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Quantitative analysis of rat liver phospholipids by a two-step thin-layer chromatographic procedure*

Chromatographic methods are essential in the isolation and quantitative determination of individual phospholipids in various tissues. A great deal of work has been done in liver tissue and a number of papers have been published presenting data on phospholipid distribution in rat liver[1–7]. Values given in Table I were obtained after silicic acid paper[1] and thin-layer chromatography[4,5,7].

TABLE I

COMPARISON OF PHOSPHOLIPID VALUES FOR RAT LIVER OBTAINED BY DIFFERENT METHODS OF SILICIC ACID PAPER AND THIN-LAYER CHROMATOGRAPHY

Phospholipid	*P as percentage of total lipid P*			
	Ref. 1	*Ref. 4*	*Ref. 5*	*Ref. 7*[a]
Phosphatidylcholine	58.1	55.0	58.5	57.7
Phosphatidylethanolamine	20.4	25.3	24.0	24.7
Phosphatidylserine	2.0	3.0	11.1[b]	3.7
Phosphatidylinositol	1.9	8.8		6.2
Sphingomyelin	8.2	1.8	5.7	2.6
Lysophosphatidylcholine	3.4	0.9	—	1.0
Cardiolipin	3.4	5.1[c]	—	4.2

[a] Recalculated.
[b] Phosphatidylserine + phosphatidylinositol.
[c] Including phosphatidic acid.

With the exception of phosphatidylcholine and phosphatidylethanolamine, considerable variations are evident in the individual phospholipid values, given by different authors. Though the differences related to strain, sex, age and dietary factors may not be neglected, in our opinion a great part of these disagreements results from an incomplete separation.

The difficulties encountered in thin-layer chromatography (TLC) of acidic phospholipids, *e.g.* phosphatidylinositol, phosphatidylserine, cardiolipin and others, could be partially eliminated by adding ammonia to the solvent system[8–10,19].

Better separation was also obtained by the use of a solvent system containing acetic acid and on silica gel plates impregnated with Na_2CO_3 (ref. 4).

Progress in the thin-layer chromatography of some acidic phospholipids (phosphatidic acid, cardiolipin and phosphatidylglycerol) was reported in a recently published paper[11] describing the use of several neutral, basic and acidic solvents in one- and two-step development procedures.

Here we describe a two-step thin-layer chromatography of phospholipids combining the possibilities of the basic and the acidic solvents.

* A short report on this investigation was presented at the Yugoslav Society of Physiology meeting in Beograd, December, 28th, 1967.

Experimental

Reference compounds and lipid extraction. Phosphatidylcholine, phosphatidylethanolamine (purified by TLC), sphingomyelin, phosphatidylserine, lysophosphatidylcholine and cerebrosides (a mixture of ceramidemonohexosides) were from Koch-Light Laboratories (Colnbrook, England); cardiolipin was obtained from Sumimoto Chemical Co., Ltd. (Osaka, Japan); monophosphatidylinositol (from wheat sprouts) was the generous gift of Dr. M. FAURE, Institute Pasteur, Paris.

Lipid extracts from livers of adult female albino rats were prepared according to the method of FOLCH *et al.*[12]

Thin-layer chromatography. Standard plates (20 cm × 20 cm) could be used, but longer plates (20 cm × 25 cm) gave better separations. The plates were coated with a layer (0.5 mm on adjustable Desaga applicator) of adsorbent prepared according to the procedure of ROUSSER *et al.*[13]. 54 g of Silica Gel H (E. Merck) and 6 g of Florisil (purchased from Serlabo, France, finely powdered by grinding in a mortar for 15 min) were slurried in 135 ml of water. The activation of the plates was accomplished just before use by heating at 110° for 30 min.

Total lipids (50–100 μl of concentrated solutions containing approx. 60 μg of phosphorus) were applied as a strip (15 mm long) 2 cm from the bottom edge of the plate.

The chromatograms were first developed in chloroform–methanol–30 % aq. ammonia–water (140:50:7:3, by vol). The solvent was allowed to run to about 3 cm from the top of the plate. After drying in a stream of air for 30 min, the chromatograms were developed in the mixture of chloroform–methanol–acetic acid–water (160:20:4:1.5, by vol.) so that the solvent front was allowed to run to the top of the plates.

The spots were detected by iodine vapour, 40 % sulfuric acid spray or ammonium molybdate–perchloric acid spray[14]. Phospholipid fractions were identified by comparison with reference compounds and by ninhydrin reagent for free amino groups[14].

Phosphorus determinations in fractions revealed with iodine was carried out by the methods of MACHEBOEUF AND DELSAL[15] and MARINETTI *et al.*[16] without the previous elution of lipids from adsorbent.

Results and discussion

Six distinct phospholipid spots were visible on the chromatograms after the two-step development (Fig. 1). A clear separation of all common phospholipids was obtained and "tailing" was not observed. Two fractions corresponding to sphingomyelin could be found on some chromatograms, as described previously in techniques involving the use of the ammonia-containing solvent[17]. Lysolecithin and lysophospatidylethanolamine which seem to be the minor fractions of liver phospholipids[7] could not be resolved by the method described above.

The phospholipid composition of rat liver, obtained by phosphorus determination in fractions isolated after TLC, is given in Table II.

Although a number of one-dimensional TLC systems, some of which are valuable in the quantitative analysis of phospholipids (see refs. 4, 5, 7, 9, 10, 14 and 18), have been described to date, as far as we are aware, the complete separation of all of the most common phospholipids in mammalian tissues has not been obtained on a single chromatogram.

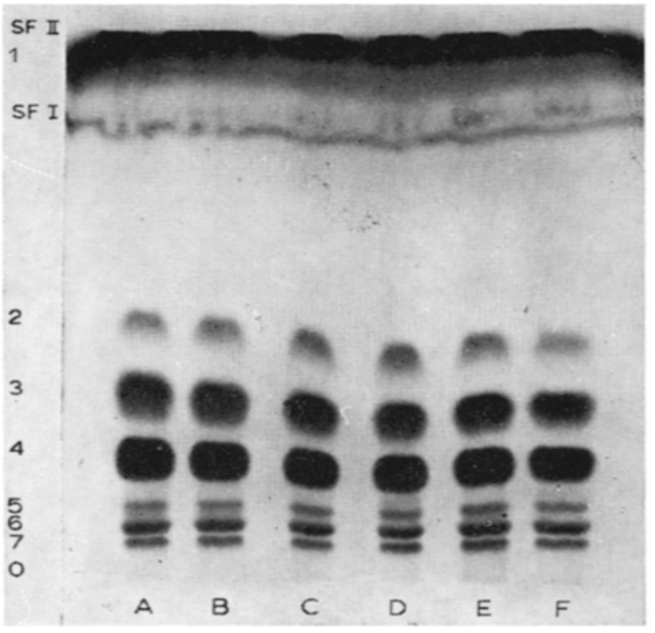

Fig. 1. Chromatogram of total lipid extracts from rat liver (A–F). Amounts applied (approx.): 60 μg P. Two-step development: I, chloroform–methanol–30% ammonia–water (140:50:7:3, by vol.); II, chloroform–methanol–acetic acid–water (160:20:4:1.5, by vol.). Detection with iodine. Identification of components: 1, neutral lipids; 2, cardiolipin; 3, phosphatidylethanolamine; 4, phosphatidylcholine; 5, sphingomyelin; 6, phosphatidylinositol; 7, phosphatidylserine; O, origin. SF I = first solvent front; SF II = second solvent front.

TABLE II

PHOSPHOLIPIDS IN RAT LIVER: RESULTS OF QUANTITATIVE ANALYSIS AFTER THE TWO-STEP THIN-LAYER CHROMATOGRAPHY

Phospholipid	*Mean value*[a]
Total	39.4 ± 2.8 μmoles P/g wet wt.
	P as percentage of total P applied
Cardiolipin	5.2 ± 0.7
Phosphatidylethanolamine	27.1 ± 2.5
Phosphatidylcholine	52.9 ± 3.7
Sphingomyelin	4.1 ± 0.7
Phosphatidylinositol	6.5 ± 0.6
Phosphatidylserine	4.3 ± 0.5
Total recovery	100.1 ± 7.1

[a] Mean values ± S.D. of six independent analyses.

The use of the two-dimensional techniques[13,19,20] makes possible the isolation of more phospholipid fraction, but often at the expense of clear separation, since diffuse spots are likely to appear and overlapping is present in many chromatograms. Another serious limitation of these methods is that they are not adequate for densitometric analysis.

The two-step development procedure described in this paper has the advantage of giving spots without streaking, which are uniform in shape and width; this is of considerable importance when direct thin-layer chromatographic–densitometric techniques[21,22] are used.

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