Studies on the Quantitative Autoradiography. IV. Quantitative Reliability of TLC-Autoradioluminography

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Phosphor Imaging plates (IPs) with high sensitivity to both low- and high-energy radiation have been on the market since 1988. The TLC-autoradioluminography (ARLG) method was developed utilizing such IPs. The conventional TLC method was suited for discrimination of microradioactive components; however, the method could only present semi-quantitative values. Conversely, the TLC-ARLG method showed completely quantitative values. That is, the TLC-ARLG method could be used to measure the radioactivity ratio between ^{14}C -radiolabeled spots A and B, which were spotted 1 mm apart on a TLC plate, after development with chromatogram, not only when the radioactivity ratio between spots A and B was 1:1, but even when it was 16:1, within the measurement error of $\pm 10\%$. The ARLG method showed sensitivity, resolution, and quantitative reliability far superior to those of the scraping-liquid scintillation counter (LSC) method or radiochromatoscanner method. In the future, the ARLG method will be widely used as a quantitative microanalytical method for unknown radioactive metabolites existing as microcomponents in organisms, especially for quantitative analysis considering mass balance to dose in biological samples, which is considered to be an important point in pharmacokinetic research.

Keywords quantitative autoradiography; TLC-autoradioluminography; Imaging plate; metabolic study

The high sensitivity of photosensitive material to radiation was first recognized in 1867. Since then, in biological studies, the microautoradiography (Micro ARG) technique has been utilized to obtain cell-division indices as a link between cytology and biochemistry. However, it had not been refined to the level of use as a quantitative technique by 1957. It is a well known fact that Ullberg¹⁾ was the first to suggest utilization of visual autoradiography, so called whole body micro-autoradiography (WAB) in pharmacokinetic studies. During the period between Ullberg's suggestion and 1990, only X-ray films were used for WAB in the field of pharmacokinetic studies. The method for reading out the photographic tone of images produced on X-ray films with a microdensitometer²⁻⁴⁾ has been called quantitative autoradiography.

Since 1988,⁵⁾ our research group has reported on the advantages of the Imaging plate (IP, Fuji Photo, Co., Ltd.) as a sensitive material for two-dimensional quantitative recording of soft β -ray radiation density.⁶⁻¹⁰⁾ Furthermore, Motoji *et al.* reported that compared with IP, X-ray films had a lower dynamic range, and their optical density was not directly proportional to radiation density because soft β -rays had a large self-absorption ratio to thickness even if the optical density of the X-ray film was in direct proportion to the number of reduced silver grains in the thickness direction of the film emulsion^{11,12)}

Thus, compared with X-ray films, IP is advantageous as a two-dimensional quantitative recording of soft β -ray radiation. However, with the increased utilization of IP, disadvantages inherent to the IP material itself have been revealed such as (1) water absorption, (2) presence of a separation layer between sample and IP (10 μm), (3) sensitivity of radiation energy dependence and (4) saturation phenomenon of each pixel. Furthermore, disadvantages of the device (BAS2000 system, Fuji Photo Co., Ltd.) used for image recording from an exposed IP were also pointed out such as (1) the flare phenomenon,

(2) limited resolution, (3) complicated procedure for region of interest (ROI) determination and (4) lack of an appropriate computer software. We have heard that manufacturers have studied some of these disadvantages with a view to improving the image recording device.

Pharmacokinetic research will certainly advance markedly using IPs appropriate for two-dimensional quantification of soft β -ray radiation density. However, even with a superior measurement device, the reliability of quantification of the samples cannot be assured if the samples to be measured are not appropriate. Therefore, in the current study, based on some actual examples, the following 4 items are examined with respect to the quantification of radiation dose in metabolites after separation by TLC, since such quantification is considered to be the most often utilized technique in pharmacokinetic studies: (1) resolution of IP, *i.e.*, discrimination of each sample within the range of resolution, (2) setting of ROI of TLC spot which is to be visually discriminated, (3) the lowest range of quantitative relative values (photo-stimulated luminescence (PSL)) using combination of IP-BAS and (4) the number of divisions in radioactive metabolites and calculation method for their radioactivity distribution ratio (%) in a sample.

MATERIALS AND METHODS

I. Preparation of Labeled TLC Plate Radioactive Specimen: Radiolabeled compound I (specimen I): L-[U- ^{14}C]tyrosine, 16.6 GBq/mmol, Amersham International plc, radiolabeled compound II (specimen II): L-[2,3,5,6- ^3H]tyrosine, 2.6 TBq/mmol, Amersham International plc.

TLC Plate: Silica gel 60 F₂₅₄, Merck Art 5626.

Spotting of Original Specimen: Specimen I was diluted with distilled water without addition of nonradioactive compound to a concentration of 20 Bq, 1200 dpm/ μl . One μl of the solution was spotted onto the origin on silica gel.

Specimen II was diluted with distilled water without addition of nonradioactive compound to a concentration of 20 kBq, 1.2×10^6 dpm/ μ l.

Preparation of Dilution Solution: Specimen I was prepared in concentrations of 600, 150 and 75 dpm/ μ l by 2-, 8- and 16-fold dilutions respectively. Specimen II was prepared in concentrations of 6×10^5 , 1.5×10^5 and 7.5×10^4 dpm/ μ l.

Location of Spot on TLC Plate: In the case of Expt. 1 and 2, each specimen in the original concentration was spotted onto nine points of origin located 1 cm apart on the plate. Specimens in the same concentration were spotted 10, 15, 20, 25, 30, 35, 40, 45 and 50 mm vertically above the nine spots, respectively; each plate was termed ^{14}C -plate 1 or ^3H -plate 1. Using the same preparation method, plates 2, 3 and 4 were prepared with specimens of 3 different concentrations.

Developing Solvent for Chromatography: BuOH/AcOH/ H_2O = 4/1/1.

Developing Distance: Development was performed by the ascending method to a distance of 100 mm. The TLC plate was completely air-dried after development. After development, positions of spots from the origin were called spots A, and positions of spots from the different distances were called spots B.

Protection of TLC Plate from Contamination after Development: Surfaces of the TLC plates were covered with thin polyvinylidene films of 4 μm thickness after development for Specimen I, though they were not covered with the films for specimen II.

II. Recording of Labeled Spots of the TLC Plate onto IP Exposure: The TLC plate covered with protection film was placed in the bottom of a cassette with the

thin-film surface up, and the exposure surface of the IP placed in contact with it. The cassette was closed and allowed to stand in a cool, dark place (inside a lead shield) for 24 h.

Recording of IP Image: Processing of IP was automatically performed by BAS2000.

Reading of Spots on IP Image: Spots of the TLC plate imaged on IP were visually discriminated. When discrimination was difficult, the image was colored and expressed so that the center of a spot showed the highest concentration; it was then determined whether the spot was in fact a single spot (Fig. 1).

Relative Intensity of Radiation so Called PSL of Spots in IP Image: PSL of a spot was determined according to the standards for effective values by Fuji Film Co., Ltd.; that is, $\text{PSL}/\text{S} \geq 1$ and $(\text{PSL} - \text{BG})/\text{S} \geq 0.8 \text{ PSL}/\text{S}$.

Background on the IP Image: A random area between spots on the same TLC plate was encircled, and PSL value per 1 mm^2 in that area was defined as background (BG). Using $(\text{PSL} - \text{BG})$ values read at spots A and B, the ratio of A/B was calculated.

III. Radiochromatography Using Radioscanner The TLC plate prepared in I was placed in a radiochromatizer (JTC-601; Aloka Co.) and a two-dimensional radiochromatogram was recorded by computer in an atmosphere of CH_4/Ar gas, then a profilogram was produced. Relative radioactivity of each discriminated spot from this profile was calculated.

IV. Silica Gel Scraping in TLC After the profile of the relative radioactivity image was produced according to the methods in II and III, using a knife, silica gel was scraped from each area (10 mm width and 2 mm length) along the lanes of each sample on the TLC plate where the protection

Fig. 1. TLC Images by BAS2000 after Development

film was detached. The scraped silica gel was transferred to a vial, and toluene-containing scintillation fluid was added. Then, using a liquid scintillation counter (LSC; LSC1000, Aloka Co., Ltd.), radioactivity in silica gel was counted as cpm, corrected by counting efficiency and expressed as dpm. Taking the total of the results obtained by the scraping method, relative radioactivity of each discriminated spot was calculated.

V. Calculation Method for Radioactivity Distribution Ratio of Each Spot Theoretical Equation: The spotted amount at the origin of each lane was assumed to be 1, 2, 8 or 16, and ratios to spotted amounts of other spots were obtained.

Actual Ratio: Based on the results of II, III and IV, the spotted amount at the origin of each lane was assumed to be 1, 2, 8 or 16, and ratios to spotted amounts of other spots were obtained.

RESULTS

I. TLC-ARLG Method The results for ^{14}C -plates 1 to 4 as well as ^3H -plates 1 to 4 are shown in Fig. 1. Plates 1 to 4 appear in sequence from left to right in the figure, each showing radioactivity ratios of 1:1, 2:1, 8:1 and 16:1, respectively, between spots A and B. The distances between two spots in one lane after development were 1 to 9 mm in numerical sequence. When using ^{14}C as a nuclear species, two spots could be visually discriminated

in all lanes except lane 1 on plates 1 to 4. However, when the image was colored at the center of the spots, two spots could be sufficiently discriminated even in lane 1 on plates 1 and 2, as shown in the results. Two distinct spots could be identified in lanes 3 to 9.

In the case of ^3H nuclear species, two spots could be visually discriminated in all lanes except lane 1 on plates 1 to 4. Coloring at the center of spots led to sufficient

Fig. 2. Three Dimensional Display Showing Resolution of Radio-TLC Scanner for Measuring Separately Different Radioactivities of 2 Spots in Different Distances

Radioactivity ratio was spot A: spot B = 1:1/8.

Fig. 3. Graphs of TLC Scraping and Counting Showing the Different Pattern According to the Distance between 2 Spots, in Counting the Radioactivity of Each Fraction by LSC

Distance between 2 spots (mm): A, 3.2; B, 5.0; C, 6.8; D, 8.5; E, 10.3; F, 12.2; G, 13.9; H, 16.0; I, 18.3. Radioactivity ratio was spot A and spot B = 1:1/8.

discrimination for plates 1 and 2, as in the case of ^{14}C . Two spots could be completely identified in lanes 2 to 9 on plates 2 to 4.

II. TLC-Radioscanner Method Figure 2 shows an actual example of ^{14}C on plate 3. In this figure, two developed spots could be discriminated using a peak as an index in lanes 4 to 9. Also, two spots could be discriminated as completely separated peaks in lanes 6 to 9.

III. Scraping and Counting Method Figure 3 shows an actual example of ^{14}C on plate 3. Two developed spots could be discriminated using a peak as an index in lanes 4 to 9. Similarly, two spots in lanes 4 to 9 were discriminated as completely separated peaks.

IV. The Results Described in I to III are Listed in Tables I to IV Table I lists discrimination of spot A from spot B after each of the ^{14}C radioactive spots was chromatographed. In the table, "yes" indicates that discrimination of the two spots was possible, and "no" indicates otherwise. Table II lists the ratio of radioactivity measured at A to that measured at B after each spot was chromatographed.

The discrimination ability for each division differed

among the three measurement methods, depending on ^{14}C theoretical radioactivity ratios between spots A and B. In the case of radioactivity ratios of 1:1 and 2:1, the distance between the two closest spots of lane 1 could not be discriminated either by scraping-LSC or radiochromatoscanner method. For the radioactivity ratio of 8:1, the above-mentioned method could not discriminate the distance between spots in lanes 1 and 2. Furthermore, in the case of the radioactivity ratio of 16:1, the distances between spots in lanes 1 and 2 could not be discriminated. In particular, discrimination of the distance between spots even in lane 3 was not possible using the scraping-LSC method.

Table II shows the theoretical radioactivity ratios between spots A and B labeled with ^{14}C , as well as actual radioactivity ratios between them. Boxes marked by diagonal lines in the table represent values which exceed the range of $\pm 10\%$ of the theoretical ratios. The number of values enclosed by these boxes is smallest in the plate with 1:1 ratio, and the number increases as the ratios of A:B increase. However, when the autoradioluminography (ARLG) measuring method was used, no measurement

TABLE I. Discrimination of Spot B to A Labelled with ^{14}C

Theoretical radioactivity ratio between A and B	Measuring method	Distance (mm) between spot A and B after developing								
		1	2	3	4	5	6	7	8	9
1:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
8:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
16:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes

TABLE II. Measured Intensity of Spot B to A Labelled with ^{14}C

Theoretical radioactivity ratio between A and B	Measuring method	Distance (mm) between spot A and B after developing								
		1	2	3	4	5	6	7	8	9
1:1	ARLG	0.96	0.92	0.99	1.03	1.04	0.96	0.95	0.95	0.97
	LSC after scraping	—	1.05	1.01	1.03	0.99	1.10	0.97	0.98	1.01
	Radiochromatoscanner	—	1.18	0.96	1.03	0.92	1.10	0.97	0.98	1.01
2:1	ARLG	2.11	1.92	1.96	1.83	1.98	2.02	1.97	2.01	1.99
	LSC after scraping	—	2.36	2.22	1.84	1.99	1.89	2.03	1.74	1.77
	Radiochromatoscanner	—	2.52	1.94	1.89	1.94	1.99	1.83	1.94	2.00
8:1	ARLG	8.47	8.24	7.89	8.37	7.45	8.08	7.59	8.40	7.98
	LSC after scraping	—	—	10.03	11.03	9.21	9.18	8.56	9.38	8.36
	Radiochromatoscanner	—	—	5.03	7.49	8.62	8.66	6.01	7.24	9.10
16:1	ARLG	16.78	16.17	16.18	16.90	16.39	16.02	16.80	15.78	17.18
	LSC after scraping	—	—	—	17.23	16.12	9.78	19.61	21.46	20.50
	Radiochromatoscanner	—	—	18.98	14.46	13.56	13.74	15.93	16.02	15.34

In the column, abbrev.; — represents the ratio over $\pm 10\%$ errors to the theoretical ratio.

TABLE III. Discrimination of Spot B to A Labelled with ^3H

Theoretical radioactivity ratio between A and B	Measuring method	Distance (mm) between spot A and B after developing								
		1	2	3	4	5	6	7	8	9
1:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
8:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
16:1	ARLG	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	LSC after scraping	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Radiochromatoscanner	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

TABLE IV. Measured Intensity of Spot B to A Labelled with ^3H

Theoretical radioactivity ratio between A and B	Measuring method	Distance (mm) between spot A and B after developing								
		1	2	3	4	5	6	7	8	9
1:1	ARLG	1.14	1.04	1.01	1.01	0.95	1.02	0.97	1.02	0.98
	LSC after scraping	—	0.94	0.94	0.81	0.85	0.87	0.84	0.79	0.61
	Radiochromatoscanner	—	1.64	1.01	1.03	0.95	0.99	0.98	0.96	0.96
2:1	ARLG	2.18	2.01	1.99	1.85	2.17	1.96	1.97	2.16	2.04
	LSC after scraping	—	1.96	1.96	1.75	2.02	1.86	1.91	2.09	1.90
	Radiochromatoscanner	—	2.92	2.18	2.37	2.19	1.97	1.90	2.21	2.03
8:1	ARLG	6.91	9.03	7.99	7.93	7.29	7.18	7.64	7.59	8.80
	LSC after scraping	—	11.85	8.43	7.30	7.04	7.86	7.60	6.36	8.02
	Radiochromatoscanner	—	—	10.97	10.64	8.92	8.61	7.02	7.58	9.15
16:1	ARLG	11.63	16.87	16.89	15.71	15.89	17.68	16.01	17.60	17.75
	LSC after scraping	—	19.61	14.93	13.73	14.85	12.90	14.07	13.41	15.30
	Radiochromatoscanner	—	—	26.99	17.29	20.12	16.82	16.60	16.49	19.80

In the column, abbrev.; — represents the ratio over $\pm 10\%$ errors to the theoretical ratio.

error exceeding $\pm 10\%$ was observed for any sample distance. A rather small number of values were enclosed by the boxes in the case using the radiochromatoscanner method when the radioactivity ratio of A:B was 2:1. However, in the samples with ratios of 8:1 and 16:1, both measurement methods showed inferior quantitative discrimination ability compared with the ARLG method.

With regard to samples labeled with ^3H , the ability to discriminate distance between spots A and B, as well as quantitative discrimination ability by three measurement methods, was compared similarly to the case of samples labeled with ^{14}C . Table III shows that in the case of radioactivity ratios of 1:1 and 2:1, the distance between spots in lane 1 can be discriminated only by the ARLG method. Also, the other two methods cannot discriminate the distance between spots in lane 1 in the cases of 8:1 and 16:1, as in the case of 2:1. Discrimination of the distance between spots in lane 2 was difficult using the radiochromatoscanner method.

Table IV lists the ratios of measured radioactivity between spots A and B labeled with ^3H . When the ARLG

method was used, no measurement error exceeding the range of $\pm 10\%$ was observed in lanes 1 to 9 for samples with radioactivity ratios of 1:1 and 2:1. However, in the scraping-LSC method, distances between spots in lane 1 could not be discriminated in the plates with radioactivity ratios of 1:1 and 2:1, and no quantitative value could be obtained. When the radiochromatoscanner method was used, no quantitative value could be obtained in lanes 1 and 2. In the case of the plate with radioactivity ratio of 8:1 using the ARLG method, a value with low reliability was exhibited in lane 1, and the measurement error slightly exceeded $\pm 10\%$ in lane 2. Using the scraping-LSC method, reliable discrimination ratios were not obtained until lane 3. Moreover, reliable values could not be obtained from lanes 1 to 5, and also lanes 9 by the radiochromatoscanner method. As to the radioactivity ratio of 16:1 between spots A and B using the ARLG method, reliability was low in lane 1, and the value in lane 9 slightly exceeded the measurement error range of $\pm 10\%$. However, when using the other two measurement methods, extremely low reliability was exhibited compared with the

ARLG method. This tendency was especially marked in the scraping-LSC method.

DISCUSSION

Using three kinds of radioactivity measurement methods, discrimination ability and quantitative reliability of the two spots with different radioactivities on the TLC plates were examined in detail.

As described in Results, when the radioactivity ratio between spots A and B was 1:1, all three measurement methods exhibited superior discrimination ability and high quantitative reliability compared with other experimental groups. This tendency was exhibited in experiments with both ^{14}C and ^3H nuclear species. As the difference in radioactivity ratio between spots A and B increased, discrimination ability and quantitative reliability tended to deteriorate. This tendency was especially obvious in the group of plates with ratios of 8:1 and 16:1. However, there was a large difference in the results among measurement methods. Using the ARLG method, especially good discrimination ability and quantitative reliability were exhibited in all four group of plates, compared with the other two measurement methods. In particular, for all groups of plates, the measured radioactivity ratios did not exceed the error range of $\pm 10\%$ in the case of ^{14}C nuclear species. Conversely, when scraping-TLC and radiochromatoscanner methods were used, quantitative reliability was extremely low in samples with the radioactivity ratios of 8:1 and 16:1. This tendency became stronger as the distance between spots

A and B decreased. In the case of ^3H nuclide specimens, the ARLG method exhibited superior discrimination ability and a little inferior reliability compared with the other two methods since ^3H specimens in radioactivity level were used about 1000 times higher than those of ^{14}C -specimens. However, the results obtained with ^3H were relatively inferior compared with those obtained with ^{14}C nuclide specimens. There was almost no difference between ^3H and ^{14}C nuclide specimens for the discrimination of 2 different radioactive spots closely each other on the processed TLC plate. However, the quantitative reliability of ^3H nuclide specimens particularly less inferior than that of ^{14}C nuclide specimens. All three measuring methods exhibited slightly inferior functions for ^3H nuclear species compared to ^{14}C nuclear species. Using ^3H , the ARLG method showed lower quantitative reliability in the two groups of 8:1 and 16:1 in lane 1. Naturally, every kind of technology has its own advantages and disadvantages; therefore it is better to utilize them according to the particular objective. Sufficient understanding of both advantages and disadvantages is required for the ARLG method, which is the newly reported technology in this paper. Table V lists various radioactivity measurement methods with their advantages and disadvantages for comparison. In the table, scraping-LSC, radioscanner, ARLG and HPLC methods are listed with the time required for processing, total man-hours, discrimination ability, quantitative reliability and quenching effects, among others.

The TLC discrimination method is advantageous in that a large number of samples can be spotted on one plate,

TABLE V. Which Method Do You Choose for Analyzing Metabolites?

Separation of radioactive samples Method for analysis	TLC (developing distance 10 cm, 1 dimension)			HPLC (analyzing for 30 min)	
	[LSC] Scraping and RI-counting	[Scanner] Radioscanner	[ARLG] IP and Bio imaging analyzer	Fractionation and RI-counting	Radio-detector
Detection and measuring	Unseparated	Unseparated	* Separated	Unseparated	Unseparated
Time required for analyzing whole samples					
Preparation I (setting up)		Developing time: 0.5—2 h (depend on a solvent)		Operation (or fractionation) and set up time: 100 h	
Preparation II (setting)	10 h	0 h	0 h	0 h	0 h
Counting or scanning	>166 h (1 min/fraction)	>50 h (30 min/sample)	* About 3 min (whole sample)	>80 h (1 min/fraction)	Real time
Quantitative analysis	About 20 h (by hand)	About 10 h (by machine)	About 1 h	About 20 h (by hand)	About 20 h (by machine)
Total working time					
Analyzer	>166 h	>50 h	* About 1 h	>20 h	>120 h
Manpower	>266 h	>20 h	>9 h	>120 h	>120 h
Quality of quantitative analysis					
Resolution	>2 mm (normally 5 mm)	3—6 mm (W1/2)	* 100 μm (graphic)	Arbitrary time (normally 0.5 mm)	W1/2 0.3 min
Sensitivity	>50 dpm	>100 dpm	* 2 dpm	>50 dpm	>10000 dpm
Dynamic range	10^2 — 10^6 dpm	10^2 — 10^6 dpm	10^1 — 10^5 dpm	10^2 — 10^6 dpm	10^4 — 10^8 dpm
Preferable time for counting	1—10 min	30—60 min	* 30 min—72 h	1—10 min	0.1 min
Statistical disintegration error	$\pm \sqrt{\frac{N}{(10)^2}}$	$\pm \sqrt{\frac{N}{(60)^2}}$	* $\pm \sqrt{\frac{N}{(72 \times 60)^2}}$	$\pm \sqrt{\frac{N}{(10)^2}}$	$\pm \sqrt{\frac{N}{(0.1)^2}}$
Accuracy of measuring	1	$\times 3$	* $\times 216$	1	$\times 0.01$
Quenching trouble	Serious	No	* No	Serious	Serious
Scintillation fluid waste	Yes	No	* No	Yes	No
Reusable	No	Yes	* Yes	No	No

Assumption: analyze 100 samples (plasma, urine, or bile) containing ^{14}C -labeled metabolites. *: advantage.

and that they can be processed by chromatography under the same conditions. However, a difficulty existed in the method of quantification of the discriminated area. The coloring method is qualitatively reliable because the result can be visualized. However, for quantitative purposes, the method is not suitable even with the use of a microdensitometer due to its sensitivity, narrow dynamic range and nonhomogeneous distribution intensity in a separated area. Therefore, with regard to radiolabeled compounds and their metabolites, a method was examined tentatively in which an autoradiographic (ARG) image was obtained by contact with an X-ray film; then radioactivity of the image was quantitatively measured by means of microdensitometry. However, the method has not been generally used because linear correlation between soft β -ray radiation and the amount of reduced silver grains produced on X-ray film could be obtained only under limited conditions. Therefore, as a quantitative analytical method for the radioactive component using TLC, the scraping-LSC method has been utilized. As measuring devices, two-dimensional radiation measurement devices such as Ambis or radiochromatoscanner have been utilized. Moreover, the HPLC method has also been used as a reliable quantitative analytical method. However, with regard to previously known components which can be discriminated, the HPLC method has a number of problems to be overcome as a method for qualitatively and quantitatively microanalyzing radioactive components in a large number of biological samples or in excretion from organisms with high sensitivity, if the conditions for discrimination of known components are satisfied.

Table V shows the results of evaluation of important points such as total manpower, man-hours, occupied hours of analyzer, resolution, sensitivity, dynamic range, actual measurement hours (short hours increase statistical variations in radioactive counting) and measurement accuracy, using 100 metabolite samples. In the experiments, qualitative and quantitative analyses were simultaneously performed after a minimum time of separation and extraction of biological samples which were a mixture of radioactive parent compounds and unknown metabolites. In the case of the TLC method, scraping-LSC requires a greater number of hours, 100 vials and a scintillator per 1 plate for LSC counting. Ambis or chromatoscanner is very reasonable in these respects, although consumption of nonpolar Ar_2 gas and CH_4 gas, as well as scanning time required for samples with low radiation dose, results in high cost. Furthermore, if we assume that analysis of 1 lane requires 30 min, the time during which devices are in operation must increase for a large number of samples, which also creates difficulties.

In the case of HPLC, the radio-HPLC method can be used for high-radioactivity substances such as ^{125}I or ^{14}C among known radioactive samples. However, when it

comes to samples with low-radioactivity substances containing unknown metabolites in components of organisms, the method cannot quantitatively measure radioactivity due to the difference in extraction rate of each unknown radioactive component at the time of extraction and purification. Furthermore, it is incapable of measuring the mass balance of components which cannot pass through a column. Thus, the HPLC method has a number of disadvantages for pharmacokinetic research, and many points should be improved. Also, the method requires that the device be in operation for a long period of time for every sample as well as a large amount of manpower; therefore, efficiency should be improved. Moreover, when the HPLC method is combined with the LSC method, it is necessary to consider the disadvantages of LSC, such as generation of waste scintillation fluid, correction of vial and quenching effects, statistical variations of high β -ray decay and short measurement time per scintillation sample.

Compared with measurement methods which have a number of disadvantages, the advantages of the TLC-ARLG method are shown in Table V. Especially in pharmacokinetic research on processes of transfer, distribution, absorption, metabolism and excretion of trace amounts of drug administered to organisms, the TLC-ARLG method will certainly be utilized as a method which can simultaneously perform qualitative and quantitative discrimination and analysis of drugs as well as unknown metabolites derived from drugs, while maintaining the mass balance of biological samples when the dose is assumed to be 100, and when omitting separation and extraction or limiting the number of separations and extractions to the minimum.

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