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Application of radioluminography to off-line counting of radioactivity in high-performance liquid chromatographic eluates

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Abstract

An off-line counting method for the determination of carbon-14 in HPLC eluates was developed using radioluminography (RLG). A succession of aliquots of the eluate were collected in the flat-bottomed wells of a polystyrene microplate, evaporated to dryness and contacted with an imaging plate, and radioactivity was determined with a Bio-image analyser. The limit of detection was 0.35 Bq per injection. The inter-assay relative standard deviation was less than 3% over the range 2–20 Bq. The RLG off-line counting method was utilized to determine [^{14}C]eicosapentaenoic acid metabolites formed by rat hepatic microsomes. The results were compared with those obtained with an off-line liquid scintillation counting method and an on-line counting method.

1. Introduction

A radioisotope tracer technique using soft β-ray emitters such as carbon-14 is widely used in biological and pharmaceutical studies. In this tracer technique, HPLC has been used for the separation of compounds [1–3]. The measurement of radioactivity has been performed by fractionating the eluate in vials, mixing with a liquid scintillation cocktail and counting with a liquid scintillation counter (LSC), *i.e.*, namely the LSC off-line counting method. In order to maintain the chromatographic resolution, a large number of fractions must be collected, which requires a large volume of liquid scintillation fluid and a considerable time for counting. This method was not easily adaptable to the large number of samples that can be generated by HPLC. Karmen and co-workers [4,5] reported the collection of eluates in wells formed in a non-wetting fluorocarbon film and off-line counting by autoradiography. However, it is difficult to obtain quantitative information directly from the autoradiograms using conventional X-ray film. Radioactivity in HPLC eluates can also be monitored in real-time with a flow-through on-line detector. The method is classified into two types, homogeneous and heterogeneous. In the homogeneous counting method, the eluate is mixed with a liquid scintillation cocktail and the mixture is passed continuously through the counting cell placed between a pair of photo-

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multiplier tubes. In the heterogeneous counting method, the eluate is passed through a cell packed with a solid scintillator. However, these types of detectors do not permit a simultaneous improvement in both the detection sensitivity and the chromatographic resolution.

Recently, radioluminography (RLG) [6,7] using an imaging plate (IP) had made possible the measurement of β-ray emitters on TLC plates and in tissue slices [8,9]. The IP is composed of polyester film coated with microcrystals of a photostimulable phosphor ($BaFBr:Eu^{2+}$) which memorizes the energy of radioactivity in the form of quasi-stable colour centres and emits photostimulated luminescence (PSL) by laser beam stimulation with its intensity proportional to the absorbed radiation energy. By digitizing the intensity of the emission, a digital image showing the radioactivity distribution is obtained. Data analysis including scanning can be completed in *ca.* 3 min for an IP. The image stored on the IP can be easily erased by irradiation with visible light, allowing repeated use. The sensitivity of IP was shown to be over 100 times higher than that of X-ray film, and further IP has a considerably wider dynamic range than X-ray film [8].

VanRollins *et al.* [10] reported the HPLC determination of [^{14}C]eicosapentaenoic acid (EPA) metabolites formed by rat hepatic microsomes using the LSC off-line counting method. As a single injection generated about 550 fractions, it required 4 days for counting and produced about 3000 ml of scintillation waste per chromatographic analysis. In the previous paper [11], we proposed a method alternative to LSC for detecting carbon-14 using RLG. The method involves placing an aqueous radioisotope sample in flat-bottomed wells of a polystyrene microplate, evaporating the solvent and determining the radioactivity in each well using RLG. This method permitted the detection of the radioactivity of several hundred samples simultaneously and required no scintillation fluids. These advantages are useful for the off-line counting of radioactivity in HPLC eluates. This paper describes the determination of [^{14}C]EPA metabolites with rat hepatic microsomes by HPLC to assess the feasibility of the RLG off-line counting method.

2. Experimental

2.1. Materials

Eicosapentaenoic acid (EPA) was purchased from GL Sciences (Tokyo, Japan). [1-^{14}C]EPA (specific radioactivity 2.05 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). This compound was diluted with non-labelled EPA to give an ethanol solution of 27.6 MBq/mmol (100 Bq per 0.1 ml). Working standard solutions were prepared by diluting the solution with ethanol to give radioactivity concentrations of 5, 0.5, 0.35, 0.05 and 0.005 Bq/μl. Polystyrene flat-bottomed microplates (85 × 127 mm) with 48 wells (11.3 mm in diameter) were purchased from Costar (Cambridge, MA, USA) and the depth of the wells was adjusted to 5.0 mm by cutting the plates horizontally.

2.2. HPLC system and conditions

A block diagram of the HPLC system is shown in Fig. 1. The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-6A pumps, a Shimadzu SLC-6B system controller, a Rheodyne Model 7125 injector and a Shimadzu SPD-6A UV spectrophotometric detector. The column was LiChrosorb RP-18 (7 μm; 250 × 4 mm I.D.) (Merck, Darmstadt, Germany). The

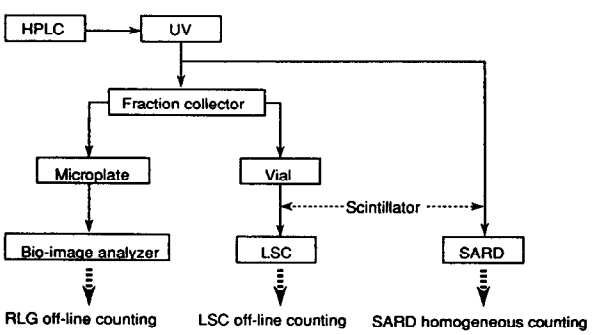

Fig. 1. Block diagram of HPLC system.

mobile phases were (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile. Elution was performed isocratically with a solution of 15% of solvent A in solvent B, except for the determination of [^{14}C]EPA metabolites, which was carried out by gradient elution as indicated in Fig. 4. The flow-rate was 1 ml/min in each instance.

2.3. RLG off-line counting

The HPLC eluate was fractionated into the wells of microplates at 10-s intervals (20-s intervals for the determination of [^{14}C]EPA metabolites) using a Gilson (Middleton, WI, USA) Model 222 fraction collector. The microplates were allowed to stand for 5–12 h at room temperature for evaporation of the solvent. The samples were overlaid with Diafoil plastic film (0.52 mg/cm^2) (Mitsubishi, Tokyo, Japan) and contacted with an IP (20 × 40 cm) in the brass chamber (30 × 50 × 3 cm; thickness 1 cm) for 24 h. The absorbed dose recorded on the IP was quantified using a BAS-2000 Bio-image analyser (Fuji Film, Tokyo, Japan).

2.4. LSC off-line counting

The HPLC eluate was collected in Minivials at 10-s intervals using a Gilson fraction collector. A 3-ml volume of a hydrophilic liquid scintillation cocktail (dioxane–toluene–ethyl Cellosolve (75:15:10, v/v/v) containing 100 g of naphthalene, 4 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis[2-(5-phenyloxazoyl)]benzene per litre) was added to each Minivial. The radioactivity was counted for 3 min per sample in an Aloka (Tokyo, Japan) LSC-1000 liquid scintillation counter.

2.5. Homogeneous counting

The HPLC eluate was mixed with a fivefold volume of the hydrophilic liquid scintillation cocktail and the mixture (6 ml/min) was passed through an Aloka synchronized accumulating radioisotope detector (SARD) [12,13]. The SARD was composed from five counting cells with a 1-ml cell volume and five pairs of photomultiplier tubes.

2.6. Cross-talk ratio between wells

A 100-Bq amount of [^{14}C]EPA in 0.1 ml of ethanol was applied to a well of the microplate and was allowed to stand for 5 h at room temperature for evaporation of the solvent. The sample was overlaid with a Diafoil and contacted with the IP in the brass chamber for 24 h. The absorbed dose recorded on the IP was measured with the BAS-2000.

2.7. Preparation of rat hepatic microsomes

Rat hepatic microsomes were prepared by the method of VanRollins and co-workers [10,14]. In brief, a Wistar male rat (*ca.* 200 g) was given daily intraperitoneal injections for 3 days of 10% phenobarbital in saline (0.1 ml per 100 g body mass). After the third injection, the rat was deprived of food overnight. The rat was decapitated 24 h after the last injection and the liver was removed. All subsequent steps were performed at 0–4°C. The liver was homogenized (0.25 g/ml) in a buffered salt solution consisting of 150 m*M* KCl and 50 m*M* Tris–HCl (pH 7.5). The homogenate was centrifuged at 10000 *g* for 10 min. The supernatant was then centrifuged at 105 000 *g* for 60 min. The microsomal pellets were washed once, resuspended in buffer (6–8 μg protein/ml) and stored at −80°C.

2.8. Incubation of [^{14}C]EPA with rat hepatic microsomes

A 1-ml volume of 1 m*M* NADPH was added to 0.5 ml of the microsomes and the solution was incubated at 37°C for 3 min. To the solution were added 8.33 kBq of [^{14}C]EPA in 50 μl of 0.01 *M* $NaHCO_3$ and the mixture was incubated at 37°C for 20 min. Reaction was stopped by the addition of 2 ml of ice-cold ethanol. The mixture was centrifuged at 1000 *g* for 20 min at 4°C and the pellets were suspended in ethanol and recentrifuged. The washing step was repeated twice and the combined supernatants were con-

centrated and acidified with formic acid to pH 3. Separation of EPA metabolites from the samples was performed as described previously [15]. In brief, the solution was applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) which was washed with 5 ml each of methanol and water immediately before use. The cartridge column was washed with 10 ml each of water, 5% ethanol in water and light petroleum and then eluted with 6 ml of methyl formate. The eluate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of ethanol and aliquots of the solution were injected into the HPLC column.

3. Results and discussion

The feasibility on the present RLG off-line counting method was determined by injecting [^{14}C]EPA into the HPLC column. Elution was performed with acetonitrile–water–TFA (85:15:0.1, v/v/v) at 1.0 ml/min and the eluate was collected in the wells of microplates at 10-s intervals. The solvents (167 μl per well) could be evaporated by standing overnight at room temperature. Fig. 2A shows a representative radioluminogram. The intensity of PSL in the images is proportional to the number of β-particles registered from the samples in the wells. As each image was placed in order of separation, a chromatogram (Fig. 2B) was obtained by processing the radioluminogram.

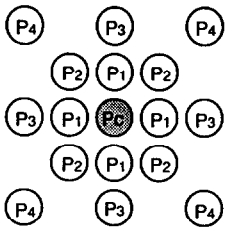

Fig. 3. Scanned area for cross-talk examination.

If high radioactivity exists in a well, an unexpected high intensity of PSL may be obtained over the adjacent wells, that is "cross-talk" between wells occurs. In order to access the cross-talk, 100 Bq of [^{14}C]EPA were applied homogeneously to a well of a microplate and the intensity of PSL in the surrounding wells (Fig. 3) was measured. The cross-talk ratios (Table 1) were negligibly small and were calculated using the following equation:

$$\text{cross-talk } (\%) = \frac{\text{PSL}_n - \text{PSL}_{\text{B.G.}}}{\text{PSL}_{\text{centre}} - \text{PSL}_{\text{B.G.}}} \cdot 100$$

The accuracy of the RLG off-line counting method was determined over the range 0.35–20

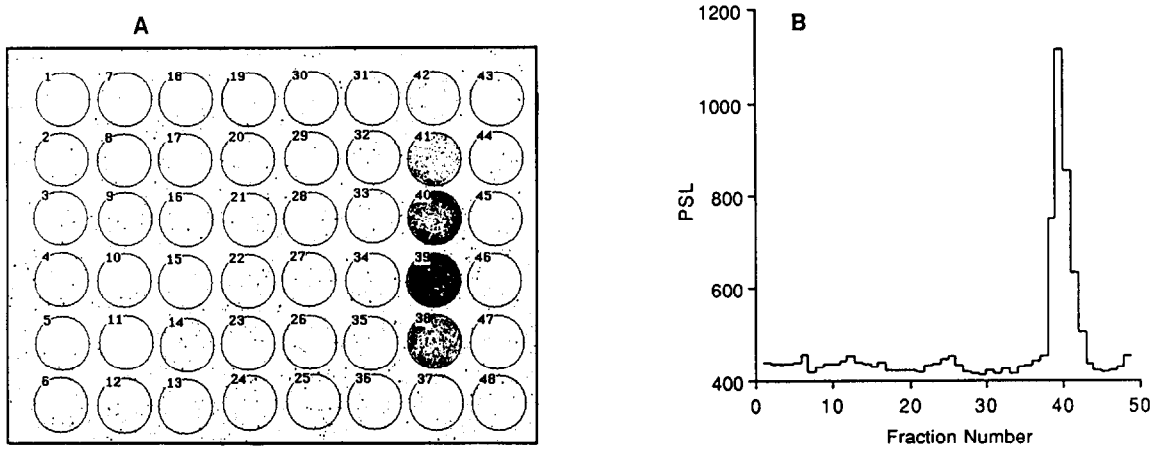

Fig. 2. (A) Radioluminogram and (B) chromatogram following the injection of 15 Bq of [^{14}C]EPA into the HPLC system. HPLC was performed on a LiChrosorb RP-18 (7 μm; 250 × 4 mm I.D.) column with acetonitrile–water–TFA (85:15:0.1, v/v/v) at a flow rate of 1 ml/min. The eluate was collected in the wells of a microplate at 10-s intervals. The plate was exposed with an IP for 24 h.

Table 1
Cross-talk ratio between wells

Position No.[a]	PLS		Cross-talk (%)
	Mean ± S.D.	R.S.D. (%)	
P_{centre}	13702.1[b]		
P_1	289.5 ± 10.8	3.7	0.098
P_2	282.4 ± 2.4	0.8	0.045
P_3	279.7 ± 4.0	1.4	0.024
P_4	276.8 ± 3.6	1.3	0.003
$P_{B.G.}$	276.4 ± 4.2[c]	1.5[c]	

[a] The numbers of the wells are shown in Fig. 3.
[b] The PSL value corresponded to 100 Bq of [^{14}C]EPA placed in the P_{centre} well.
[c] The value was obtained with the wells of the same microplate except P_{centre} and P_n ($n = 1–4$).

Bq of [^{14}C]EPA. There was strict linearity between the values of PSL and the radioactivity injected into the HPLC column. The results were compared with those from other methods (LSC off-line counting method and a homogeneous counting method by SARD), as shown in Table 2. The inter-assay relative standard deviations (R.S.D.s) in the RLG method were constant and less than 3% regardless of the radioactivity over the range 2–20 Bq of [^{14}C]EPA injected. On the other hand, the R.S.D.s in the LSC off-line counting method and the homogeneous counting method became progressively greater as the amounts of radioactivity decreased. The reason may be that the number of radioactive decays for a constant period of 24 h in the RLG method is so high as to make statistical variations irrelevant. In the RLG method, the detection limit was reported to be *ca.* 0.2 Bq for ^{14}C [11]. Further studies demonstrated that the background was reduced by exposure in a brass chamber and the detection limit could be decreased to 0.05 Bq. The limit of detection for [^{14}C]EPA in the present HPLC method was *ca.* 0.35 Bq injected. This is smaller than those of the LSC off-line counting method (0.7 Bq) and the homogeneous counting method (2 Bq).

Table 2
Comparison of accuracy ($n = 3$) among the RLG off-line counting method, the LSC off-line counting method and the SARD homogeneous counting method

Injected amount (Bq)	RLG		LSC[a]		SARD	
	PSL	R.S.D. (%)	dpm	R.S.D. (%)	cpm	R.S.D. (%)
0.35	39.6	4.6	n.d.[b]		n.d.[b]	
0.7	66.0	3.1	57.3	6.4	n.d.[b]	
2.0	189.2	3.0	132.4	12.9	122.0	9.7
5.0	561.1	2.8	304.6	5.0	264.3	7.3
10.0	1138.2	1.7	550.6	8.0	531.0	2.6
15.0	1720.2[c]	2.0[c]	807.6[c]	3.7[c]	732.4[c]	2.1[c]
20.0	2292.5	2.6	1068.2[d]	1.4[d]	885.7	2.0

[a] Counting time 3 min.
[b] Not detected.
[c] $n = 5$.
[d] $n = 4$.

To assess the feasibility of using the RLG off-line counting method, the determination of [^{14}C]EPA metabolites formed by rat hepatic microsomes was performed. [^{14}C]EPA metabolites were prepared by the method of VanRollins *et al.* [10]. Sep-Pak C_{18} cartridges were used to separate EPA metabolities from biological samples as described previously [15]. About 833 Bq of the sample were injected into the HPLC column. As the eluate was collected in the wells of microplates at 20-s intervals from 15 min to 225 min after injection, twelve sheets of the microplates (576 wells) were used per injection. After the solvents (333 μl per well) had evaporated, the samples were exposed with two sheets of IP because an IP can be contacted with six sheets of the microplates. On the other hand, equal amounts of [^{14}C]EPA metabolite sample were analysed by SARD, in which an improvement by a factor of $\sqrt{5}$ in detection accuracy was achieved without sacrificing the chromatographic resolution as compared with conventional detector [12,13]. Fig. 4 shows the chromatograms obtained by the RLG off-line counting method and the homogeneous counting method. Although there was no appreciable difference between the two chromatograms for the separation of the metabolites, the signal-to-noise ratio in the RLG off-line counting method was better. The homogeneous counting method has the advantage that it can yield immediate access to data. However, it produced about 1200 ml of liquid scintillation waste per injection. In the RLG off-line counting method, no scintillation fluid was required and the used plates were disposed of easily because they were flammable and inexpensive. Although about 2 days were required to obtain the data (5–12 h for evaporation, 24 h for contact and 3 min for data analysis including scanning with the BAS-2000, many chromatograms could be obtained in this period because the time for evaporation and contact could be kept almost constant regardless of the number of samples. This suggested that the RLG off-line counting method is a useful method for the routine analysis of radioactive samples requiring long analysis times.

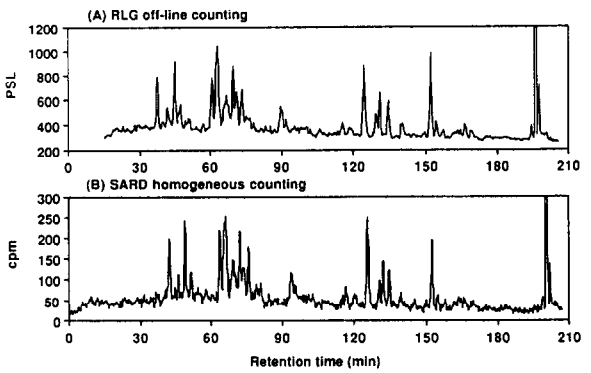

Fig. 4. Chromatograms obtained by (A) RLG off-line counting method and (B) SARD homogeneous counting method after injecting 833 Bq of [^{14}C]EPA metabolities formed by rat hepatic microsomes. Chromatographic conditions: column, LiChrosorb RP-18 (7 μm; 250T × 4 mm I.D.); mobile phase, (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile; flow-rate, 1 ml/min. Elution was carried out with a stepwise linear gradient from 30 to 50% solvent B over 180 min, from 50 to 100% solvent B over 10 min and isocratic at 100% solvent B for 30 min. In (A) the fraction interval was 20 s.

The proposed method provided high sensitivity and a reliable procedure for determining the radioactivity of several hundred fractions simultaneously. As no scintillation cocktail is required in this method, the volume of radioactive waste produced per analysis is much smaller than that in the LSC off-line method. In addition, the radioactive compounds can be recovered from the wells and re-analysed by other methods.

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