CHAPTER 12

Whole body autoradiography

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12.1 *Introduction*

The technique of whole body autoradiography (WBA) was introduced in 1954 by Sven Ullberg, in Upsala, Sweden. WBA involves the cryosectioning of whole animals such as mice, rats, rabbits and monkeys and was developed to study the distribution of compounds in the intact animal's body. In the early days, cryosectioning was performed by technicians dressed in fur coats in a cold room maintained at −15 °C using a hand-driven sledge microtome (Ullberg, 1954, 1958). Today, the technique has been refined substantially, and highly specialised equipment has been designed for all aspects of WBA techniques. WBA was developed in part to overcome the technical problems encountered in traditional methods of studying compound distribution. Many compounds are soluble in water or other liquids used in the histological preparation of tissue specimens and may therefore be extracted from the specimen during processing. WBA allows for the fixation of the compound in the intact animal by freezing, thereby preventing any tissue preservation liquids from coming into contact with the test compound. As such, the localisation of the compound is preserved (Ullberg, 1977). WBA has many applications, but its most frequent use is to generate comprehensive information about the distribution

pattern of new drug candidates. For this application, WBA data are used to support histopathology data from toxicity testing, and to generate radiolabelled dose estimates for human studies. The technique may also be used for a variety of other applications, including the identification of target tissues for a test compound, receptor identification, to investigate blood–brain barrier or placental permeability, or to generate samples for micro-autoradiographic studies.

Although we have attempted to cover as many of the applications of WBA as possible, our experience is limited to use of this technique within the pharmaceutical industry, and as such the information contained within this chapter is from that perspective. The methods presented are intended to serve as general guidelines for the design of WBA studies.

12.2 *Historical background*

The published observation of Niepe de St Victor (1867) of the autoradiographic (ARG) phenomenon pre-dated the discovery of radioactivity per se and assisted in the discovery and awareness of radioactive principles by Henri Bequerel (1896) and Pierre and Marie Curie (1898). The first macro ARG was thought to have been produced by London (1904). The first systematic use of the ARG phenomenon was by Lacassagne and Lattes (1924). Advances in biology, chemistry and physics during and after the 1940s gave scientists access to ever increasing numbers of radioisotopes. These were used to study biological distribution of both endogenous and exogenous substances. Prior to 1954, most macroscopic ARG studies were performed using either Lamholt (1930) or Libby's (1947) method. Lamholt method was only suitable for preparing individual organs, whilst Libby was cutting 5 mm thick whole body sections using a bandsaw. Both methods involved impregnating the sectioned tissue with liquid paraffin, which made it unsuitable for working with soluble compounds. In 1954 Ullberg's method was published, and most autoradiographers today work to a variation of this method for studying the distribution of radiolabelled compounds *in vivo*.

12.3 *Methodology*

For a typical WBA study, a radiolabelled compound is administered to an experimental animal, and the compound is allowed to distribute for various periods of time. At each time point, each animal is euthanised, immediately frozen in a bath of solvent (usually hexane or heptane) and dry ice and the frozen animal embedded in a chilled solution of carboxymethylcellulose. Using this process, a frozen block is formed on a large microtome stage and the block is mounted in a large cryomicrotome. Sagittal sections are taken, collected onto tape and

freeze-dried. The dried sections are then exposed to X-ray film or storage phosphor film, and the latent or digital image generated is quantified using densitometry software. In general, the darkest areas indicate the highest radioactivity concentrations. It is important to note that WBA allows for the detection of radioactivity, which may correlate with parent compound, metabolites or impurities.

12.4 *Study design*

12.4.1 Animal selection

In general, for WBA studies performed to generate tissue distribution data for a test compound, animals are chosen to correlate with the rodent species used in the toxicity testing of the compound under investigation, which supports the interpretation of toxicity data. Usually, the toxicological species is an albino rodent strain. Another consideration in the selection of animals is the evaluation of the potential for melanin binding. If a compound is found to bind to melanin, this may limit the radiolabelled dose in a human study. It is therefore recommended to include pigmented animals in the study design. Consideration of the current regulatory requirements of each country where the drug is to be marketed must also be considered; for example, the Japanese authorities require data from albino animals only and do not investigate melanin binding.

12.4.2 Dose selection

For WBA studies performed to generate tissue distribution data for a regulatory submission, the dose is often chosen to correlate as closely as possible with the low dose used in toxicity testing and the effective clinical dose. This practice ensures that the WBA data can be used with more confidence to support toxicity data and to generate human radiolabelled dose estimates. The dose vehicle should also reflect that used in toxicity testing and in clinical studies. It is critical to use the purest radiochemical possible (>98 per cent), because an impurity could bind preferentially to a particular tissue, resulting in misleading data. Since WBA allows for the study of the tissue distribution of radioactivity, if a radiolabelled impurity were to bind exclusively to a particular tissue, this could be interpreted as the distribution of the parent compound.

12.4.3 Time point selection

In the selection of time points for a WBA study, the compound T_{max}, half-life and pharmacokinetic profile in the rodent species in which WBA will be performed

should be considered. Time points chosen for studies should provide adequate data to allow for the calculation of tissue half-lives, which will be used to calculate the radioactivity exposure for each tissue. Time points chosen for the study should also illustrate that the compound of interest has entered the body and is subsequently excreted. A reasonable course of action is for the early time point to coincide with the T_{max} of the compound in blood. The final time point should be based on the period of time in which it is expected that little or no compound will remain in the body, and unless the compound half-life is very long, or very short, a reasonable choice is seven days post-dosing. Additional time points are chosen based on the pharmacokinetic profile. Different laboratories study different numbers of time points; however, it is generally agreed that five time points are sufficient to provide comprehensive data on the distribution pattern of the compound, while allowing for the calculation of tissue half-life data for most tissues.

12.5 *Obtaining whole body sections*

12.5.1 Freezing and embedding

To prepare animals for WBA, the freezing mixture (usually dry ice added to hexane or heptane) and the embedding agent (2 per cent w/v carboxymethyl cellulose) should be prepared in advance. Animals should be frozen immediately after euthanasia to minimise the diffusion of test compound between tissues. The body should be straightened and positioned to facilitate sectioning, tissue identification and to obtain a pleasing image. The animal should be placed within a freezing frame, and chilled CMC solution poured around the carcass and worked into the fur and crevices before the embedded animal is re-frozen. A 250–300 g rat should be entirely frozen in the freezing mix for a minimum of 20–30 minutes to ensure that ice crystals, which are detrimental to the imaging process, do not form. Once the animal has been frozen, the tail and limbs are trimmed away.

12.5.2 Sectioning

The frozen animal embedded in a solid CMC block is mounted on a cryomicrotome (Figure 12.1), and the animal is trimmed until a level of interest appears. When tissues or organs of interest have been identified, a strip of transparent tape is adhered to the surface of the block. A section is obtained as the microtome knife cuts swiftly under the tape, and the section is lifted away from the block. The thickness of the section is an important factor in the accurate quantification of radioactivity within tissues. It is suggested that 40 µm is the optimum section thickness as there are adequate amounts of radioactivity within the section, but

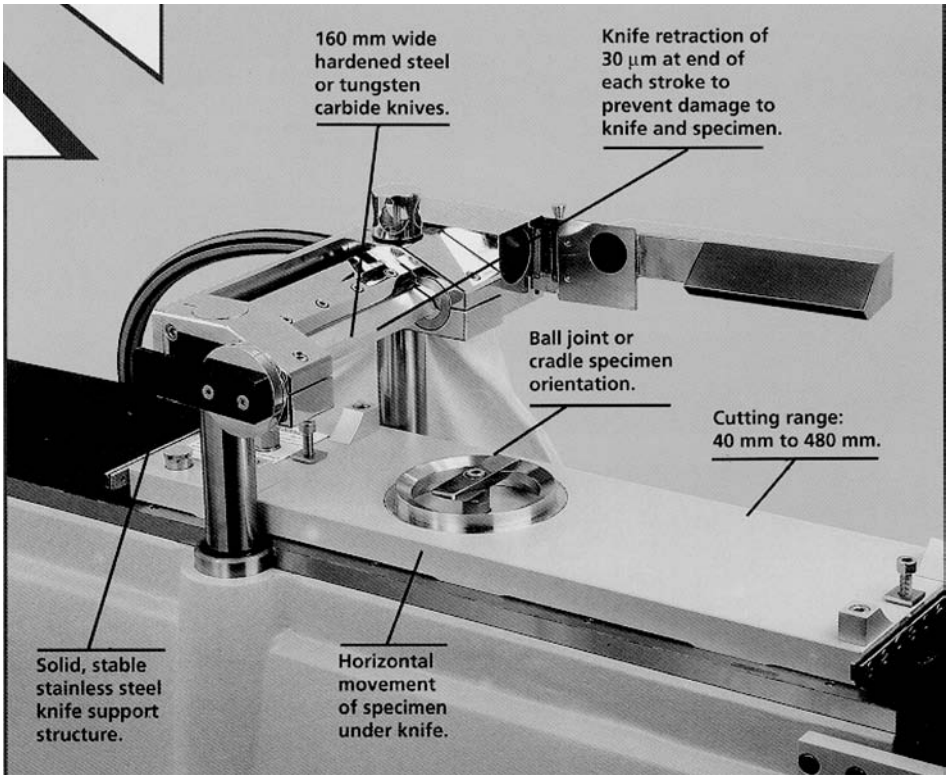

FIGURE 12.1 *Microtome.*

tissue self absorption is minimised and resolution maximised. In this fashion, sections are obtained which contain tissue samples from all tissues of interest for the particular study. After the sections are obtained, they are freeze dried by sublimation at $-20\,^{\circ}C$ in the microtome. Freeze-drying for four days minimises density differences between the tissues, which occur as a result of tissue water content. A light dusting of talc can be applied to the edges of each section prior to exposure, to prevent the tape from adhering to the imaging media.

12.6 *Imaging*

The sections are exposed to the surface of X-ray film or a phosphorimaging plate along with standards. In general, sections are exposed for 4–10 days, depending on the radioisotope, specific activity and time point. Studies show, that after ten days of exposure to a ^{14}C phosphorimaging plate, the signal to noise ratio begins to decrease, so there is no value in exposure times of longer than ten days.

12.6.1 Principle of imaging plate methodology

In the late 1960s, X-ray photography was the only available means for medical imaging diagnostics (Miyahara, 1989). The development of imaging plate technology was made in the 1970s by the Fuji Film company of Japan. Commercially available instruments were introduced in 1981, exclusively for the medical industry, and it was not until the 1990s that analytical phosphorimagers were available globally.

Phosphors are powdered substances that emit light when exposed to radiation, UV rays or an electron beam, or when heated, mechanically hit or stimulated by chemical reaction (Miyahara, 1989). A phosphor emits light when stimulated, for example, by radiation. The light disappears instantaneously when the stimulation ceases. This phenomenon is called 'fluorescence'. Some of the phosphors continue to emit light for a time after the stimulation has stopped, this is 'phosphorescence'. 'Luminescence' incorporates both of these light emission phenomena. The phosphor used for the imaging plate utilises 'photostimulable luminescence' (PSL) which is neither fluorescence nor phosphorescence, but involves a substance that emits light again upon the second stimulation by light having a longer wavelength than the luminescence wavelength of the first stimulation, e.g. radiation.

The imaging plate (IP) is a flexible image sensor in which groups of very small crystals of photostimulable phosphors of barium fluorobromide, containing a trace amount of bivalent europium as a luminescence centre are uniformly coated 150–300 μm thick on a polyester support film (Motoji *et al.*, 1995).

Exposure of samples to the IP is performed in a manner similar to that of X-ray film (Gahan, 1972; Shindo, 1979). The exposed IP is scanned with a He–Ne laser beam of red light (633 nm) whilst the plate is conveyed with high accuracy through a phosphor reader. Resolution and reading density are dependent upon the specification of the machine, and with current technology are 25–100 pixels/mm^2. The reading sensitivity and sensitivity range can also be selected based on the objective. A bluish purple (400 nm) PSL released upon laser excitation is collected via the light collecting guide and passed through the photo multiplier tube (PMT) where it is converted to an analogue signal and then to a digital image. The higher the radioactivity within the sample, the higher the PSL value. This shows up with increased tissue density in the image displayed on the screen.

12.7 *Quantitative whole body autoradiography*

Once the image has been obtained, visual analysis of the image can give qualitative information of the distribution, retention and excretion of radiolabelled material. However, prior to administering the test compound to humans it is

important to be able to obtain quantitative information on the concentrations of drug that each tissue will be exposed to. To perform quantitative WBA, standards of known radiological activity are exposed to the IP along with the sections. Commercially available polymer standards, or blood standards prepared in the laboratory can be used for quantitative WBA. It is essential that the calibration standards are of the same thickness as the sections, or suitably calibrated, because direct comparison of the two on the same imaging plate is central to the quantitative process.

A standard curve is prepared which plots the PSL values against the known concentration of radioactivity. The PSL of each area of interest within the sample can be measured and when plotted against the standard curve, enables the radioactive content of the tissue to be calculated. By knowing the specific activity of the source and the amount administered, the radio-concentration of a tissue can be equated to the concentration of drug that has accumulated in that tissue. The information obtained from QWBA studies can be used to calculate a safe dose of radiolabelled compound for administration to human volunteers.

12.8 *Applications of quantitative whole body autoradiography*

WBA studies provide a rapid way to provide preliminary information on the ADME properties of radiolabelled compounds. WBA is the only method available that can allow the scientist to evaluate the tissue distribution of a drug without having to make prior assumptions regarding the tissue distribution of the compound under evaluation. Although limited to detecting total radioactivity, WBA can none-the-less provide valuable information in many areas relevant to preclinical drug development. In all of the following images, darker areas of the image represent higher levels of radioactivity.

12.8.1 Absorption/distribution

WBA studies provide a rapid method to determine the extent to which a compound is absorbed, and where it distributes within the body. Figure 12.2 is an autoradiogram following an oral dose of a drug candidate at 24 hours post-dose. The compound was well absorbed and distributed into all tissues. Significant distribution into the brain tissue is evident, along with fairly high levels of radioactive drug-related material in the liver, salivary gland and adrenal gland. Non-absorbed or excreted radiolabelled material is also seen clearly in the faecal pellets found in the colon. Note the small structures in the brain (pineal body) where distribution can be visualised and quantified.

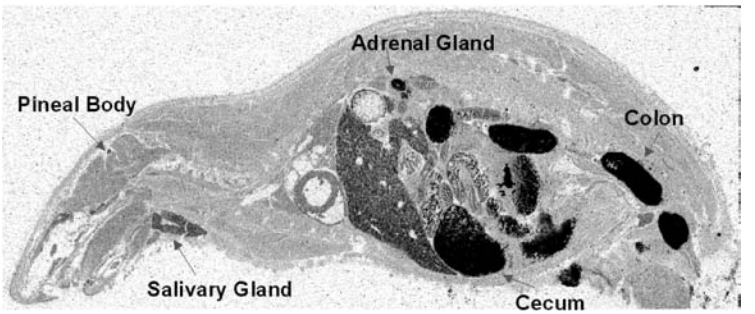

FIGURE 12.2 *Distribution of a ^{3}H-drug candidate in the male albino rat, 24 hours after oral administration.*

12.8.2 EXCRETION

During pre-clinical development, it is important to determine the various routes of elimination for the compound under evaluation. Pre-clinical metabolic excretion balance studies often require the investigator to account for the majority (90 per cent or greater) of the administered dose. Information from WBA studies can identify unsuspected routes of elimination (i.e. skin, bile, saliva, CO_2) which may increase the potential for complete recovery in the metabolic studies. WBA studies can also provide information on retention of radiolabelled material in the tissues or organ systems.

Figure 12.3 is an image following intravenous administration of a drug candidate to a rat, even at 15 minutes post-dose, extensive uptake by the liver is observed with some distribution in the lung and kidney. Distribution into the other tissues of the body, with the exception of the skin is minimal. The presence of radiolabelled material in the stomach suggests that the drug candidate may be directly secreted into the stomach, even at this early time point.

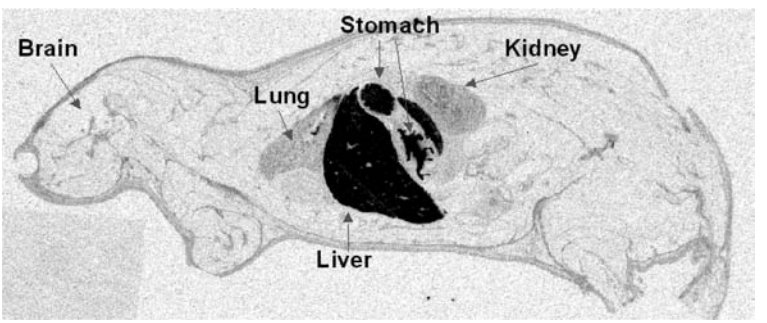

FIGURE 12.3 *Distribution of a ^{14}C-drug candidate in the male albino rat, 15 minutes after intravenous administration.*

12.8.3 Sites of metabolism

High concentrations of radioactivity in a specific organ system (e.g. liver, kidney or lung) may suggest a possible site of metabolism that could be confirmed using *in vitro* tests. If radiolabelled metabolites are available, the distribution of parent and metabolite(s) can be evaluated. Figure 12.4 depicts a drug candidate with high amounts of drug-related material present in the liver at 24 hours post-dose. In this case the presence of the radioactivity correlated with liver toxicity. Subsequent extraction of liver tissue and identification of the radiolabelled material suggested a toxic metabolite.

WBA can also differentiate between the distribution of a metabolite generated *in vivo* following administration of the parent, or when it is administered as a separate entity. Plate A in Figure 12.5 represents the distribution of a ^{14}C drug candidate at 24 hours following IV administration to a male albino rat. Renal toxicity was associated with this compound in the rat species. Pre-clinical studies demonstrated that this compound was rapidly and extensively metabolised to a single metabolite, eliminated in the urine. Other species used in the toxicology studies did not produce this metabolite, and did not demonstrate renal toxicity. A study of the distribution of the metabolite in the rat was performed (Plate B, Figure 12.5) and demonstrated that the distribution of the radioactivity 24 hours post-dose was dramatically different. Subsequent toxicology studies indicated that no renal toxicity was observed when the metabolite was administered.

12.8.4 Residues and toxicity

WBA studies provide a method for correlating the distribution and/or retention of radiolabelled material with observed or potential toxicity. The potential for WBA studies to evaluate unanticipated sites of distribution and/or retention is one of the most appealing aspects of this technique. An example of this is a study where radiolabelled drug was administered to male albino rats by both the intravenous (IV) and oral (PO) routes. The distribution of total radioactivity to various organ

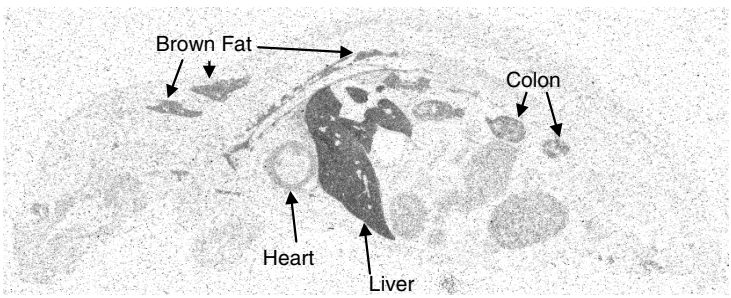

Figure 12.4 *Distribution of a ^{14}C-drug candidate in the male albino rat, 24 hours after intravenous administration.*

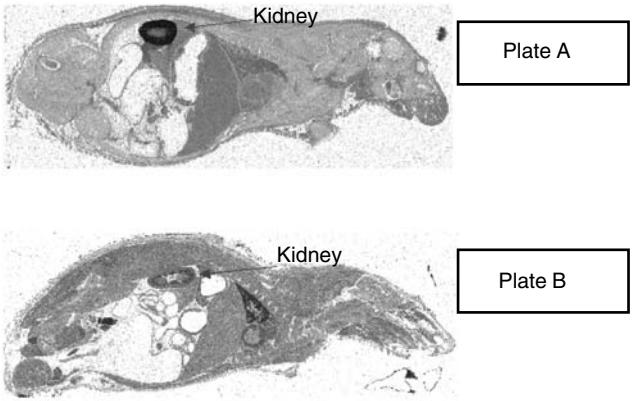

FIGURE 12.5 *Differentiation distribution of parent and metabolite following intravenous administration in the male rat. Plate A – ^{14}C parent drug candidate, Plate B – ^{14}C metabolite.*

systems was monitored for up to 96 hours after dosing. The data was then used to support the micronucleus test by indicating that there was continuous and increased exposure (relative to blood) of the bone marrow to the test material and its metabolites. In this case, the quantitative WBA data also demonstrated that there were no differences between the IV and PO routes in bone marrow exposure.

This information would also be critical in the design of the human radioactivity studies, since it suggested that drug-derived material may accumulate in the bone marrow, and this could limit the amount of radioactivity which could be administered.

Figure 12.6 provides an excellent example of how visualisation and quantification of radiolabelled material can impact upon the development of a drug candidate. During the development of this drug, QWBA studies were performed in albino animals. In the toxicology studies in dogs, histopathology work suggested

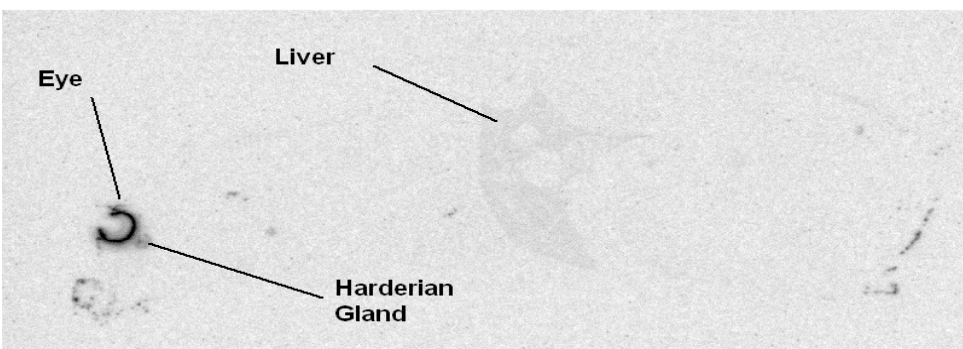

FIGURE 12.6 *Distribution of a ^{14}C-drug candidate in the male pigmented mouse, 13 days after oral administration.*

a potential structural alteration in the eye. QWBA studies in albino rats did not give any evidence for distribution into the eyes of these animals. However, when QWBA studies were performed in pigmented animals a different picture emerged. Pigmented rats demonstrated a significant amount of distribution and accumulation in the uveal tract (retina) of ^{14}C drug-related material. This observation resulted in further efforts to evaluate the time course of elimination from this site and the nature of the binding. These efforts cumulated in the identification of the metabolite responsible for the binding, and impacted the clinical program by the inclusion of ophthalmologic evaluations during the initial clinical studies.

12.8.5 Therapeutic activity

Quantitative WBA can also be used to support the potential of therapeutic activity for development compounds. Often during a drug's development the question is raised as to whether the drug is or can distribute to the site of action. QWBA provides a method to answer this question and quantitative information on the time course of the drug. Figure 12.7 demonstrates the tumour penetration and retention in mice of an agent designed to enhance the effect of anti-cancer drugs. In addition to demonstrating good tumour penetration, the agent appears to be retained in the tumour longer than in the rest of the tissues, especially blood. This could translate into a sustained effect of the compound, which can often be beneficial when developing compounds of this type.

CNS penetration of new chemical entities is also readily and rapidly evaluated using WBA. In this example, WBA was used to demonstrate a change in distribution due to the interaction of two compounds. A *p*-glycoprotein inhibitor, was administered to two groups of male mice once a day for four days prior to administration of a protease inhibitor. Following a single ^{14}C-labelled dose of protease inhibitor to each group of animals, the autoradiograms obtained at 2 hours

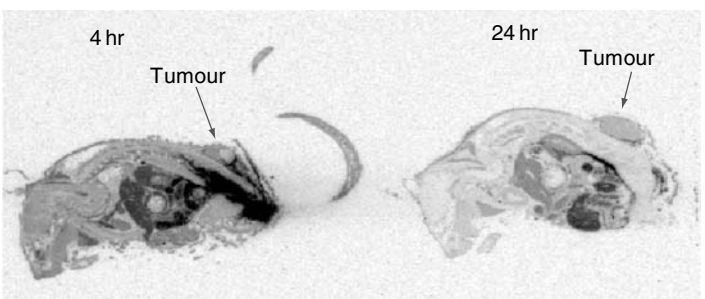

Figure 12.7 *Tumour penetration and retention following a single oral dose of ^{14}C candidate to male SCID mice.*

post-dose are shown in Figure 12.8. This time point was chosen because metabolism studies in mice indicated that the majority of the ^{14}C-labelled material circulating at this time was parent compound. Plate A is the control animal, and Plate B is a section from one of the animals that was pre-treated with the *p*-glycoprotein inhibitor. Quantification of these images revealed that there was an approximately 8-fold increase in the brain:blood ratio in the presence of the protease inhibitor, and a 2-fold increase in the CSF:blood ratio. The studies readily demonstrated the ability of *p*-gp inhibitors to dramatically alter the distribution *p*-gp substrates. It was also noted that blood levels were not substantially different between the two groups, suggesting that the absorption/elimination was not affected to any great extent (Polli *et al*., 1999).

In the second example (Figure 12.9), WBA was used to demonstrate the lack of CNS distribution for a neuromuscular blocker under pre-clinical development. In this set of studies, the ^{14}C-labelled neuromuscular blocker was administered by continuous IV infusion to male cynomolgus monkeys (*Macaca fascicularis*) for 30 minutes. The animals were anaesthetised and ventilated during the infusion period. Quantification of the processed samples demonstrated that only very low levels of radioactivity (just above the limit of detection) were observed in brain tissue, while the meninges and cerebro-spinal fluid had slightly higher levels of radioactivity, suggesting that the compound and its metabolites have very limited penetration across the blood–brain barrier.

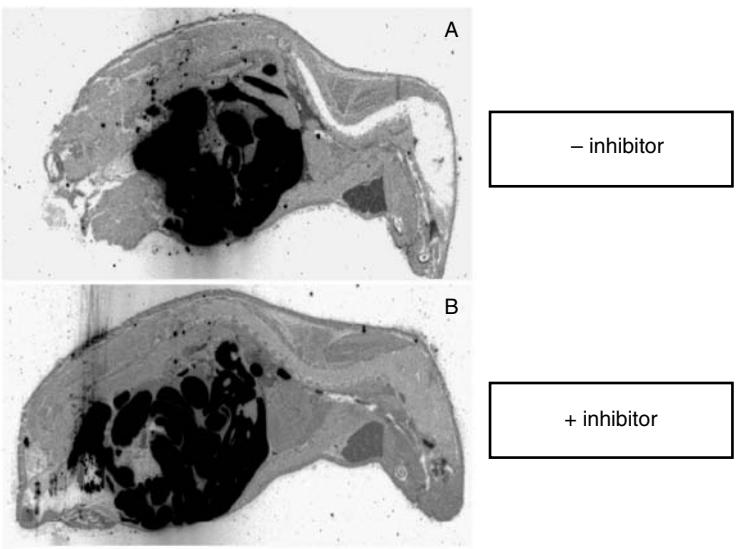

FIGURE 12.8 *Distribution of ^{14}C-p-gp substrate in male CD-1 mice pretreated with a p-gp inhibitor.*

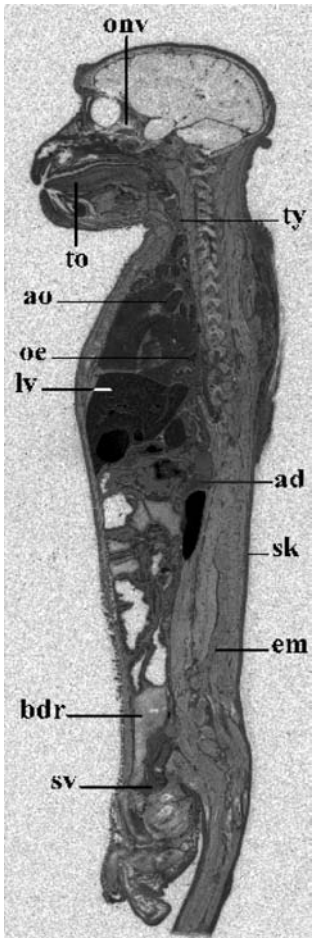

FIGURE 12.9 *Autoradiogram of a ^{14}C neuromuscular blocker in the male cynomolgus monkey following a single 30 minute intravenous infusion. ONV = optical nerve, TO = tongue, TY = trachea, OE = oesophagus, AO = Aorta, LV = liver, AD = adrenal, SK = skin, EM = epimysium, BDR = bladder, SV = Seminal vesicle.*

12.8.6 REPRODUCTIVE TOXICOLOGY STUDIES

QWBA studies can also be used to provide information to support reproductive toxicology studies, by indicating the potential for accumulation in relevant target tissues, including the placenta and developing foetus. Figure 12.10 demonstrates the distribution of a ^{14}C-drug candidate following oral administration to a pregnant rat. The foetal unit (fs), uterus (ut) and placenta (pl) can be readily identified, allowing the distribution of radiolabelled drug-related material to be quantified and the drug exposure estimated.

Figure 12.11 is an enlargement of Figure 12.10 providing further information on the distribution of the radiolabelled material within the foetal unit. At this higher

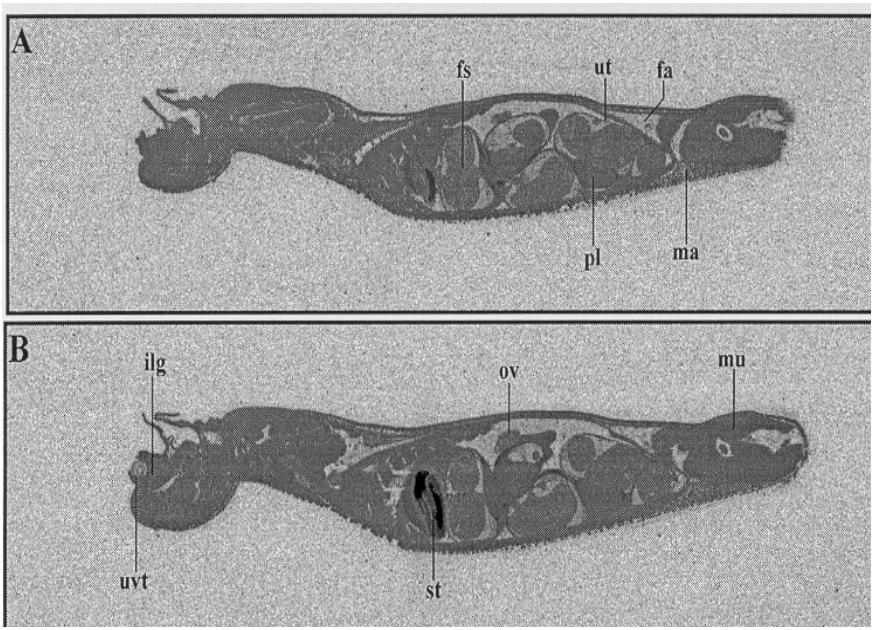

FIGURE 12.10 *Whole-body autoradiograms of a pregnant albino rat 1 hour following a single oral administration of ^{14}C-drug candidate. FS = foetus, UT = uterus, ILG = intraorbital lachrymal gland, UVT = Uveal tract, OV = ovary, ST = stomach, MU = muscle, PL = placenta, MA = mesenteric artery, FA = (white) fat.*

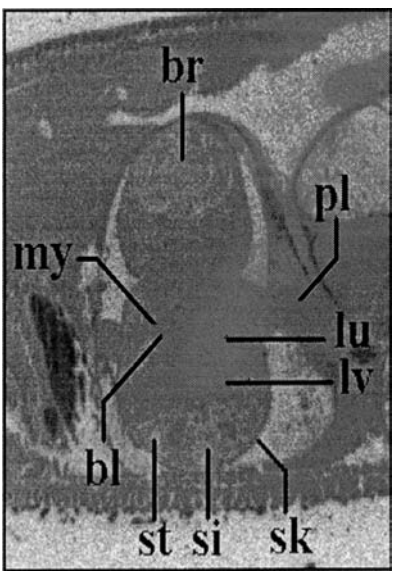

FIGURE 12.11 *Enlargement of Figure 12.10 to show additional foetal distribution detail. BR = brain, BL = blood, MY = myocardium, ST = stomach, SI = small intestine, SK = skin, LV = liver, LU = lung, PL = placenta.*

resolution, additional organs such as the brain (br), myocardium (my), blood (bl), lung (lu) and liver (lv) can be identified and ^{14}C levels quantified. Information such as this can serve to support the reproductive toxicology tests by demonstrating foetal exposure to ^{14}C-labelled drug material.

12.8.7 Human dosimetry studies

QWBA studies are often used to estimate the quantity of radiolabelled material that can be administered to humans. The amount of radioactivity is quantified in individual organs or tissues at various time points to account for the disposition and elimination of the radiolabelled material. This quantitative information is then used to estimate the exposure of the organ or tissue to the radioactivity in the animal model. This exposure in the animal model is then scaled to humans using physiologic modelling on various computer software packages such as MIRDOSE 3.0. Figure 12.12 provides an example where a compound exhibits extensive binding to melanin following oral administration in the rat. Although binding was extensive in the rat, retention was minimal, and the persistence did not adversely affect the amount of radioactivity that could safely be administered to humans.

Figure 12.6 (residues and toxicity section) gave an example of where the retention of radiolabelled material would affect the administration of radiolabelled material to humans. The extent of binding in all melanin-containing tissues and the persistence over time in the eye impact on the amount of radioactivity that could be administered in the human study. Additional WBA studies at later time points suggested that the elimination half-life in the retina of the eye was greater than 1,000 days.

Over the past ten years, QWBA has increasingly replaced traditional tissue dissection experiments undertaken to investigate the tissue distribution and retent-

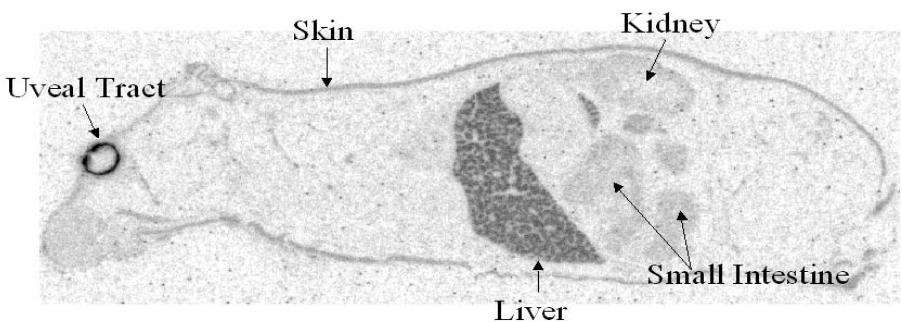

Figure 12.12 *Retention of ^{14}C-labelled material 7 days after an oral dose.*

ion and/or elimination of radiolabelled test materials. WBA offers several advantages over tissue dissection studies, including:

- Preservation of drug candidate localisation within the animal.
- Determination of the distribution in all organs utilising relatively few animals.
- Permitting the observation of unforeseen localisation and retention locations.
- Allowing for determination of the differential distribution within an organ system.
- Providing quantitative and qualitative information.

The primary disadvantages in the technique are that it allows for visualisation and quantification of only radiolabelled material and it is difficult to estimate the actual percent of dose within a specific organ.

However, future advances may remove some of these disadvantages. Recent experiments (Troendle *et al.*, 1999) suggested that by using a quadrupole ion trap mass spectrometer laser microprobe instrument and matrix-assisted laser desorption/ionisation (MALDI) detection, pharmaceutical compounds can be detected in intact tissue. In these studies, MALDI MS–MS was used to detect physiological relevant concentrations of paclitaxel in a human ovarian tumour. Further development of this technology may offer the ability to image the distribution of parent and metabolites of all tissues.

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