

Quantitative Whole-Body Radioluminography—Future Strategy for Balance and Tissue Distribution Studies

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The present routine to conduct balance and/or tissue dissection distribution studies has now and then been questioned, because the way they are generally conducted does not produce information in proportion to the spending of animal and personnel resources. Usually only total radioactivity is measured and due considerations are not always taken to the metabolic fate of the label. In this study a different strategy is presented—integrating quantitative whole-body radioluminography and different chromatographic methods on extracts of tissue pieces punched from the whole-body sections. In addition to the saving in cost and time, the proposed integrated whole-body radioluminographic/metabolic profile protocol will provide (i) a detailed picture of the distribution of radioactivity at selected dose levels and time points in male, female, and pregnant animals; (ii) the time course of radioactivity in blood/plasma and tissues selected from the images (approximate half-life and AUC); (iii) accumulated urinary and fecal excretion of radioactivity and an estimate of the proportion of radioactive metabolites; (iv) tissue information about the proportion of parent drug versus metabolites of pieces punched from the whole-body sections; and (v) indications of possible tissue binding.

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INTRODUCTION

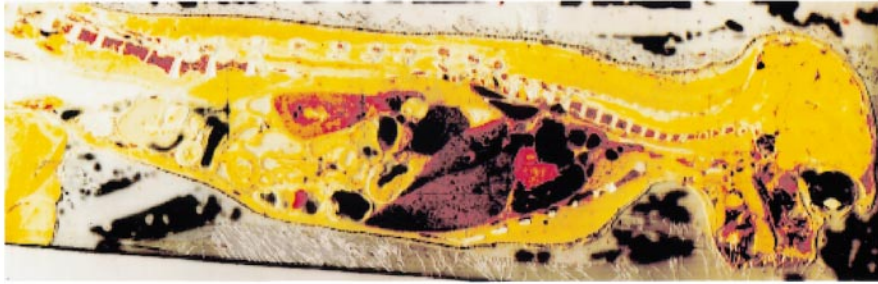
In connection to the preparation of harmonized guidelines for preclinical biodisposition studies the present routine to conduct balance/tissue dissection distribution studies was questioned (Campbell, 1994; Monro, 1994; Chamberlain, 1994). The way they are generally conducted and the information obtained are not in proportion to the spending of animals and other resources. Usually only total radioactivity is measured and due considerations are not always taken to the stability of the label. The dose (or systemic exposure) is often not defined vis-à-vis pharmacological or toxicological effects produced, which may affect several variables. It was argued that the most valid reason to

perform these studies is for dosimetry before giving the radiolabeled drug to humans. A different strategy integrating whole-body radioluminography and metabolic profiling may give more useful information with fewer animals in addition to savings in time and efforts.

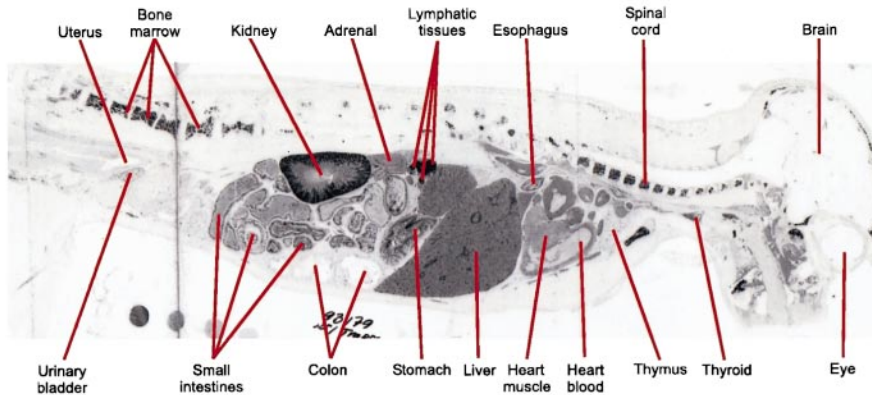
The autoradiographic technique (Ullberg, 1954, 1977), has been further developed by utilizing image analysis programs for converting the obtained gray scale images into quantitative data (Gooche *et al.*, 1980; Yonekura *et al.*, 1983; Ramm *et al.*, 1984; d'Argy *et al.*, 1990), and several other new applications of this technique have been presented (for a review see Dencker *et al.*, 1991).

Over a couple of years we have used quantitative whole-body autoradiography (WBA) as a possible substitute for conventional tissue distribution studies. Our experience is that the accuracy of the quantification of the film image can be similar to that based on liquid scintillation of tissue homogenates. The major disadvantages are the time taken for exposure (usually 1 week to 3 months) in addition to the narrow dynamic range of the film, which often calls for multiple exposures. The advantages, however, are several; the anatomical localization of the (nonvolatile) radioactivity in freeze-dried whole-body sections minimizes diffusion artifacts. The contamination of parenchyma by body fluids is also avoided, which is inevitably the case by tissue dissection methods. Whole-body autoradiograms may also help to demonstrate access of the drug molecules to target cells for both primary and possible secondary actions. Areas of interest can be selected and quantified with very high accuracy after a visual inspection of the image. Thus, radioactivity in structures that may be very difficult to dissect such as small endocrine organs, layers of the eye, nerve ganglia, different parts of the brain, lymph nodes, bone marrow, joints, and fetal tissues can be measured by quantitative whole-body autoradiography. In addition the heterogeneous distribution of radioactivity

a



b



c



FIG. 1. Whole-body autoradiogram (b) and whole-body radioluminogram (c), illustrating the distribution of radioactivity with a ^{125}I -labeled protein in the cynomolgus monkey. For comparison the corresponding whole-body section is shown in its native color above (a).

in tumors can both be characterized and quantified. We have obtained good results with deducing pharmacokinetic parameters based on quantitative whole-body autoradiography. Tissue concentrations and AUC have been determined for blood (heart, liver, carotic, sinus) and for major tissues including placenta and fetus. By studying two or several dose levels nonlinear kinetics of total radioactivity have been discovered using quantitative autoradiography (d'Argy, unpublished results.)

RESULTS AND DISCUSSION

With the introduction of phosphor imaging plates instead of film for detection of radioactivity (Miyahara,

1989; Hamaoka, 1990; Ahr and Steinke, 1994; Motoji *et al.*, 1995), many disadvantages of autoradiography have been eliminated. With this new imaging technique called radioluminography (RLG), the exposure time has been decreased to usually 1–5 days, and in contrast to film, the phosphor imaging plates show a linear response over a wide range of radioactivity concentrations (for ^{14}C -labeled compounds from 0.15 to about 1000 nCi/g tissue with exposure times between 1 and 5 days). This implies that the plates are 10 to 100 times more sensitive than ordinary X-ray films, having a limit of detection of 0.9 dpm/mm²/h (^{14}C). The major disadvantage is a slightly lower resolution (about 100 μm) compared to that of X-ray film (30 μm). These

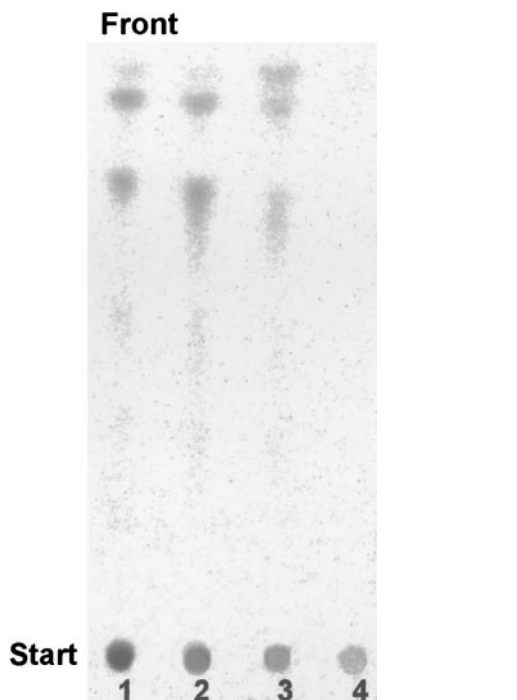


FIG. 2. TLC of tissue extracts prepared from discrete regions of a whole-body section. \varnothing 4-mm tissue samples were punched from a 100- μ m whole-body section. Two samples each from liver, kidney, lung, and blood were extracted with 0.4 ml water. The proteins were precipitated with 1.0 ml acetone (recovery about 80% as revealed by combustion and liquid scintillation of the pellet). The extracts were evaporated and the residue dissolved in about 100 μ l of methanol. The radioactive spots were detected and quantitated by RLG (BAS 2000, TINA). The total radioactivity of each tissue sample was 1000 (liver), 200 (lung), and 50 (blood) dpm.

	Parent compound (%)	Lipophilic metabolites (%)	Polar metabolites (%)	Total activity in PSL
1. Liver	9	8	80	27,000
2. Kidney	20	23	50	13,000
3. Lung	24	20	47	6,000
4. Blood	26	—	74	1,200

PSL, photo-stimulated luminescence.

features are illustrated in Fig. 1 and show in cynomolgus monkey a comparison in resolution of a conventional whole-body autoradiogram on X-ray film (Fig. 1b) and a radioluminogram (Fig. 1c) using the Fuji BAS 2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). The exposure time was 40 days on the X-ray film and 4 days on the imaging plate. Recently a phosphor imaging scanning device for a large specimen has been developed with a stated resolution of about 50 μ m or even 25 μ m, thus making the difference in resolution between X-ray film and phosphor imaging plates still smaller.

Our experience based on ^{14}C - and ^{125}I -labeled compounds has been that this new technique is extremely convenient and has very high precision and reproduc-

ibility. When tissue levels from several sections from the same animal are compared the standard deviation for most tissues is below 10% of the mean (Busch *et al.*, 2000). In addition cross-validation of quantification of blood and various tissue radioactivity in radioluminograms compared to liquid scintillation from animals given a ^{14}C -labeled compound shows that radioluminography supplies highly reliable and reproducible distributional data (Steinke *et al.*, 2000). The use of phosphor imaging plates for quantitative whole-body radioluminography is rapidly spreading, and there has during the past couple of years been an ambitious validation program ongoing with the participation of several European pharmaceutical companies (Busch *et al.*, 2000). These programs have also included a between laboratory comparison.

One of the serious limitations of radioluminography, autoradiography, and conventional tissue dissection studies is that they are unable to distinguish between parent compound and metabolites. However, under certain conditions samples of tissue pieces punched from whole-body sections, which may be cut up to 100 μ m thick, can be sufficient for separation of radioactivity using, e.g., thin-layer chromatography (TLC). Autoradiography or radioluminography can then detect radioactive spots on the chromatogram (d'Argy, 1977). With the application of radioluminography of the TLCs the opportunities for rapidly obtaining results are likely to be considerable, as the plates are up to 100 times more sensitive, e.g., the film. In this way the time course of parent compound in relation to metabolites in major tissues may be explored from routine whole-body sections. An example of quantification of parent compound from a chromatogram obtained from punched tissue pieces is given in Fig. 2. Also high-pressure liquid chromatography (HPLC) can be applied to extracts obtained from punched tissue pieces. Coupling of HPLC to photo diode array detectors may further provide structural information of the radioactive metabolites. In Fig. 3 an example of applying HPLC on punched tissue pieces is shown. In the case of iodine-labeled peptides or proteins such a tissue sampling would also allow measurement of free iodine in different tissues in relation to parent compound, e.g., using electrophoresis. In order to register firmly bound radioactivity, e.g., metabolites of xenobiotics bound to the tissues, some of the tape-fastened sections can be washed in various solvents before the exposure (Miyazaki *et al.*, 1978; Hellman, 1984).

In order to obtain balance data some of the animals intended for autoradiography or radioluminography could alternatively be housed in metabolism cages for say 72 h before sacrifice. The obtained urine and feces thus collected for cumulative urinary excretion data may as well be used for chromatographic or electrophoretic separation of the radioactivity. Samples of urine or feces could also be taken more frequently in

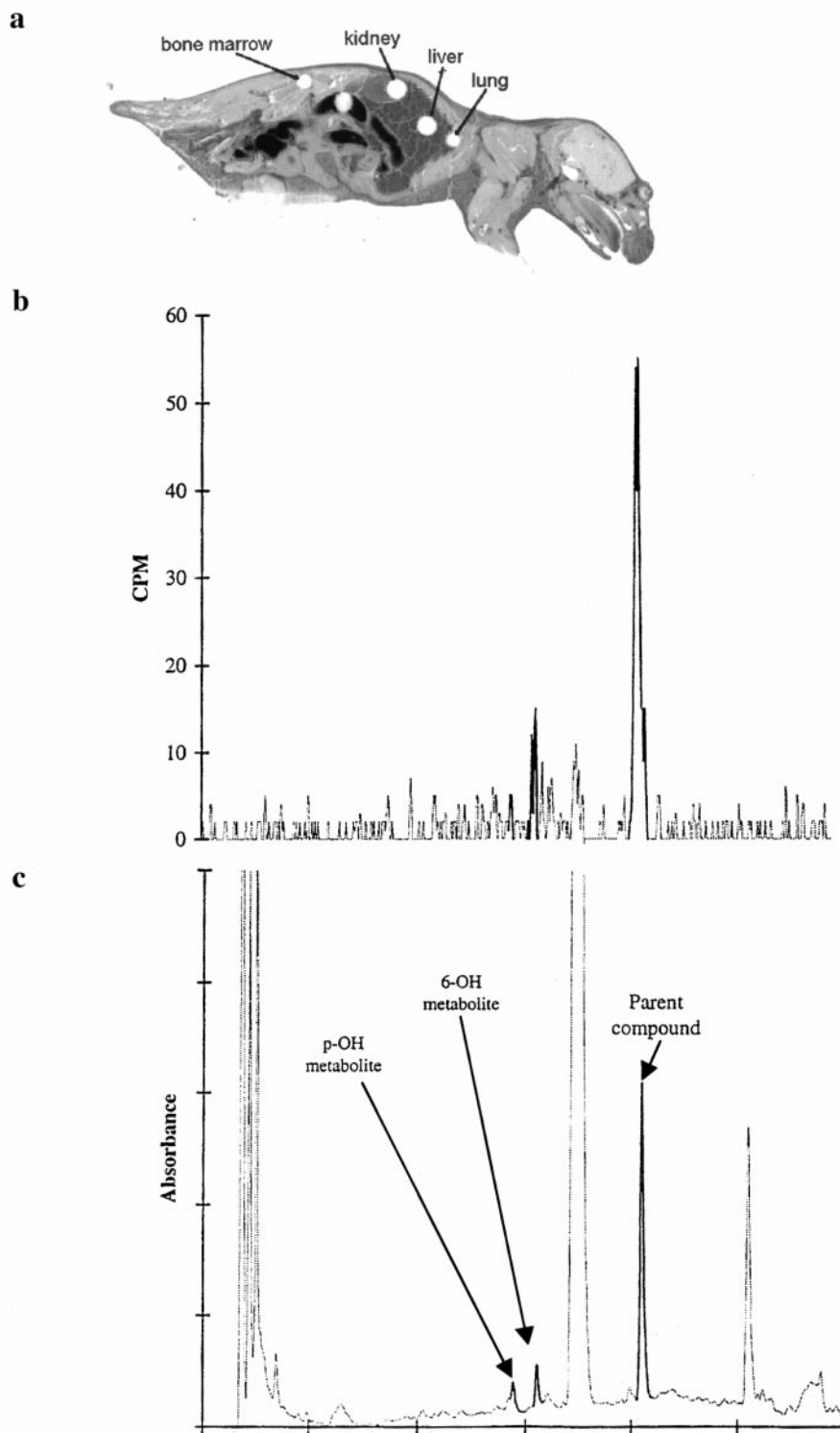


FIG. 3. Whole-body radioluminogram of a ^{14}C -labeled drug showing the holes where different punched pieces have been taken from the section (a). A HPLC radiochromatogram obtained from an extract of a punched liver piece is illustrated in b. Coupling of the HPLC to photo diode array detectors for simultaneous detection of the corresponding UV spectra may also provide structured information of the metabolites as illustrated in c.

TABLE 1

Example of a Possible Standard Protocol Showing an Integrated Radioluminographic/Metabolic Profiling Study, Illustrating the Different Types of Information That May Be Obtained from a Set of Animals Primarily Selected for Mapping the Distribution

Type of study	Time schedule				
	15 min	1 h	4 h	24 h	72 h
Distribution (WBA/RLG)	+	+	+	+	+
Balance, urine/feces		(+)	(+)	+	+
Expired air ^a			+	(+)	(+)
Blood/plasma	+	+	+	+	+
Metabolism ^b (TLC, HPLC)			+	(u, f)	(u, f)

^a ¹⁴C and/or ³H₂O.

^b Tissue extraction from whole-body sections (4 h), urine (u) and feces (f) obtained from the balance (24, 72 h).

order to obtain patterns in metabolite formation, and if a recovery of >95% is the aim, the sampling time maybe had to be extended.

An integrated radioluminographic study could give pharmacokinetic information such as time course, C_{max} , and approximate half-life and AUC of radioactivity could be calculated in selected tissues, in addition to a detailed picture of the distribution of radioactivity. Utilizing samples from tissue sections an estimate of the time course of parent compound in relation to metabolites may also be obtained for different tissues. In addition, quantitative measurements of the amount of radioactivity excreted in urine and feces could be sampled as in ordinary balance studies and by chromatographic methods the amount of unchanged parent compound in relation to metabolites calculated. Although the scene is rapidly changing, many companies still carry out a whole series of balance/distribution studies with a fairly large number of animals not taking advantage of an integrated quantitative whole-body autoradiographic or radioluminographic protocol. A standard protocol could include 7–21 animals, five time points with 1–3 male rats per time point, 1–3 female, and 1–3 pregnant female rats at one of the time points. By using more than 1 rat per time point a feeling for the biological variability will be obtained. A protocol as that outlined in Table 1 might suffice to get a fairly good picture of the fate of a compound.

This type of integrated protocol could be very useful at an early stage of a project—candidate drug selection or in conjunction with single-dose toxicity studies when disposition in relation to the spectrum of pharmacological and toxicological effects and time course of events could be studied. The results might provide a good base for the detail design of the preclinical development plan. Therefore, a carefully designed integrated whole-body radioluminographic study could reduce time, cost, and number of animals. Not only rodents but due to the high sensitivity of the method also cynomolgus monkeys, which are increasingly used as the nonrodent species in the building of the preclinical safety dossier,

will be suitable for producing whole-body radioluminograms and be used in an integrated RLG approach.

CONCLUSIONS

An integrated radioluminographic study will in addition to the saving in cost and time, provide:

- A detailed picture of the distribution of radioactivity at a carefully selected dose level and time point in male, female, and pregnant animals.
- Time course of radioactivity in blood/plasma and tissues selected from the images in one sex (half-life, AUC).
- Accumulated urinary and fecal excretion of radioactivity as well as an estimate of the proportion of radioactive metabolites.
- Information about the proportion of parent drug versus metabolites by TLC, HPLC, or electrophoresis (peptide drugs) of tissue samples punched from the sections
- Indication of possible tissue binding.

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