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QUANTITATIVE LIQUID SCINTILLATION RADIOASSAY OF PHOSPHOLIPIDS FROM THIN-LAYER CHROMATOGRAMS

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SUMMARY

1. Several elution techniques for the quantitative recovery of phospholipids from thin-layer chromatograms were compared. Chloroform–methanol (1:1) containing 10% water was a most effective elution agent.

2. A methanol, Cab-O-Sil in toluene–fluor radioassay technique and a water–Aquasol radioassay technique for the quantitative recovery of ^{14}C-phospholipids from thin-layer chromatograms is described.

3. Dual-labelled (^{3}H:^{14}C) phospholipids were radioassayed, after thin-layer chromatography, by the water–Aquasol technique. A quantitative recovery was obtained for several phospholipid classes examined, including phosphatidylcholine and lysophosphatidylcholine. The position of the labels had little effect, and the ratios of ^{3}H:^{14}C had no effect on the recovery rate.

INTRODUCTION

Several methods are available for the radioassay of lipids separated by thin-layer chromatography (TLC), of which the simplest and most sensitive is the direct counting of TLC scrapings by liquid scintillation spectrometry[1,2]. However, the recovery of radioactive lipids by several widely used techniques is low, and disconcertingly, variable for the different lipid classes[3,4].

In order to overcome these difficulties we compared the quantitative radioassay of natural phospholipids in the presence of silica gel, using a number of conventional techniques. Our successful attempts to significantly improve on these rates of recovery, using both single- and dual-labelled lipids, form the basis of this report.

EXPERIMENTAL AND RESULTS

Preparation of labelled compounds

[1-^{14}C]oleic and palmitic acids, and [U-^{14}C]glucose were obtained from Amersham Searle, Toronto, Canada. [2-^{3}H]glycerol and [9,10-^{3}H]oleic acid were obtained from New England Nuclear, Boston, Mass., U.S.A. They were purified by TLC immediately prior to use. Labelled lipids were prepared as described previously[5].

Extracted phospholipids were separated by TLC into major lipid classes[6], visualized with iodine and sprayed with water. Phospholipids were extracted from

the layer three times with a variety of solvents whilst the adsorbent was still moist. The combined supernatants and silica gel were taken to dryness in scintillation vials and the disintegration rates calculated[5].

The elution technique of ROUSER *et al.*[7], utilizing chloroform–methanol (1:1), containing 10% water, gave a 94.2% recovery* of total phospholipids, 98.7% recovery of phosphatidylcholine and 96.4% recovery of lysophosphatidylcholine. Methanol alone with 10, 20 or 30% water gave 96.3% recovery of phosphatidylcholine and 96.8% recovery of lysophosphatidylcholine. The chloroform–methanol–water method was therefore used to prepare larger quantities of pure phospholipids for the second series of experiments.

Radioassay of phospholipids on silica gel using conventional scintillation fluors

Equal quantities of pure phospholipids were pipetted directly into scintillation vials and onto activated silica gel in scintillation vials. 15 ml of toluene–fluor was added to each vial, shaken, counted and corrected for quenching and counter efficiency. The disintegration rates obtained with lipid added directly to the vials

TABLE I

RECOVERY OF ACTIVITY FROM PHOSPHOLIPIDS ADSORBED ONTO SILICA GEL BY RADIOASSAY

[1-^{14}C]oleate-labelled phospholipids; radioassay in (A) toluene–fluor; (B) 1.5 ml of methanol, toluene–fluor; (C) 15 ml 4% Cab-O-Sil in toluene–fluor; (D) 1.5 ml of methanol, and C.

Compounds	*Radioassay treatment*	*Recovery (%)*[a]	*S.E.*
Total lipids	A	78.06	0.99[b]
Total lipids	B	74.47	1.2[b]
Total lipids	C	85.07	3.1[b]
Total lipids	D	95.03	0.98[b]
Phosphatidylethanolamine	D	92.55	1.2[c]
Phosphatidylserine and phosphoinositide	D	94.56	0.77[c]
Phosphatidylcholine	D	93.76	1.6[c]
Lysophosphatidylcholine	D	98.69	0.73[c]

[a] $\%\ \text{recovery} = \frac{\text{d.p.m. eluted lipid}}{\text{d.p.m. eluted lipid} + \text{d.p.m. lipid remaining in silica gel}} \times 100.$

[b] $n = 5$.

[c] $n = 4$.

were considered to be 100% recovery of activity. The percentage recovery from phospholipids adsorbed onto silica gel was only 78.06 ± 0.99%. The addition of 1.5 ml of methanol to the silica gel prior to the addition of the toluene–fluor did not improve the recovery rate (see Table I).

Cab-O-Sil, obtained from Packard Instrument Co., has been widely used for the radioassay of insoluble particles in suspension[8]. 15 ml of 4% (w/v) Cab-O-Sil in toluene–fluor was added to samples of phospholipid adsorbed onto silica gel. This

* $\%\ \text{recovery} = \frac{\text{d.p.m. eluted lipid}}{\text{d.p.m. eluted lipid} + \text{d.p.m. lipid remaining in silica gel}} \times 100.$

resulted in a significant increase in the percentage recovery of activity, which was still, however, far from quantitative.

1.5 ml of methanol was added to phospholipids adsorbed onto silica gel, shaken, and left to stand for several minutes. 15 ml of Cab-O-Sil in toluene–fluor was then added, shaken, and the vials counted. This treatment resulted in a further significant increase in the count rate to an almost quantitative 95 % recovery.

The latter procedure was then tested on several pure phospholipids prepared as described above. High recovery rates, consistently greater than 92 % (see Table I), were achieved for all the phospholipids tested. This represents a considerable improvement over conventional counting methods.

Radioassay of phospholipids with Aquasol

Aquasol, obtained from New England Nuclear, Boston, Mass., U.S.A., a multipurpose, xylene-based, scintillation fluor, has the capacity to take up to 26 % water, yet remains a homogeneous mixture. Water, being extremely polar, should deactivate the active sites on the silica gel and replace those occupied by the adsorbed lipid. When Aquasol is added, the phospholipids should then be free to dissolve in the xylene base. This hypothesis was tested by the addition of 1 ml of water to phospholipids adsorbed onto silica gel, which was then shaken rapidly on a Vortex mixer, followed by the addition of 10 ml of Aquasol.

The results in Table II show that with the water–Aquasol procedure an almost quantitative recovery of activity may be obtained for both phosphatidylcholine and lysophosphatidylcholine, the percentage recovery being significantly higher than with Aquasol alone. Also good percentage counting efficiencies were obtained with the water–Aquasol technique: a gain of 20 %, 50–1000 window gave a percentage efficiency of 84 %, *cf.* Aquasol alone; gain 14 %, window 50–1000 gave a percentage efficiency of 86 % for ^{14}C.

Radioassay of dual-labelled phospholipids

Dual-labelled phospholipids were bio-synthesized and purified as described above. Equal quantities of pure [^{3}H]glycerol-[^{14}C]palmitate phospholipids were spotted onto TLC plates and into scintillation vials. The TLC plates were treated as described above and the lipid areas scraped off into scintillation vials. The vials were then treated as follows. To one series 1 ml of water was added, followed by 10 ml of Aquasol. To a second series, 1.5 ml of methanol was added followed by 15 ml of Cab-O-Sil in toluene–fluor. The vials into which the pure phospholipids had been directly added were counted in either toluene–fluor or Aquasol, the calculated disintegration rates being taken to represent 100 % recovery of activity*.

The percentage recovery of ^{3}H and ^{14}C using two scintillation fluors, is shown in Table III. These results show that whilst good recovery rates of ^{14}C were obtained

* $$^{14}C \text{ d.p.m.} = \frac{(\text{c.p.m. green channel} - \text{background green channel})}{\%\ \text{efficiency } ^{14}C \text{ green}} \times 100$$

$$^{3}H \text{ d.p.m.} = \left[\frac{(\text{c.p.m. red channel} - \text{background red channel})}{\%\ \text{efficiency } ^{3}H \text{ red}} - \frac{\%\ \text{efficiency } ^{14}C \text{ red} \times (\text{c.p.m. green} - \text{background green})}{\%\ \text{efficiency } ^{14}C \text{ green} \times \%\ \text{efficiency } ^{3}H \text{ red}}\right] \times 100.$$

TABLE II

RECOVERY OF ACTIVITY FROM PHOSPHOLIPIDS ADSORBED ONTO SILICA GEL BY RADIOASSAY

[1-^{14}C]oleate-labelled phospholipids; radioassay in (A) 11 ml of Aquasol and (B) 1 ml of water, 10 ml of Aquasol.

Compound	*Treatment A*		*Treatment B*	
	Recovery (%)[a]	*Relative S.D.* ($n = 4$)	*Recovery* (%)[a]	*S.E.*
Phosphatidylethanolamine	98.1	0.48	98.22	0.34 ($n = 5$)
Phosphatidylserine and phosphoinositide	99.57	1.04	100.78	1.68 ($n = 4$)
Phosphatidylcholine	84.03	0.52	96.13	0.44 ($n = 6$)
Lysophosphatidylcholine	82.9	1.11	96.23	0.74 ($n = 6$)

[a] Formula in Table I.

TABLE III

RECOVERY OF ACTIVITY FROM DUAL-LABELLED PHOSPHOLIPIDS ADSORBED ONTO SILICA GEL BY RADIOASSAY

[2-^{3}H]glycerol-[1-^{14}C]palmitate-labelled phospholipids; radioassay in (A) 1 ml of water, 10 ml of Aquasol and (B) 1.5 ml of methanol, 15 ml of 4% Cab-O-Sil in toluene-fluor.

Compounds	*Treatment A*				*Treatment B*				*Relative activity ratio* (^{3}H:^{14}C)
	^{3}H		^{14}C		^{3}H		^{14}C		
	Recovery (%)[a]	*S.E.* ($n = 4$)	*Recovery* (%)[a]	*S.E.* ($n = 4$)	*Recovery* (%)[a]	*S.E.* ($n = 4$)	*Recovery* (%)[a]	*S.E.* ($n = 4$)	
Phosphatidylethanolamine	106.06	0.66	101.48	2.25	84.2	1.44	96.53	1.52	1.14
Phosphatidylserine and phosphoinositide	105.05	0.55	102.03	1.83	76.18	1.81	97.95	1.10	1.60
Phosphatidylcholine	105.9	4.0	94.7	1.37	82.68	3.75	96.23	1.59	0.31
Lysophosphatidylcholine	106.88	3.62	96.72	2.88	67.85	1.43	101.47	3.22	0.78

[a] % Recovery-formula in Table I; for ^{14}C d.p.m.- and ^{3}H d.p.m.-formulae, see footnote in text.

with the methanol, Cab-O-Sil in toluene–fluor procedure, the recoveries obtained for ^{3}H were low. Good recovery rates for both ^{14}C and ^{3}H were obtained with the water–Aquasol technique.

The recovery rates obtained for ^{3}H with the water–Aquasol technique were high. It has been found that spurious counts may arise in the red channel, presumably from fluorescence, resulting in excessive recovery rates. However, when the vials were left in the refrigerated compartment of the counter for 24 h prior to counting, repeated counting over several days gave consistent but still high results.

To determine if the position of the label or the relative activities of ^{14}C and ^{3}H had any effect on the recovery rates, phospholipids labelled with [9,10-^{3}H]oleic acid and [U-^{14}C]glucose were prepared and purified by TLC. Equal quantities were spotted onto TLC plates and directly into scintillation vials. The TLC plates were developed, subjected to iodine vapour, the lipid areas scraped off into scintillation vials, and then counted by the water–Aquasol procedure. The percentage recovery

TABLE IV

RECOVERY OF ACTIVITY FROM DUAL-LABELLED PHOSPHOLIPIDS ADSORBED ONTO SILICA GEL BY RADIOASSAY

[9,10-^{3}H]oleic acid [U-^{14}C]glucose-labelled phospholipids; radioassay in 1 ml of water, 10 ml of Aquasol.

Compounds	*Water + Aquasol*				*Relative activity ratio* (^{3}H:^{14}C)
	^{3}H		^{14}C		
	Recovery (%)	*S.E.* ($n = 3$)	*Recovery* (%)	*S.E.* ($n = 3$)	
Phosphatidylethanolamine	98.77	0.92	101.92	2.56	4.59
Phosphatidylserine and phosphoinositide	102.01	2.09	97.84	0.52	38.7
Phosphatidylcholine	89.64	0.74[b]	97.72	0.74[b]	48.0
Lysophosphatidylcholine	93.15	2.22[b]	102.78	0.68[b]	4.27

[a] For formulae, see Table III.
[b] $n = 9$.

of both ^{14}C and ^{3}H is shown in Table IV. The results show that good recovery rates were obtained with both ^{3}H- and ^{14}C-labelled phosphatidylethanolamine and phosphatidylserine plus phosphoinositide. Whilst the recovery rates obtained with ^{14}C-labelled phosphatidylcholine and lysophosphatidylcholine were also good, the percentage recovery with ^{3}H was slightly lower.

DISCUSSION

The above results show that chloroform–methanol (1:1), containing 10% water, is a suitable eluent for the recovery of phospholipids from TLC. For the direct radioassay of [^{14}C]fatty-acyl phospholipids the methanol, Cab-O-Sil in toluene–fluor technique provides a rapid and quantitative radioassay method. Whilst partial sedimentation of the suspended material will occur within short time, such sedimentation has little effect on the recovery rates, as the external standard, when "in", is positioned

beside the vial[9]. However, the methanol, Cab-O-Sil in toluene–fluor technique is of little use for the radioassay of dual-labelled phospholipids, because the ^{3}H recovery rates were not only low, but the yield decreased with sedimentation of the Cab-O-Sil and silica gel in the fluor. Cab-O-Sil is somewhat difficult to handle, and some batches give excessively high background count rates[10]. This technique is, however, rapid, simple and relatively inexpensive.

Aquasol was shown by KRITCHEVSKY AND MALHOTRA[3] to be a useful all-purpose-scintillator for the direct radioassay of phospholipids, with the exception of phosphatidylcholine. The more tightly adsorbed phospholipids, such as phosphatidylcholine and lysophosphatidylcholine may, however, be radioassayed in water–Aquasol as described above. In this new technique, not only the count rates are significantly greater than in Aquasol alone, but the yield is virtually quantitative. Other phospholipids may also be radioassayed by the water–Aquasol technique, which therefore allows the use of one single counting procedure and one quench correction curve. Excellent low background count rates were obtained with Aquasol, thereby making the radioassay of low-activity-samples considerably more accurate.

Dual-labelled phospholipids may also be quantitatively assayed in the water–Aquasol scintillator, though some attention has to be paid to tritiated phospholipids. Somewhat high count rates were obtained when tritiated phospholipids, labelled in the glycerol moiety, were assayed; low count rates were observed when assaying tritiated-fatty-acylphospholipids. Sedimentation of the silica gel in the vials had little effect on either ^{3}H or ^{14}C count rates; neither did the ^{3}H:^{14}C ratios. Good ^{14}C recovery rates were observed with both saturated and monounsaturated fatty-acyl-labelled phospholipids. Under the conditions in which the water–Aquasol scintillator was used, a considerable degree of quenching may occur, yet have little effect on the count rates. The water–Aquasol scintillator may therefore be used for the direct quantitative radioassay of both single and dual-labelled phospholipids. The technique is simple, rapid and effective.

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REFERENCES

1 F. SNYDER, *Anal. Biochem.*, 9 (1964) 183.
2 F. SNYDER AND D. SMITH, *Sep. Sci.*, 1 (1966) 709.
3 D. KRITCHEVSKY AND S. MALHOTRA, *J. Chromatogr.*, 52 (1970) 498.
4 H. G. ROSCOE, R. GOLDSTEIN, B. A. RICCARDI AND M. J. FAHRENBACH, *Arch. Biochem. Biophys.*, 138 (1970) 329.
5 R. A. WEBB AND D. F. METTRICK, *Can. J. Biochem.*, 49 (1971) 1209.
6 V. P. SKIPSKI, R. F. PETERSON, J. SANDERS AND M. BARCLAY, *J. Lipid Res.*, 4 (1963) 227.
7 G. ROUSER, G. KRITCHEVSKY AND A. YAMOMOTO, in G. V. MARRINETTI (Editor), *"Lipid Chromatographic Analysis"*, Vol. 1, Marcel Dekker Inc., New York, 1967, p. 154.
8 E. RAPKIN, *Packard Tech. Bull. No. 5*, Packard Instrument Co., Inc., revised 1963.
9 N. W. H. HOUX, *Anal. Biochem.*, 30 (1969) 302.
10 A. F. MCEVOY AND W. G. HARRIS, *Anal. Biochem.*, 43 (1971) 123.