QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS BY THIN-LAYER CHROMATOGRAPHY*

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Thin-layer chromatography (TLC) has been extensively used in the last few years for the study of different lipid compounds. In spite of this, there are very few papers published with reference to its quantitative aspects, and furthermore, earlier papers only give very brief information about the quantitative analysis of phospholipids.

Among the various publications, the procedures used by PURDY[1], DOIZAKI[2] and AMENTA[3] seem to be, in our opinion, adequate for quantitative studies. Nevertheless, the method that we are presenting here has the advantage of being relatively quick, easy to carry out, reproducible, sensitive and accurate. The use of 1 *N* hydrochloric acid in methanol enables us to extract quantitatively, from chromatoplates, lipid compounds containing between 1 and 10 μg of phosphorus.

MATERIALS AND METHODS

Redistilled analytical solvents were used throughout.

Lipid extraction

Lipids were extracted from rat liver and brain according to FOLCH *et al.*[4] and concentrated by evaporation on a water bath at 50° under a stream of nitrogen. The final volume was calculated in such a way that approximately 4 μg of P would be present in 25 μl of lipid extract.

Thin-layer chromatography

Chromatoplates were prepared using a Desaga applicator and following the technique described by STAHL[5]. The slurry was prepared from 30 g of Silica Gel (Merck G.F.$_{254}$) and 65 ml of water by shaking vigorously for 45 sec. After spreading the silica, the plates were air-dried for 20 min and activated prior to using at 110° for 30 min. They were divided into 2 cm wide lanes and the sample was applied 1.5 cm from the lower edge of the plate using a 25 μl Lange-Levy pipet. The sample was applied

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in successive small drops placed one after the other in a straight line parallel to the lower edge of the plate. This system gave a better resolution for each spot on the chromatogram.

The plates were developed in the alkaline solvent mixture described by MÜLDNER *et al.*[6], which has the advantage of giving a good separation between sphingomyelin and phosphatidyl serine. The chromatographic jar was allowed to equilibrate overnight. The chromatoplates were placed in the chamber and the solvents were allowed to ascend until they reached a distance of 1–2 cm from the top of the plate. The plates were then placed in an oven at 100° for 10 min to evaporate the remaining solvent.

Detection of the spots was carried out with iodine vapours, which according to BARRET[7] do not chemically affect the structure of the phospholipids. The area of each spot was delineated by transparency and marked on the clean side of the glass plate using a glass marker pen. The plate was left at room temperature until residual iodine had disappeared and the zone corresponding to each phospholipid, as indicated in Fig. 1, was scraped off the plate using the edge of a piece of microslide of adequate size. The silica was collected over glazed paper and carefully transferred to centrifuge tubes and eluted.

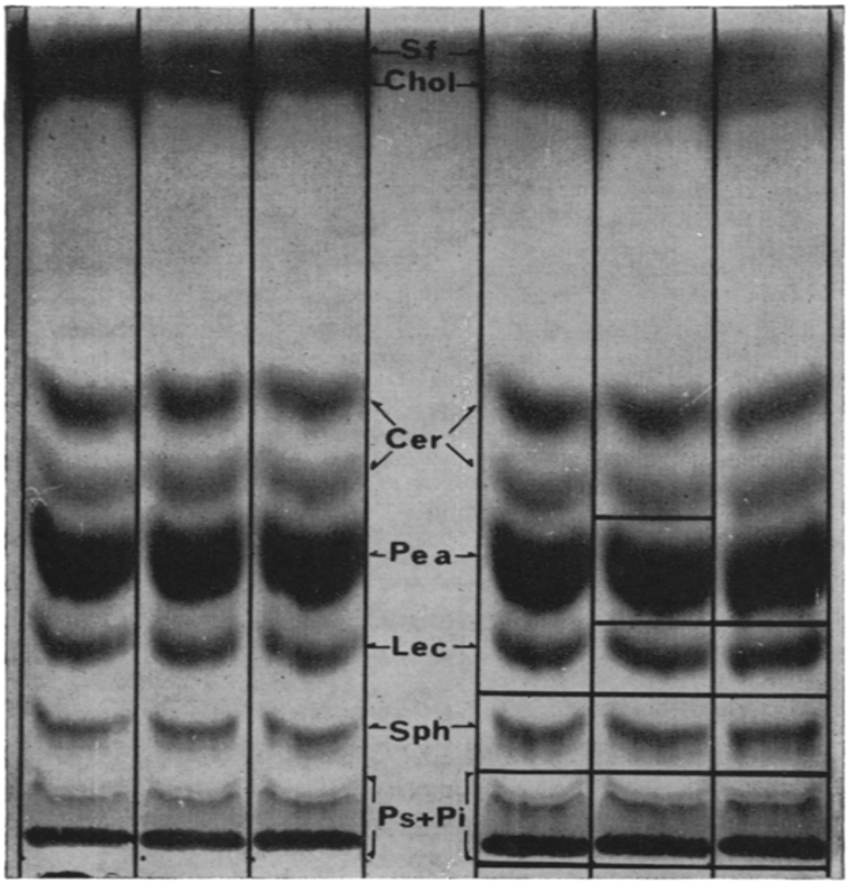

Fig. 1. TLC of rat brain lipid extract. Developing solvent: Chloroform–methanol–30% NH_3 (14:6:1). Abbreviations: Ps + Pi = phosphatidyl-serine plus phosphatidyl-inositol; Sph = sphingomyelin; Lec = lecithin; Pea = phosphatidyl-ethanolamine; Cer = cerebrosides; Chol = cholesterol; Sf = solvent front. Areas shown on the right-hand side of the chromatogram were used for the determination of P in each individual phospholipid.

Elution of the spots

The solvent used was 1 *N* HCl in methanol, which has already been employed by MARINETTI[8] for chromatography on siliconized paper. As will be seen, this solvent was the only one that gave good results among the different ones that we tried. Amounts of 4 ml of the eluting solvent were added to each centrifuge tube. The tubes were placed in a water bath at 50–60° for 15 min, stirring periodically with a glass rod. The tubes were then centrifuged for 15 min at 2000 rev./min and the supernatant was carefully decanted, the passage of the silica particles which might interfere with the colorimetric reaction being avoided. The whole procedure was repeated once more and the combined supernatants were evaporated to dryness in a phosphorus tube.

Phosphorus determination

Because of its sensitivity and accuracy, the method of CHEN *et al.*[9] was used throughout. The determination was carried out in triplicate samples. Each sample was selected in such a way that the equivalent of 1–4 μg of P would be present in each reaction tube. For this purpose, the spot given by each individual phospholipid was adjudged according to its colour intensity with the iodine vapours and when the colour was weak, two or more corresponding areas were used for each phosphorus determination (see Fig. 1). Elution and phosphorus determination were also carried out in areas of silica from the same plate in which no sample was applied (silica blank). To determine the per cent recovery obtained by our method, one of the lanes of the chromatogram was used for the determination of total phosphorus and compared with the total phosphorus in the extract.

RESULTS

In our first experiments we investigated the elution of the phospholipids from the silica. The method used by DAVISON[10] in column chromatography and later applied to TLC by the same investigator[11] did not allow us to obtain recoveries better than 80 %. Although methanol had been recommended for the same purpose[12] we were not able to obtain complete recovery of the phospholipids. Chloroform–methanol mixtures in various proportions were also tried with similar results. The highest recovery obtained with these solvents was 88 %. The results obtained using combinations of two solvents can be seen in Table I.

When 1 *N* HCl in methanol was first used two extractions were performed with 7 ml of this mixture on a water bath (50–60°) for 30 min. The mixture was agitated

TABLE I

RECOVERIES OBTAINED USING METHANOL AND OTHER MIXTURES SUCCESSIVELY FOR EXTRACTION*

First solvent	*Second solvent*	*Recovery*
Methanol	0.04 *N* KOH–ethanol–ether (10:7:5)	88 %
Methanol	Ethanol–ether (5:5)	50 %
Methanol	Ethanol–ether–chloroform (5:2:2)	60 %
Methanol	Methanol–chloroform (7:3)	65 %

* 10 ml of each solvent or solvent mixture were used in each extraction.

periodically, then centrifuged and the supernatant was filtered through sintered glass funnels. In view of the excellent results obtained with this method, we tried to simplify it, and finally adopted the procedure described under "Methods". Table II summarizes the results obtained using different elution techniques with the same solvent. Although the absorption given by silica is low, it should be pointed out that it is necessary to run a silica blank in all cases.

TABLE II

COMPARISON BETWEEN THE PHOSPHORUS VALUES OBTAINED IN ALIQUOTS OF RAT BRAIN LIPID EXTRACTS USING DIFFERENT ELUTION TECHNIQUES WITH 1 *N* HCl IN METHANOL

Amount of solvent (ml)	*Time of extraction (min)*	*Number of extractions*	*μg P*
7	30	2	5.65
4	30	2	5.59
7	15	2	5.53
4	15	2	5.59

In experiments carried out with different areas of silica, scraped off a plate, the readings obtained were always very close to 0.030 units of optical density. We have also carried out phosphorus determinations in the area where no phospholipids should be present (*i.e.* the area immediately below the front), with negative results.

When the mean value obtained from four experiments where the total rat brain phospholipids were eluted, chromatographed, and determined after removal from the plate was compared with the value for total rat brain phospholipids found in aliquots of total lipid extract, the recovery obtained was 96 %. A similar recovery was obtained for rat liver. To study the reproducibility of the method, nine aliquots of rat liver extract were run in separate lanes on a single plate, eluted and determined by our

TABLE III

DISTRIBUTION OF LIPID PHOSPHORUS IN RAT BRAIN*

*Phospholipid fraction***	*μg of lipid phosphorus per g of fresh tissue*										*% of lipid phosphorus*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*mean*	
TLE	2500	2112	2313	2580	2580	2220	1810	1930	2200	2250	—
Pea	1080	962	1027	915	870	710	770	850	—	898	39.8
Lec	1050	980	1015	1040	815	640	730	850	—	890	39.7
Sph	180	301	278	190	110	130	170	—	—	194	8.6
Ps + Pi	330	473	485	290	370	280	310	—	—	362	16.1

Recovery: 104.2 %***

* Results of nine different experiments.

** TLE = total lipid extract; Pea = phosphatidyl-ethanolamine; Lec = lecithin; Sph = sphingomyelin; Ps + Pi = phosphatidyl-serine plus phosphatidyl-inositol.

*** $\frac{\mu g \text{ lipid P/g fresh tissue in Pea + Lec + Sph + Ps + Pi}}{\mu g \text{ lipid P/g fresh tissue in TLE}} \times 10^2$ (recovery was calculated, using mean values).

method. The mean total lipid P value obtained was 11.3 mg per g of fresh tissue with a standard deviation of 0.3. We have also verified that the mean phosphorus value for total phospholipids eluted and determined from chromatoplates is the same as that obtained by adding the phosphorus value of the individual phospholipids eluted and quantitated in the same way. Table III shows the results for rat brain, and Table IV

TABLE IV

DISTRIBUTION OF LIPID PHOSPHORUS IN RAT LIVER*

*Phospholipid fraction***	*μg lipid phosphorus per g of fresh tissue*			*% of lipid phosphorus*
	1	*2*	*mean*	
TLE	9830	10420	10125	—
Pea	2342	2500	2421	24.0
Lec	5950	5890	5920	58.5
Sph	564	583	574	5.7
Ps + Pi	1117	1130	1124	11.1
Recovery: 99.3 %***				

* Results of two different experiments.
** Abbreviations as in Table III.
*** Calculated as in Table III.

the results for rat liver. We have also applied this method to rat brain subcellular fractions separated by a procedure previously described[13]. The results obtained are shown in Table V.

TABLE V

DISTRIBUTION OF LIPID PHOSPHORUS IN DIFFERENT SUB-CELLULAR FRACTIONS OF RAT BRAIN*

*Phospholipid fraction***	*μg lipid phosphorus per g of fresh tissue*			
	Total homogenate	*Nuclear fraction*	*Mitochondrial fraction*	*Microsomal fraction*
TLE	2376	542	692	460
Pea	996	200	236	146
Lec	1021	187	264	190
Sph	253	64	88	51
Ps + Pi	429	104	120	70
Recovery***	113 %	102 %	102 %	100 %

* Mean values of seven experiments.
** Abbreviations as in Table III.
*** Calculated as in Table III.

DISCUSSION

Although several methods have been described for the quantitative study of phospholipids separated by paper chromatography[14–16], we were interested in finding a suitable procedure for TLC. As was mentioned above, literature references to quan-

titative determination by TLC were scanty[17–20]. However, PURDY[1] had published an algebraic method for quantitative analysis of lipids separated by TLC, based on the well known linear relationship between intensity of the colour of the spot and lipid content. The main disadvantage of this method is, in our opinion, that it is difficult to use for a great number of determinations, as is the case in the study of the lipid composition of subcellular particles. Furthermore, it requires the use of lipid standards in all cases. It is a well known fact that "chromatographically pure standards" are not very pure when studied by TLC, and that lipid standards free from contaminating substances are very difficult to obtain.

The method described by DOIZAKI[2] seemed to be much easier to carry out, but it has the disadvantage of requiring a correction factor proportional to the amount employed in the determination of phosphorus (silica interferes with the colour reaction and lowers the final optical density). Furthermore, as there is no previous elution of the spots, it is difficult to carry out radioactive measurements. AMENTA[3] has recently published a very useful method for the quantitative determination of various lipids by TLC. From our point of view, the main disadvantage of this method is that it requires the use of a special type of silica gel (free of reducing compounds) and that the determination might in some cases be inaccurate, as one has to rely on the R_F values obtained in one lane developed with iodine vapour to locate the same spots in the other lanes (since residual iodine might interfere with the analysis by acting as a reducing agent if used on the other lanes).

The use of special equipment for the elution of silica, as described by MILLET[21] and GOLDRICK[12] does not seem to be necessary in our estimation because of the low standard deviation obtained by us.

The method reported in this paper uses a very simple elution procedure. Excellent recovery values can easily be obtained by extracting twice small volumes of a single solvent mixture. Of all the solvent combinations investigated, only 1 *N* HCl in methanol gave a 100 % recovery. 88 % was the highest recovery obtained using those solvents described by DAVISON[11]. Our method is also quick and very simple.

The values that we have found for phospholipids in rat liver agree with those of SCHMIDT *et al.*[22] and BIEZENSKY[23]. With reference to phospholipids in rat brain and its subcellular fractions, our data agree with those of PETERSEN AND SCHOU[24] and BRANTE[25], except for lecithin, where a difference can be observed.

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SUMMARY

A method for the quantitative analysis of phospholipids by thin-layer chromatography, based on the use of 1 *N* HCl in methanol as eluting solvent, is described.

The method is very simple, quick and reproducible. The mean value obtained for rat liver total phospholipids is 11.3, with a standard deviation of 0.3.

Data for total and individual phospholipids, determined by our method, in rat liver and brain as well as in its subcellular fractions are presented.

ADDENDUM

During the preparation of the manuscript a paper on quantitative determination of phospholipids by thin-layer chromatography by SKIPSKI *et al.*[26] has been published. The steps that we have used are similar to those which these investigators have followed. Their data agree with ours, although the method of elution and the thin-layer techniques used are different from ours.

REFERENCES

1 S. J. PURDY AND E. V. TRUTER, *Analyst*, 87 (1962) 802.
2 W. M. DOIZAKI AND L. ZIEVE, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 91.
3 J. S. AMENTA, *J. Lipid Res.*, 5 (1964) 270.
4 J. FOLCH, M. LEES AND G. H. S. STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
5 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962, p. 5.
6 H. G. MÜLDNER, J. R. WHERRET AND J. N. CUMMINGS, *J. Neurochem.*, 9 (1962) 607.
7 G. C. BARRET, *Nature*, 194 (1962) 1171.
8 G. V. MARINETTI, M. ALBRECHT, T. FORD AND E. STOTZ, *Biochim. Biophys. Acta*, 36 (1959) 4.
9 P. S. CHEN, Jr., T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
10 A. N. DAVISON AND M. WAJDA, *Biochem. J.*, 82 (1962) 113.
11 A. N. DAVISON AND E. G. WOLFAARD, *Biochem. J.*, 83 (1963) 31P.
12 B. GOLDRICK AND J. HIRSCH, *J. Lipid Res.*, 4 (1963) 482.
13 L. M. SEMINARIO, N. HREN AND C. J. GOMEZ, *J. Neurochem.*, 11 (1964) 197.
14 R. M. C. DAWSON, *Biochem. J.*, 68 (1958) 357.
15 R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45.
16 G. J. M. HOOGHWINKEL AND H. P. G. A. VAN NIEKERK, *Koninkl. Ned. Akad. Wetensch. Proc.*, B63 (1960) 258.
17 E. HABERMANN, G. BANDTLOW AND B. KRUSCHE, *Klin. Wochschr.*, 39 (1961) 816.
18 K. HUHNSTOCK AND H. WETCKER, *Klin. Wochschr.*, 38 (1960) 1249.
19 N. ZÖLLNER AND G. WOLFRAM, *Klin. Wochschr.*, 40 (1962) 1098.
20 W. SCHLEMMER, *Boll. Soc. Ital. Biol. Sper.*, 37 (1961) 134.
21 M. A. MILLET, W. E. MOORE AND J. E. SAEMAN, *Anal. Chem.*, 36 (1964) 491.
22 G. SCHMIDT, J. BENOTTI, B. HERSHMAN AND S. J. THANNHAUSER, *J. Biol. Chem.*, 166 (1946) 505.
23 J. J. BIEZENSKI, *J. Lipid Res.*, 3 (1962) 120.
24 P. V. PETERSEN AND M. SCHOU, *Acta Physiol. Scand.*, 33 (1955) 309.
25 G. BRANTE, *Acta Physiol. Scand.*, Suppl. 63 (1949) 104.
26 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.