Warren in 1955 [99] revised that technique and dipped slides

into photographic emulsion, which gained wide use because of its ease of manipulation. This technique has survived the years and is sometimes used today. However, if diffusible radiolabeled compound are utilized, the results can be useless owing to the relocation of the radiolabeled substance, which produces telltale artifacts that can invalidate experiments and discourage investigators. During the 1940s, methods utilizing strips of dried emulsion were developed where the histological sample containing a radioactive substance was placed in direct contact with the dried emulsion strip for a finite exposure time. This method proved cumbersome because maintaining the precise position of the strip on the samples proved cumbersome during exposure, development, and staining. Often alignment could not be maintained, ending the entire process in failure. However, in 1964, Appleton [89] first developed the technique of collecting cryosections onto slides covered with strips of dried emulsion using a thaw mounting technique. This required tissue sectioning and collection to be conducted in a darkroom and under safelight conditions, which required a high level of skill.

The use of cryopreservation and cryosectioning remains critical to the study of diffusible substances because it maintains the spatial locale of the radiolabeled substance in the matrix, whereas liquid tissue fixation steps often solubilize and relocate diffusible test articles. However, when substances are tightly bound to cellular structures (e.g., receptor proteins), positive results may still be obtained from samples processed using conventional histology techniques. Further refinement of MARG techniques occurred during the 1960s by Caro [100], and shortly after that Stumpf and Roth [91] made additional improvements to establish receptor ARG as a more reliable technique. This established the basis for the current MARG techniques. Numerous elaborations on the techniques have been presented by different investigators [101], but the basic principals have remained unchanged for >40 years. Today, as in the past, the MARG technique is very difficult to master, which continues to hamper its use. Researchers must use caution when reviewing the literature and relying on articles that used the emulsion dipping technique and claim quantitative data. The conclusions may be questionable and disputed in some cases. Validation of the technique is lacking in most laboratories and results can be very subjective. This is due to the lack of thickness uniformity of both the tissue sections and the emulsion detection media used. Additionally, there are few, if any, instances where calibration and/or quality control standards have been co-exposed within the samples, which is the only way to clearly prove quantitation across different types of tissue samples.

13.5.2 Strengths and Limitations of MARG

The main strength of the MARG technique is the ability to provide researchers with a high resolution imaging tool for determining the cellular and to a certain extent subcellular, localization of radiolabeled compounds in animal tissues. It is also a very sensitive technique that can theoretically detect the actual number of molecules in a preparation [90], although those claims are difficult to substantiate, and so the technique is mostly used as a qualitative tool. ARG techniques can also be applied in specimens prepared for electron microscopy. However, because of the high degree of tissue processing, it does not lend itself to the localization of diffusible substances, which can be easily solubilized and relocated from tissue during fixation, infiltration, and embedding procedures; however, when the radiolabeled substance is stable in the tissues, such as DNA, RNA, structural proteins, and/or covalently or tightly bound substances, then

the technique can be very powerful and useful. *In situ* hybridization [102] is a good example of how molecular biologists have applied ARG techniques to reveal many aspects of cellular processes, which has been used for many years. Another strength of MARG is limited tissue samples processing and thus accurate capture of the localization of test articles in space and time. The main assumption is that exsanguination and dissection of the tissue samples used do not adversely effect localization of the radiolabeled test substance. Tissues can be removed and frozen relatively quickly and so qualitative localization is believed to be accurately reflected.

Several limitations have impeded the progress and wider use of MARG in drug discovery and development. These include the high rate and ease of artifact production and difficulties of tissue section collection under darkroom conditions. The processing time to obtain results by MARG is difficult to gauge as 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 or even months to obtain optimal results. 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. It may also help to develop easier methods of collecting uniformly thick tissue sections that can be automatically mounted onto slides for processing, although this would be quite a challenge because of the varying matrices to be sectioned (e.g., hard bone, adipose, and eyes). Dependable microsized calibration and quality control standards that can be co-exposed with every section would also need to be developed to assure reproducibility of quantitation. Finally, the new methods would need to enable a significant reduction in the amount and types of artifacts that are produced. Currently, the following types of artifacts [90] must be controlled: (i) effects on emulsion by slight variations in light, humidity, temperature, tissue characteristics, fixation, freezing, chemicals, pH, developer, fixer, and miscellaneous debris in developer solutions; (ii) tissue condition (e.g., freezing technique, fixation, autolysis, sectioning temperature, improper section mounting); (iii) light leaks; (iv) latent image fading; (v) folding and shrinking of emulsion; (vi) positive chemography, which is exposure of the emulsion by endogenous chemical effects such as tissue enzymes; (vii) negative chemography; (viii) deviations of pH in processing fluids; (ix) pressure artifacts; (x) ice crystals on knife; and (xi) crystalline deposits from developing process. Some of these are more easily controlled than others, but together they require a high level of skill by the analyst to overcome. The current technique will remain qualitative and too daunting for routine use in pharmaceutical discovery and development until methods and/or technologies can be developed to better control for variables and artifacts. The lack of new developments in MARG methods has 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.

13.5.3 MARG in Drug Discovery and Development

Despite the difficulty in mastering the technique and high risk of artifacts, MARG has made important contributions to drug discovery and development over the years and has provided insight into the localization of pharmaceuticals to support proof of concept studies, mechanisms of toxicity, efficacy, physiology of hormone action, and cell regulation. For example, MARG is useful to study skin penetration of various

compounds and is routinely used in the cosmetic industry, physiology research, pharmacology, and safety studies. Unilever has developed MARG techniques to examine skin penetration in different *in vitro* test models in rats, pigs, and human skin sample to demonstrate efficacy for consumer skin care products using MARG coupled with confocal microscopy and other techniques (Minter 2007, Personal communication). Linoleic acid (LA) is commonly used in cosmetics, but its *in vivo* human skin penetration characteristics were not very well demonstrated. However, in 2006, Rauvast and Mavon [103] used a unique, *in situ*, "virtual" microautoradiographic slide to examine transfollicular delivery of LA in human scalp. They combined their MARG data with an *in vitro* permeation experiment and compartmental analysis to show that most of the LA was localized to the hair sheath but none was present in the dermal compartment and that 10% of the total LA recovered was found in the stratum corneum and dermis after 6 h. This supported the notion that the diffusion of LA occurred by a transfollicular route. It also demonstrated the value of high resolution MARG for providing detailed cellular localization of the molecule. Skin receptor MARG techniques have also been used to study the absorption, penetration, and cellular localization of ^{3}H-Maxacalcitol, which is a vitamin D analog used for the treatment of psoriasis. Hayakawa *et al*. treated the dorsal skin of rats with a ^{3}H-Maxacalcitol ointment and examined skin exposed for periods of 0.5 through 168 h [104]. They discovered two routes of skin penetration; one via epidermal cell layers and the other via hair follicles. They were also able to distinguish very fine regions of cellular localization, which supported theories on the mechanism of efficacy. Young *et al*. [105] used ^{14}C-iodoantipyrine as a tracer to study intrarenal blood flow in nephrectomized rats, and they used their microautoradiographs and standards to determine blood flow rates. MARG provided a means to identify the morphological location of blood flow, and they concluded "that medullary blood flow was dependent on local prostaglandin production and is also influenced by sympathetic nervous supply." Figure 13.9 shows an example of the distribution of ^{14}C-azidothymidine (^{14}C-AZT) in the glomerulus of a rat kidney after a single IV dose [36]. This example demonstrates the fine detail of localization that can be obtained with MARG. Both renal function and localization of various substances in the kidney have been studied by MARG. Another laboratory used *in vitro* MARG to show localization and density of atrial natriuretic peptide (ANP) in nephrectomy biopsy samples obtained from patients with renal disease [106]. These investigators used [^{125}I]-α-human (1–28) ANP and found localization in the glomerulus and tubular regions in the human biopsy specimens. They also observed that density of ANP binding generally decreased in patients with renal dysfunction and hypertension. Overall, though, their study established an *in vitro* MARG method to assess ANP binding in human biopsy specimens.

More recent work by Yamamoto *et al*. [107] used MARG in combination with immunohistochemistry, macroautoradiography, and PET to examine intestinal ulceration and healing in the rat. They used ^{18}F-FDG to examine ulcerations in the small intestine of rats, which were induced using indomethacin. MARG combined with immunohistochemistry showed an accumulation of ^{18}F-FDG in inflammatory cells as well as in granular tissue-forming cells, forming granulation tissue, and around ulcers. ^{18}F-FDG was also found to be present in proliferating intestinal crypt cells and intact intestinal tissue taken from indomethacin-treated and control animals. They concluded that ulceration could be visualized early by the prominent uptake of ^{18}F-FDG by inflammatory cells and by the formation of granulation tissue by cells in and around

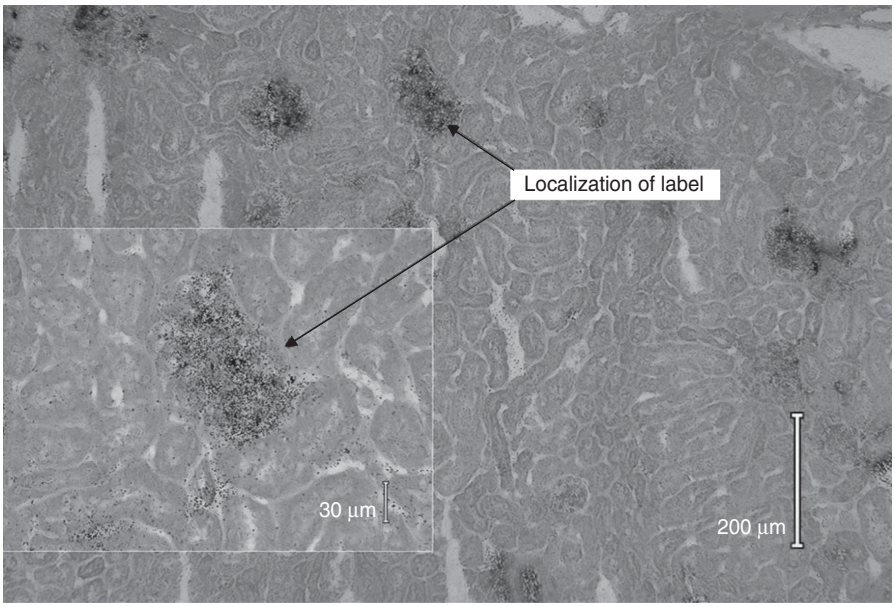

Figure 13.9 Microautoradiography results of rat kidney after a single administration of ^{14}C-azidothymidine in a rat kidney after a single IV dose. The location of the drug-derived radioactivity is made apparent by the black specks that appear over the section. In this case, a high concentration of the drug is shown localized to the glomerulus. (See color insert.)

ulcers. This work also demonstrated the value of combining both *in vivo* and *ex vivo* imaging techniques, which provided robust data sets for analysis.

13.6 CONCLUSIONS

Molecular imaging has grown dramatically over the last 50 years, and most recently, *in vivo* imaging modalities such as PET [108] and fluorescence [109] imaging has 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 PKs 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 degradants is also a limitation of these modalities. However, with the recent developments in matrix-assisted laser desorption ionization mass spectrometric imaging (MALDI MSI), desorption electrospray ionization mass spectrometry (DESI), and secondary ion mass spectrometric imaging (SIMS) technologies, 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 QWBA is expected to remain the technique of choice for tissue quantitation. To these ends, *ex vivo*/*in situ* imaging modalities such as QWBA and MARG will undoubtedly be relied on for many years to come to provide the kind of detailed tissue distribution information required for drug discovery and development programs.

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FURTHER READING

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