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Title: Practical Application of Quantitative Autoradiography in Pharmacokinetic, Metabolic, and Biochemical Studies for the Development of New Drugs.

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Introduction

The main points of pharmacokinetic and metabolic studies (hereafter referred to as "studies") are well described in "Guidelines for Pharmacokinetic and Metabolic Studies."[1] In order to realize these studies, new technologies have been required in addition to conventional ones. These new technologies are mainly related to "qualitative and quantitative microanalyses."

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).[2,3] 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)[4,5] following pretreatment with the "Paste-Mold"[6,7,8] method of Motoji et al. was developed.

Qualitative and structural microanalyses of parent compounds and their metabolites for drugs contained in plasma or other tissues, as well as their quantitative microanalysis without extracting those components from organisms, are required for realizing the guidelines for studies. TLC has been utilized as a simple qualitative method to process a large number of samples within a short period of time. However, TLC could not be used for quantitative microanalysis of radioactivity due to the occurrence of polymorphous spots, except in the case where spots are scraped off from TLC plates for LSC. In the current study, an excellent quantitative microanalytical method which can overcome these difficulties was reported; it is TLC-ARLG,[9,10,11] developed by Okuyama et al. This method is advantageous in terms of its simplicity, energy efficiency, and capability of processing a large number of samples, compared with conventional HPLC methods.

I. Paste-Mold method (pretreatment method for ARLG measurement of radioactivity in tissues): Method and results

1. Labeled compounds, animals, administration route, dose: [U-^{14}C] glucose (Amersham International plc.) with 10.7 GBq/mmol, 56 MBq/mg was used as the radiolabeled compound. [U-^{14}C] glucose was diluted with distilled water

without addition of cold glucose, and three kinds of solutions with different radioactivities, that is, 1.85 (50 μCi), 3.7 (100 μCi) and 7.4 MBq (200 μCi) per 1 ml, were prepared for administration. The solution was administered through the tail vein of a Wistar strain male rat (0.1 ml per 100 g of body weight). Ten minutes after administration, blood was drawn from the inferior vena 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.

2. Preparation of tissue paste: Paste-making: Approximately 50 mg of a tissue sample was transferred to Eppendorf tubes (1.5 cc) 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 hours 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 1.3 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.

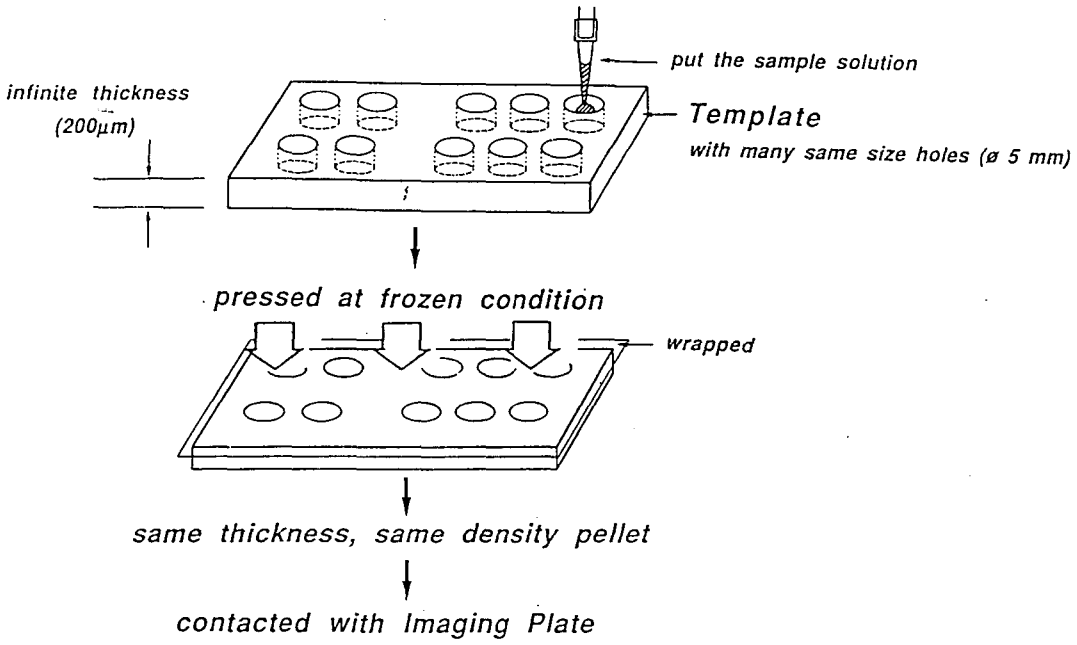

Fig 1. Preparation of Paste and Mold

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 cardbord.

3. 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 x 400 mm). IP (BAS2000 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.

4. Computer recording and imaging of relative intensity of radiation (photo-stimulated luminescence (PSL)) from IP: Using BAS2000 film (Fuji Film Co., Ltd.) for recording and imaging, the 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).

5. Relative intensity of radiation in samples (PSL/S (1 mm^2)) and background (BG/S (1 mm^2)): 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.

6. Results of Paste-Mold method: Figure 2 shows the recorded image (ARLG) obtained after 24-hour exposure. Each ARLG appearing in the upper, middle

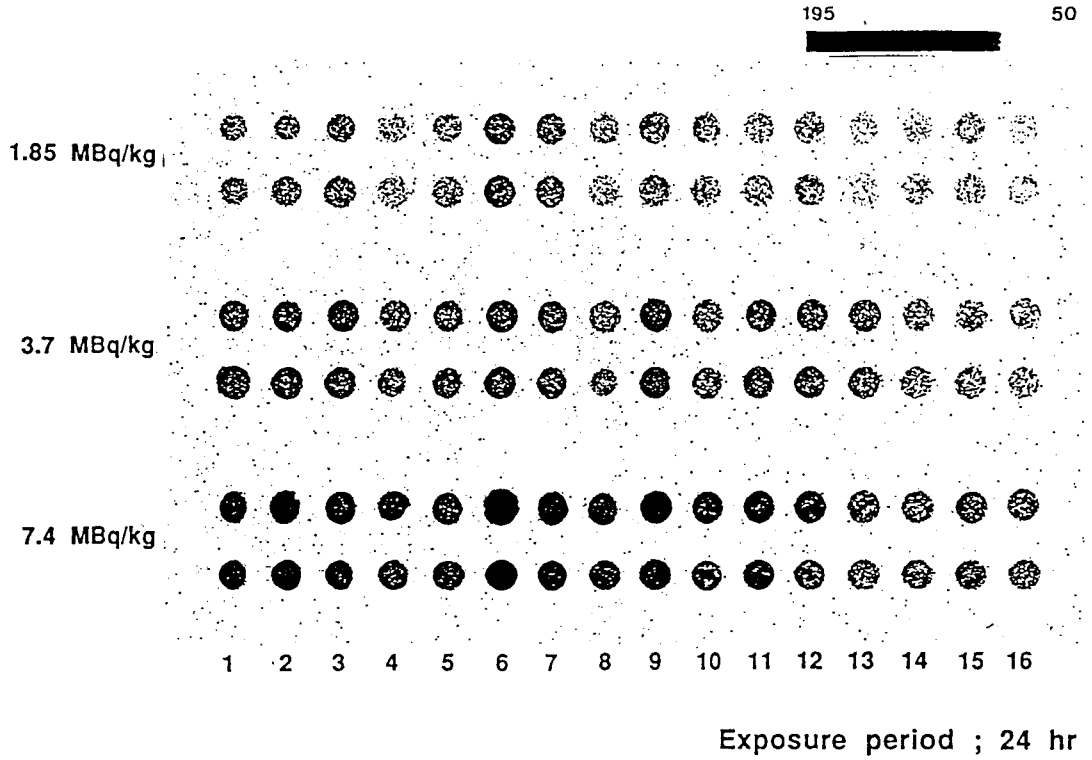

Fig 2. Autoradioluminographs for quantitative tissue balance of the radioactive distribution by a combination of Paste-Mold and infinite thickness methods

Observed tissues and organs: 1.blood, 2.plasma, 3.brain, 4.pituitary, 5.eye ball, 6.Harderian gands, 7.sublingual gands8.thyroids, 9.heart, 10.lung, 11,liver, 12.kidney, 13.adrenal glands, 14.seminal cyst, 15.skin, 16.muscle

Table I. Relative stimulated intensity (PSL) of each tissue pellet by Paste-Mold method

No.	Tissue Pellet	(PSL-BG)/S			A/A	B/A	C/A
		* A	B	C			
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.

*A, B and C represent respecive experiment with 3 kinds of radioactive doses; 1.85, 3.7 and 7.4 MBq/kg.

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 (1 mm^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-hour 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.

In the current study, each tissue section was divided into two parts. One part was treated with the "Paste-Mold" method and the (PSL-BG)/S value per g of tissue was obtained; this value was then divided by (PSL-BG)/S of blood. The other was treated with a general "LSC" solubilization method: the dpm value indicated by the manufacturer was obtained and used to calculate for the radioactivity per g of tissue (dpm/g). This value was then divided by the radioactivity (dpm/g) of blood. Regarding the ratio of relative radioactivity of each tissue to blood, the existence of a linear correlation between "Paste-Mold" method and "LSC" was examined. From the results shown in Fig. 3, the existence of a correlation between the two methods was confirmed.

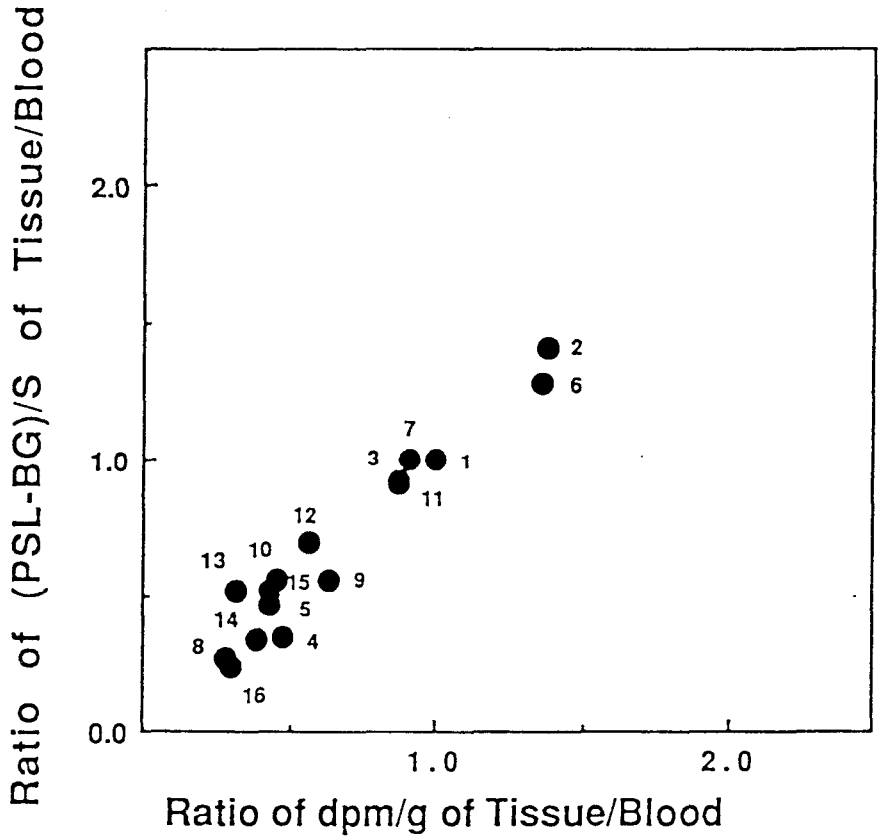

Fig 3. Correlation between (PSL-BG)/S and LSC values of the tissue/blood concentration

Dissected tissues respectively from the corpse rats were devided 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.

II. TLC-ARLG method (quantitative microanalysis of drugs and their unknown metabolites in biological samples): Methods and results

1. Labeled compounds, animals, administration route, dose: As a radiolabeled compound, [U-^{14}C] palmitic acid ([U-^{14}C]PA), 2.068 GBq/mmol, (55.6 mCi/mmol) was purchased from Amersham International plc. It was suspended with lecithin and glycerin emulsifier without addition of nonlabeled PA. The thus-prepared solution was administered through the tail vein of a Wistar strain male rat in the amount of 21.6 μCi/0.1 ml/100g body weight. Blood was drawn from the rat at 1, 3, 5, 10, 15, 30, 45, 60, 120 and 180 minutes after administration.

2. Pretreatment for preparation and measurement of samples: Using an injection tube for heparin treatment, 50 μl of blood was withdrawn from the same animal at each of the above-described sampling times to obtain plasma according to the usual method.

The standard radiation source for the calibration curve was prepared by adding a predetermined concentration of [U-^{14}C]PA to rat plasma with no radioactivity.

Five μl of plasma was spotted along a straight line on a 100 x 200 mm Merck TLC plate (Silica gel 60 F_{254}). As the developing solvent for TLC, 2 kinds of solvent systems, an ethyl acetate solvent system and a petroleum ether solvent system, were selected for separation of organic acid and ester-type metabolites such as TG, respectively. The former was obtained from the upper layer of an

ethyl acetate/ toluene/ water/ formic acid system (6/ 6/ 6/ 0.2), and the latter was a petroleum ether/ diethyl ether/ acetic acid system (80/ 18/ 1). In both cases, the development process involved an irregular method in which the TLC plate was developed to 10 mm from the origin and then dried, repeating the same process 5 times; finally the plate was developed all the way up to the solvent front.

3. Contact exposure of the developed TLC plate and IP inside the cassette: The developed TLC plate fixed onto a mount was placed inside the ready-made cassette (200 x 400 mm); then either IP was attached onto it or a Mylar membrane (4 μm) was inserted between the TLC plate and IP as a separation layer to prevent contamination. Next, the cassette was closed and left in a cool and dark place for a predetermined period of exposure time. Computer recording and imaging of relative intensity of radiation (PSL) from IP after exposure were performed according to the designated method.

4. Reading of computer-recorded image: Determination of the significance of PSL was performed within a given range to satisfy the following two equations, under a fixed condition which was set up on the widest dynamic range (10^4 latitude) and strict gradation.

$$PSL \geq 1.0 \quad (1)$$

$$(PSL-BG)/S \geq 0.8 \times PSL/S \quad (2)$$

However, each ROI was limited to a narrow area where visually significant PSL was assumed to appear.

5. Results of TLC-ARLG method: The relationship between (PSL-BG) and dpm in plasma labeled with [U-^{14}C]PA standard radiation source for a calibration curve obtained from 2 kinds of solvent systems is shown in Fig. 4. As to (PSL-BG) values on the PA spot, samples of solvent A showed a tendency to exhibit slightly higher PSL due to the different ratio of components in the solvent system; however, no large difference was found between them. Dpm values showed a good linear correlation with (PSL-BG) values within the range of 2 dpm (LSC count) to 2000 dpm (LSC count) for the 72-hour exposure. In the case of the shorter exposure time of 6 hours, a good linear correlation with (PSL-BG) values was shown within the range of 20 dpm to 20000 dpm.

In an in vivo metabolic experiment, blood samples were withdrawn periodically from the same animal, and parent compounds as well as unknown metabolites were separated from plasma; then distribution ratios of radioactivity between each divided area were determined. Results are shown in Figs. 5 and 6. The fate of PA in plasma was greatly affected by the dietary condition of the rat; i.e., whether it was subjected to fasting or fed. In fasting rats, succinic acid appeared as a metabolite of PA; conversely triglyceride was detected in fed rats. Thus, from the standpoint of future pharmacokinetic and metabolic studies, it will definitely be necessary to clarify the distribution of radioactivity in each component, in addition to measuring the radioactivity in plasma. The above-mentioned results revealed that by combining this method with TLC-ARLG method, various kinds of information regarding the radioactivity of parent

compounds in plasma as well as their metabolites can be obtained through periodic blood sampling from rats after the administration of [U-^{14}C]PA.

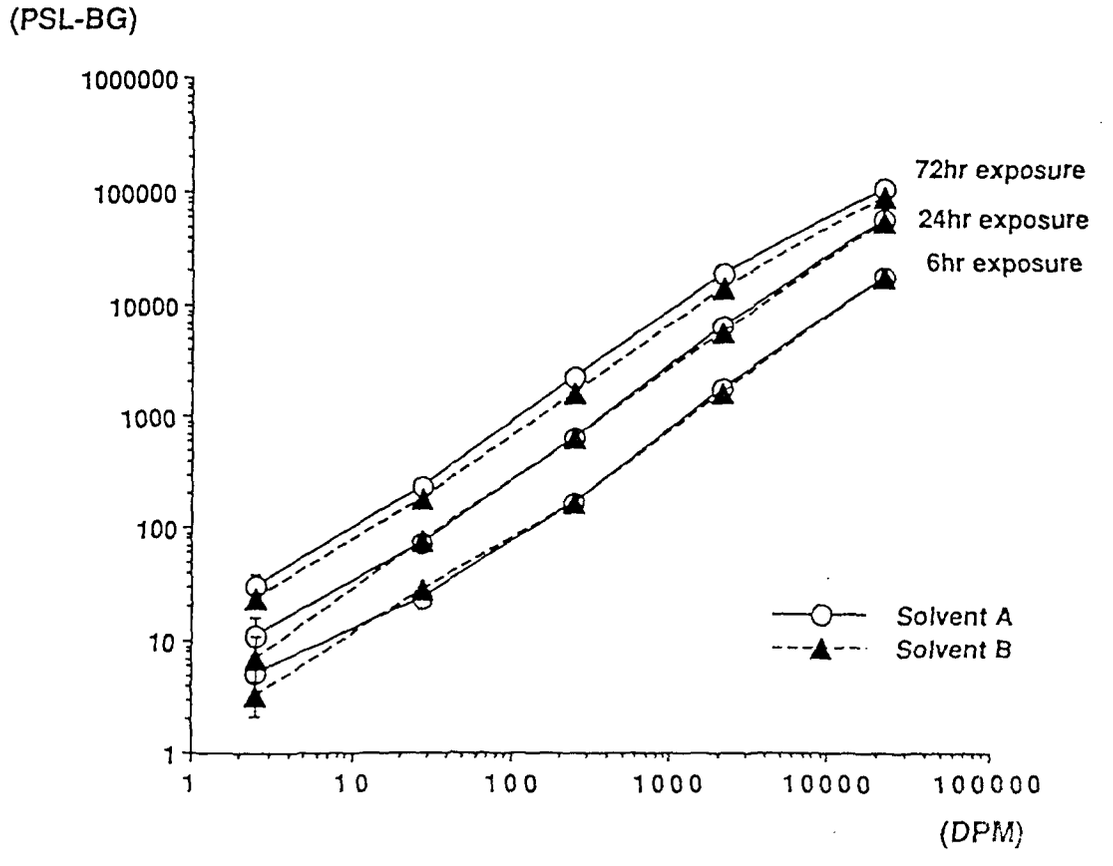

Fig 4. Relationship between PSL and radioactivity of [1-^{14}C] palmitic acid in plasma following TLC-ARLG analysis

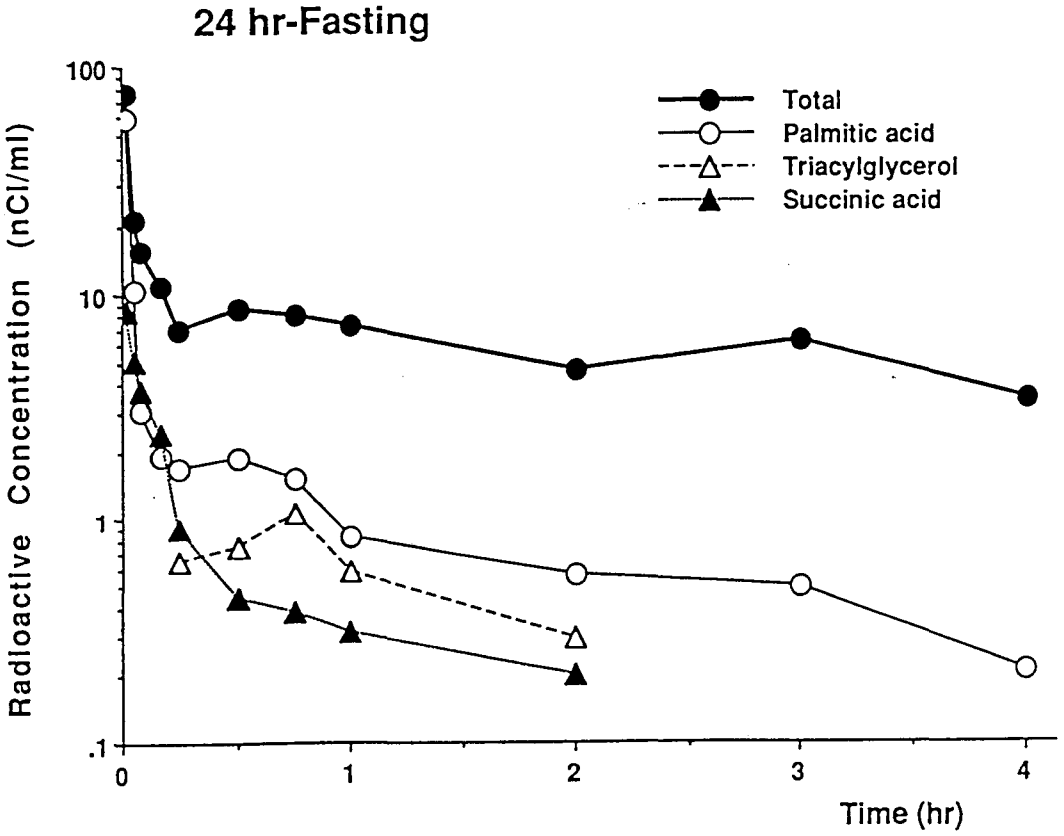

Fig 5. Fate of injected [1-^{14}C] palmitic acid in fasting rat

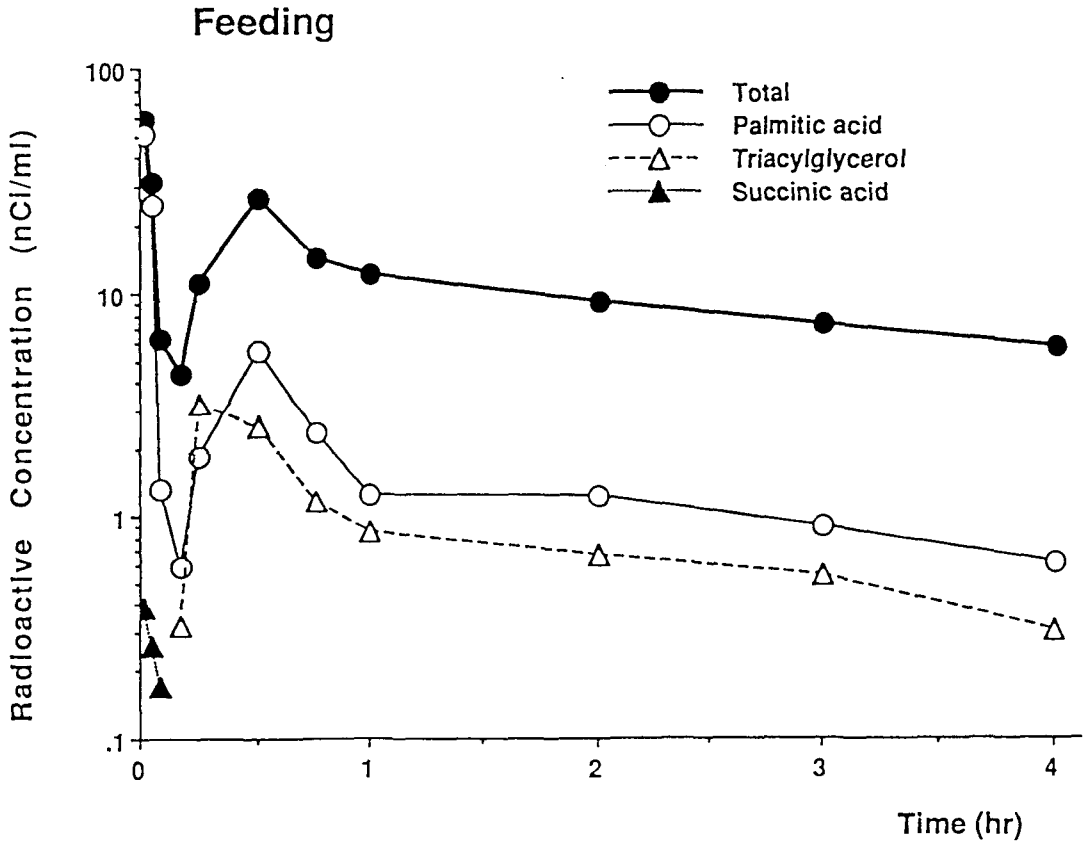

Fig 6. Fate of injected [1-^{14}C] palmitic acid in feeding rat

III. Discussion

The most sought-after result in studies is to clarify the "mass balance." Administered drugs are distributed through the circulatory system, passing though 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.

Liquid scintillation counting (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 combination of the "Paste-Mold" method with "TLC-ARLG" 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 5min 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 hours. (5) minimum measurement error, (6) no requirement for correction of

unknown sample thickness and b-ray self-absorption, and (7) reduction in the amount of radioactive waste.

The next hurdle to clear in the realization of studies is a quantitative microanalysis of the concentrations of parent compounds and metabolites in minute amounts of biological samples.

The drug-conversion mole for a radiation dose of 2 dpm varies depending on the kind of nuclear species which labels the drugs administered, as well as its location and relative radioactivity. If we assume that the relative radioactivity of [U-^{14}C]-palmitic acid (M. W. 256) is 800 mCi/mmol (1.78×10^{12} dpm/256 mg), then 2 dpm corresponds to 2.9×10^{-10} mg or 290 fg. This assumption indicates that microdetection of approximately 290 fg can be performed by TLC-ARLG, using nuclear species of ^{14}C. Two processes mentioned above were developed since high-sensitivity materials of IP can quantitatively record two-dimensional distribution of soft b-rays. The development of these complex technologies is expected to contribute to the clarification of drug toxicity and the expression mechanism of drug effects through pharmacokinetic research.

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