Studies on the Quantitative Autoradiography. III. Quantitative Comparison of a Novel Tissue-Mold Measurement Technique “Paste-Mold Method,” to the Semiquantitative Whole Body Autoradiography (WBA), Using the Same Animals

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A novel preparation technique, so called “Paste Mold,” was devised for organ and tissue distribution studies. This is the most powerful by joining with autoradioluminography (ARLG), which was established and validated recently in the working group of Forum '93 of Japanese Society for study of xenobiotics. A small piece (10–50 mg) of each organ or tissue was available for measuring its radioactive concentration and it was sampled from the remains of frozen carcass used for macroautoradiography (MARG).

The solubilization of the frozen pieces was performed with mixing a suitable volume of gelatine and strong alkaline solution prior to mild heating kept at 40°C for a few hours. After that, the tissue paste was molded in template pattern to form the small plates. The molded plates were contacted with Imaging plate (IP) for recording their radioactive concentration. The recorded IP was processed by BAS2000.

The molded plate was formed in thickness of 200 μm , so called infinit thickness against soft β rays, and therefore the resulting relative intensities, represented by $(\text{PSL} - \text{BG})/\text{S}$ values, indicated practically responsible ratio of the radioactive concentration in organs and tissues, without any calibration for β -self absorption coefficient. On the other hand, the left half body of the frozen carcass was used for making whole body autoradiography (WBA) before the Paste-Mold preparation. Comparison was performed for difference in $(\text{PSL} - \text{BG})/\text{S}$ values of organs and tissues between frozen and dried sections. A good concordance in relative intensities, $(\text{PSL} - \text{BG})/\text{S}$ by the Paste-Mold preparation was given with those by the frozen sections rather than dried sections.

The combination of the Paste-Mold preparation prior to ARLG and WBA method must be the most powerful to give a quantitative and reliable data in the tissue distribution study.

Keywords quantitative autoradiography; Paste-Mold method; radioluminography; Imaging plate

In order to realize of pharmacokinetic and metabolic studies new techniques, to measuring the radioactive concentration in organs and tissues, have been required in addition to conventional ones. In studies, it is first of all necessary to measure change of drug concentration with respect to time in blood, plasma and tissue. The usual method of measuring the concentration of drug-related components in tissues is liquid scintillation counting (LSC).^{1,2)} In this method, complete dissolution and absence of quenching of sample molecules in solvent are required for quantification. However, only a few samples meet these requirements. Therefore, the development of a simple method to quantitatively measure radioactivity in microradiolabeled samples with high sensitivity has been strongly desired. In response to this, a technique to measure radioactivity using autoradioluminography (ARLG)^{3,4)} following pretreatment with the “Paste-Mold”^{5–7)} method of Motoji *et al.* developed has been presented.

This method has particularly useful to save animal numbers for developing to save animal numbers for developing new drugs. In the Japanese guideline, both LSC and whole body autoradiography (WBA) data are essential to file for the tissue distribution study. Generally, the tissue-dissection experiments are undertaken by different protocol from that of the WBA experiments, based on different animal numbers used in both experiments. However, the up-to-date progress of quantitative ARLG as reported by previous papers suggests that this

technique will be powerful to evaluate accurately the WBA data, even if they indicated semiquantitative concentrations in tissues less inferior than those of LSC data. The half part (usually left side) of the body is used for the WBA preparation, and the opposite half part is used to be discarded. Therefore, we can use the unnecessary part for measurement by Paste-Mold method. In this paper, the detailed description of Paste-Mold method and its applied examples are reported in comparison to the WBA data which were obtained from the same experimental animals.

MATERIALS AND METHODS

Animals Male, 6 weeks, Wistar rats were purchased from Charles River Co.

Labeled Compound [$\text{U-}^{14}\text{C}$]Glucose (Amersham International plc.) with 10.7 GBq/mmol, 56 MBq/mg was used as the radiolabeled compound.

Administration Fluid, Route and Dosage [$\text{U-}^{14}\text{C}$]Glucose was diluted with distilled water without addition of cold glucose, and three kinds of solutions with different radioactivities, that is, 1.85 MBq (50 μCi), 3.7 MBq (100 μCi) and 7.4 MBq (200 μCi) per 1 ml, were prepared for administration. The solution was administered through the caudal vein of a wistar strain male rat (0.1 ml per 100 g of body weight).

Preparation of Tissue Paste Ten minutes after administration, blood was drawn from the inferior vena

Fig. 1. Preparation of Paste and Mold

cava; then specific tissues (blood, plasma, brain, pituitary, eyeballs, Harderian glands, sublingual glands, thyroid glands, heart, lung, liver, kidney, adrenal glands, seminal cyst, skin and muscle) were obtained as samples for radioactive measurement. The other batch was frozen under anesthesia after the administration as described below.

Approximately 50 mg of a tissue sample was transferred to Eppendorf tubes (1.5 ml) and weighed. Prior to the measurement, approximately 20% gelatin solution of the same weight was added to the tubes. This gelatin solution was prepared with 2N KOH solution. The above tubes were immersed in warm water at 60°C for 1 to 2 h to produce a homogenized paste.

Injection of the Paste into a Template Using Thermally Insulated Injector (Fig. 1) A thin film made of polyvinylidene chloride (thickness 4 μm) was attached to the bottom of a plastic sheet (thickness 200 μm) with small holes (diameter 5 mm); then the sheet was placed on a hot plate at 60°C supported underneath by a glass plate. A sufficient amount of the above-mentioned tissue paste was injected into each hole of the sheet to completely fill the holes.

Cooling of the Paste The above-mentioned glass plate was transferred to a refrigerator to solidify the tissue paste. After cooling, the sheet was attached onto a piece of cardboard.

Contact Exposure of the Tissue-Paste Plate with an Imaging Plate (IP) Inside a Cassette The tissue-paste plate was placed bottom-side up into a ready-made cassette (200 × 400 mm). IP (Fuji Photo Co., Ltd., IP-2040) was brought into direct contact with the plate, and the cassette was closed. The cassette was left in a dark and cool place for a predetermined period of exposure time.

Computer Recording and Imaging of Relative Intensity of Radiation (Photo-Stimulated Luminescence (PSL)) from IP: Using BAS2000 Film (Fuji Film Co., Ltd.) For re-

cording and imaging, the region of interest (ROI) to be measured on an appearing image was indicated by a small circle and each tissue sample was identified by its assigned number. The diameter of the ROI circle was set to be slightly smaller than the diameter of tissue paste (diameter 5 mm). PSL value per unit area of each ROI, as well as radioactivity from an undefined contaminant during IP exposure (background) per unit area, was recorded. The background was recorded in a randomly divided area without tissue paste.

Examples of Biological Specimens for Macroautoradiography (MARG) The following experiments were performed using the combination technique (called as "radioluminography" by the developer) of IP and BAS2000.

Preparation of MARG [^{14}C]Glucose solution, as mention above, were administered through the caudal vein of male rats and the animals were sacrificed under anesthesia 10 min after administration.

Preparation of Whole Body Sectioning The carcass was packed in a suitable box and placed in a freezer kept at -150°C. The frozen carcass was placed on a cryomicrotome maintained at -30°C. Preparation of thin sections was carried out according to Ullberg's method. The 15- μm -thick sections, were divided into 2 groups: one was kept in a specially designed freezer and dark box, while the other was gently freeze-dried under vacuum for a week. The dried sections were used at room temperature.

Contacting IP with Biological Specimen Sections Under normal light conditions, an IP was placed in a specially designed cassette after wiping off its background fog, and the thin section was put on the IP and exposed for 1, 2, 6, 24, 48 and 72 h, for reading and recording the images of radioactivity distribution on each IP was then read in a BAS2000.

In order to measure radioactive concentration of organs and tissues, the remaining part of the carcass after preparation of the thin sections was used for determination

Fig. 2. Autoradioluminographs for Quantitative Tissue Balance of the Radioactive Distribution by a Combination of Paste-Mold and Infinite Thickness Methods

Observed tissues and organs: 1, blood; 2, plasma; 3, brain; 4, pituitary; 5, eye ball; 6, Harderian glands; 7, sublingual glands; 8, thyroids; 9, heart; 10, lung; 11, liver; 12, kidney; 13, adrenal glands; 14, seminal cyst; 15, skin; 16, muscle.

of radioactivity. A specimen weighing 50 to 100 mg was removed from the tissue and emulsified with detergents. After addition of a scintillation cocktail, the emulsion was finally analyzed in a liquid scintillation counter LSC1000 (Aloka Co.).

RESULTS

Results of Paste-Mold Method Figure 2 shows the recorded image (ARLG) obtained after 24-h exposure. Each ARLG appearing in the upper, middle and lower parts in the figure represents samples containing 1.85, 3.7 and 7.4 MBq [$U-^{14}C$]glucose solution, respectively. The $[PSL-BG/S (1mm^2)]$ of each sample recorded by BAS2000 from this image is shown in Table I.

As shown in Table I, a satisfactorily quantitative relative intensity of radiation which indexed by PSL unit could be obtained after a 24-h exposure even at the lowest concentration of 1.85 MBq/kg among these 3 concentrations of radioactive glucose administered. Quantitative values could be obtained even in tissues from small organs such as pituitary and thyroid glands. Moreover, as shown in Table I, these $(PSL-BG)/S$ values increased in direct proportion to the dose in each tissue. Figure 3 shows relation between the quantitative data of Paste-Mold method and LSC method. This value of each tissue radioactivity was divided by the radioactivity of blood. From the results, the existence of a correlation between the two methods was confirmed.

Examples of Biological Specimens Figure 4 shows black-and-white hard copies of 3 kinds of autoradiographs by frozen thin sections and 3 different administration doses; the upper, middle, and bottom photos, represent 5, 10, and 20 μCi of the administered doses, respectively. By visual estimation, these autoradiographs show that their

Fig. 3. Correlation between $(PSL-BG)/S$ and LSC Values of the Tissue/Blood Concentration

Dissected tissues respectively from the corpse rats were divided into 2 parts and each part was used for measuring of radioactive intensity by 2 measuring methods of ARLG and LSC. The ratio of tissue/blood was calculated with regard to every tissues.

autoradiographic densities are dependent on the radioactive doses. However, it is difficult to determine the ratio of visual densities for the 3 photos as 1:2:4. Figure 5 shows colored hard copies of the same samples shown in Fig. 4. Differences in color gradations on the autoradiographs lead to the ease of determination of the ratio of radioactive doses.

The effect of freeze drying on autoradiographic intensity recorded on the IP was studied and results are shown in Fig. 6. The left and right photos show two macro-autoradiographs under dried and frozen conditions, respectively, while the exposure period was the same. Each photo is accompanied by a display table showing the

Fig. 4. Black-and-White Hard Copies of IP Autoradiographs of $[U-^{14}C]$ Glucose 10min after i.v. Injection in Rats
Conditions of the ARG preparation: radioactive dose: 5, 10 and 20 µCi, manner of contact: IP was made to contact 15-µm-thick frozen sections in a dark box kept at -25 °C, exposure time: 24h, display manner: black-and-white hard copy. Please refer to the text for other details of preparation.

Fig. 5. Colored Hard Copies of IP Autoradiographs of $[U-^{14}C]$ Glucose
All ARGs were prepared in the same manner as that described in Fig. 4. Thin sections: the same as shown in Fig. 4.

Fig. 6. Colored Hard Copy and Display Table of Dried and Frozen Thin Sections Labelled with [U-¹⁴C]Glucose of 20 μ Ci after a 24-h Exposure
All labelling preparations were the same as those in Fig. 4. Left photo: dried sections: kept at -25 $^{\circ}$ C during drying under vacuum, prior to contact of the sections with an IP. Right photo: frozen sections: kept at -25 $^{\circ}$ C during period of contact of the sections with an IP after a 24-h exposure.

TABLE I. Relative Stimulated Intensity (PSL) of Each Tissue Pellet by Paste-Mold Method

No.	Tissue pellet	(PSL - BG)/S			Ratio of (PSL - BG)/S		
		1.85 MBq/kg	3.7 MBq/kg	7.4 MBq/kg			
1	Blood	6.576	12.585	26.081	1.0	1.9	4.0
2	Plasma	8.129	16.691	32.474	1.0	2.1	4.0
3	Brain	8.635	16.223	33.584	1.0	1.9	3.9
4	Pituitary	4.132	8.162	16.407	1.0	2.0	4.0
5	Eye balls	5.604	11.449	23.173	1.0	2.0	4.1
6	Harderian glands	11.521	23.352	47.169	1.0	2.0	4.1
7	Sublingual glands	8.546	15.823	33.727	1.0	1.9	3.9
8	Thyroids	4.023	8.101	16.145	1.0	2.0	4.0
9	Heart	5.619	11.414	22.304	1.0	2.0	4.0
10	Lung	5.908	11.289	22.934	1.0	1.9	3.9
11	Liver	5.945	11.663	23.138	1.0	2.0	3.9
12	Kidney	5.862	10.331	23.101	1.0	1.8	3.9
13	Adrenal glands	2.660	5.401	10.351	1.0	2.0	3.9
14	Seminal cyst	3.558	7.273	14.139	1.0	2.0	4.0
15	Skin	4.359	8.370	17.302	1.0	1.9	4.0
16	Muscle	2.324	4.498	8.971	1.0	1.9	3.9

The (PSL - BG)/S value was given by 24 h exposure. Tissue pellets were prepared from 3 kinds of different radioactive doses to rats as shown in the text.

Fig. 7. Relationship between Relative Intensity of (PSL - BG)/mm² of IP Autoradiographs after a 24-h Exposure and 3 Different Doses of [U-¹⁴C]Glucose Administered in Rats

All data were obtained from the display memories in Fig. 4. ○, brain; ●, liver; □, kidney; ■, muscle; △, blood.

Fig. 8. Relationship between Relative Intensity of (PSL - BG)/mm² of IP Autoradiographs of Rats and Exposure Time

A dose of 20 μCi of [U-¹⁴C]glucose was administered in rats. ○, brain; ●, liver; □, kidney; ■, muscle; △, blood.

relative intensity of (PSL - BG)/mm² and other data.

From the computational analysis of both hard copies, differences in the images of the brain, tongue, heart, and liver between the two conditions were observed, while little difference among other tissues was apparent. According to the display data, the ratios of the muscle, blood, kidney, liver, and brain are 1:2.16:1.70:2:10:3.52 for frozen condition and 1:2.05:1.60:2.04:3.77 for dried condition, respectively. Furthermore, the relative intensities of the muscle in both photos show 63.495 and 78.219 for frozen and dried conditions, respectively.

The relationship between relative intensity of (PSL - BG)/mm² and radioactive dose is shown in Fig. 7 for the frozen condition. A linear relationship was found for all organs and tissues. A linear relationship between relative intensity and exposure time was also found for the frozen thin sections from all organs and tissues (Fig. 8).

The relative intensity of (PSL - BG)/mm² was compared with the concentration of radioactivity (dpm/g) obtained from conventional liquid scintillation counting for several

Fig. 9. Relationship between Relative Intensity of (PSL - BG)/mm² and Radioactive Concentration (dpm/g) of the Brain, Liver, Kidney, Muscle and Blood of Rats 10 min after the i.v. Injection of [U-¹⁴C]Glucose of 20 μCi

All ARGs were prepared in the same manner as that in Fig. 4. The frozen sections were 15 μm thick. ○, brain; ●, liver; □, kidney; ■, muscle; △, blood.

Fig. 10. Relationship between Relative Intensity of (PSL-BG)/mm² and Radioactive Concentration (dpm/g) of the Frozen and Dried Rat Brain

All preparations were made in the same manner as described in Fig. 4. ○, frozen brain; ■, dried brain.

Fig. 11. Relationship between Relative Intensity of (PSL-BG)/mm² and Relative Exposure (dpm/g × h) of Represent Relative Radioactivity Concentration of the Brain, Liver, Kidney, Muscle and Blood

○, brain; ●, liver; □, kidney; ■, muscle; △, blood.

organs. As shown in Fig. 9, a linear relationship between the two factors was obtained.

The relationship between relative intensity and radioactivity for both dried and frozen brain specimens was examined. As shown in Fig. 10, the two lines representing the dried and frozen brain specimens were not parallel, indicating different slopes. In the frozen thin section, the relationship of (PSL-BG)/mm² versus relative exposure (radioactive concentration (dpm/g) × exposure time (h)) was linear as shown in Fig. 11. This relationship was independent of organ specificity when the specimen section used was frozen; however, this is not so for dried specimens. These results suggest that frozen specimens are recommended for studying the relationship between radioactive concentration and the relative intensity of (PSL-BG)/mm².

DISCUSSION

The most sought-after result in studies is to clarify the mass balance. Administered drugs are distributed through

the circulatory system, passing through tissues or finally being absorbed by them. The dynamics of a drug in a stage unaffected by metabolites is described only by the change of the drug-concentration distribution in the entire body with respect to time, when the drug dose is assumed to be 100. LSC has generally been used as a technology for quantification to realize the objectives of studies. However, LSC has two disadvantages which decrease the reliability of radioactivity quantification in biological samples: (1) phosphor can only be dissolved in nonpolar solvents and not in water, and (2) absorption only occurs during penetration of luminous light through the liquid. Under these circumstances, a "method for measurement of radioactivity in tissues" which has high reliability is expected to become an extremely important tool.

The important merits of the Paste-Mold method are considered to be (1) the absence of quenching, (2) capability of performing measurement of a large number of samples under the same conditions, (3) measurement of approximately 100 samples at one time for only 5 min after insertion of the IP into a computer system (BAS2000). (4) Low back counting by contacting of the IP with the tissue-paste plate for up to 72 h. (5) Minimum measurement error, (6) no requirement for correction of unknown sample thickness and β -ray self-absorption, and (7) reduction in the amount of radioactive waste.

Theoretically, the radioactive concentration (dpm/mg) of each organ or tissue should be in direct relation to PSL-BG/mm² as obtained from a frozen thin section, because the thickness of tissues in a whole-body section was the same. Upon freeze drying however, each tissue will have different thicknesses, and this will influence the correlation between the radioactive concentration and the relative intensity of each tissue. As shown in Fig. 6, the left photo shows a higher intensity than the right photo. This is an indication that a decrease in thickness of the section but to freeze drying will increase PSL-BG/mm², due to self-absorption of soft β energy. In our experiments, a comparison of autoradiographic images prepared from both frozen and dried specimens was performed and the results are shown in Fig. 6. There was a marked difference in thickness between frozen and dried specimens. In the frozen specimens, the thickness was entirely predesignated, and a designated thickness was regulated within a few standard deviations. Furthermore, it seems practical that the designated thicknesses of TLC plates or polyacrylamide gel slabs be kept within a certain standard level under a circumstance of humidity. On the other hand, the thickness was caused to vary according to the dryness or wetness of the frozen specimens. The most efficient application of this method will be represented by joint use of MARG. Usually, the left half side of the whole frozen body is used for macroautoradiographic preparation. And the remained right half side will be used to be discarded. Therefore, the joint use of the same frozen animal body will be possible for MARG and ARLG quantitation following Paste-Mold. Thus, both data by MARG and Paste-Mold must be complements each of the other short points, quantitation in wide dynamic range and radioactive accumulation specially localized on a small tissue. As previously described, the macroautoradiographic data

are essential for readily distinguishing of specially radioactive location from the other tissues. On a longitudinal section a macroautoradiographic image is the most efficient to represent the radioactive concentration of a certain small tissue not only independent secretory organs but also mucosal membrane or connective tissues in distinction from that of blood or other tissues side by side. Even though a comparison of the radioactive concentration between the small limited tissue and the other tissues would be semiquantitative in small dynamic range of photodensity, a macroautoradiographic data must be one of the most reliable elucidation methods rather than the other radioactivity counting methods, because the comparison can be indicated in a view of the whole body section under the back-ground level and the same

condition of animal preparation and photoprocessing.

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