CHROM. 3879

A SIMPLE METHOD FOR THE QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS SEPARATED BY THIN LAYER CHROMATOGRAPHY

J. KAHOVCOVÁ AND R. ODAVIĆ*
*Institute for Cardiovascular Research**, *Budějovická 809 Prague 4 – Krč, (Czechoslovakia)*
(Received November 15th, 1968)

SUMMARY

A rapid, simple and reproducible method for the quantitative determination of phospholipids after separation by thin layer chromatography has been described. The method involves spraying the developed chromatograms with 50 % w/w sulphuric acid, direct mineralization at high temperature and subsequent determination of the liberated inorganic phosphate with HAHN AND LUCKHAUS' reagent. The range of applicability of the method is from 0.05 to 0.5 micromoles of phospholipid phosphate. The method was compared with the generally cited methods used for the same purpose.

INTRODUCTION

Simplicity, good separation ability and quickness characterize thin layer chromatography as an excellent tool for the qualitative examination of phospholipids. Quantitative evaluation of the compounds separated in this class of polar lipids on thin layer chromatograms is, however, a more difficult task. There are two general approaches to the problem[1] of quantitative analysis of phospholipids separated by TLC***:

(1) Elution of the spots from the thin layer plate and subsequent analysis[2–4].

(2) Photodensitometry of the charred chromatogram[5,6].

Both there methods have their disadvantages. Elution is time-consuming (several extractions and centrifugations, evaporation of eluents). On the other hand, elution can be a critical step in this determination because it is not necessarily, in our experience, quantitative.

The photodensitometric method has its limitations which are basically represented by the unequal photoelectric response to the same molar concentrations of different phospholipids. The reason for this discrepancy is the unequal number of carbon atoms in the molecules of individual phospholipids, which are *de facto* deter-

* Present address: Department of Physiology and Biochemistry, Medical Faculty, University of Sarajevo, Yugoslavia.

** Head Prof. J. BROD, M.D., DSc.

*** Abbreviations: TLC = thin layer chromatography, P_i = inorganic phosphate, PE = phosphatidylethanolamine, PC = phosphatidylcholine, SP = sphingomyelin, Lyso PC = Lysophosphatidylcholine.

mined by this method. This limitation can be, to some extent, solved by a separate calibration curve for each phospholipid, but the remaining difficulty is the variety of the fatty acids esterified in the naturally occurring phospholipid molecules.

In the following we present a rather simple procedure for the determination of the phospholipids separated by the TLC. The principle of this procedure involves the direct charring of the separated phospholipids on the thin layer plates (with their simultaneous detection) and the subsequent determination of the liberated inorganic phosphate.

The method is simple, reproducible and sufficiently sensitive to be applied to the quantitative determination of phospholipids on TLC plates.

MATERIALS AND METHODS

Standards

Lecithin. This was isolated from egg yolk and purified by passing it first through a column of alumina and than one of silicic acid according to RHODES AND LEA[7]. The final purity of this compound was established from the ester/phosphorus ratio and by thin layer chromatography.

Lysolecithin. This was obtained by the action of cobra venom ("Kobratoxin" — Institute of Sera and Vaccines, Prague) on lecithin according to LONG AND PENNY[8]. After incubation the lysolecithin obtained was treated as described by LONG *et al.*[9] and finally purified by passing through a silicic acid column. The purity of the fractions eluted was monitored by TLC.

Phosphatidylethanolamine. This was obtained and its purity established in the same way as in the case of lecithin. For estimation of the ester/phosphorus ratio we determined the ester moiety by SHAPIRO's[10] method and the phosphorus by the method described by BERENBLUM AND CHAIN[11] as modified by LONG[12].

Reagents

HAHN AND LUCKHAUS reagent[13] was prepared by dissolving 6.85 g sodium molybdate dihydrate and 400 mg hydrazine sulphate in 100 ml of distilled water. To this solution, 100 ml of concentrated sulphuric acid and 500 ml of water were added. After cooling the volume of the solution was adjusted to 1000 ml with water.

Thin layer chromatography

The plates were coated with a layer of silicic acid, 0.5 mm thick. We used Silica Gel CH (5–30 μ), a product of Lachema-Brno with 10 % $CaSO_4$ added. For five plates a slurry of 41 g silica gel in 95 ml distilled water was used. Before use the plates were activated by heating at 115° for half an hour. The developing system was chloroform–methanol–water (65:25:4, v/v).

Mineralization

To obtain phosphorus from phospholipids in the form of inorganic phosphate we ashed the organic matter on the thin layer plates. The developed and dried chromatograms were sprayed with sulphuric acid. Optimal conditions of direct mineralization of thin layer plates were examined and treated statistically. For the evaluation of the influence of temperature, time of mineralization and composition of the spray-

TABLE I

VARIABLES STUDIED IN LATIN SQUARE EXPERIMENT

Factors and units	*Levels*		
Temperature (°C)	I = 180	II = 200	III = 240
Time (min)	1 = 20	2 = 30	3 = 60
Composition of spraying mixture (% of H_2SO_4, w/w)	A = 30	B = 50	C = 70

TABLE II

LATIN SQUARE EXPERIMENTAL DESIGN AND THE PERCENTAGE RECOVERY OF P_i AFTER MINERALIZATION

	I	*II*	*III*	*Percentage recovery*		
1	A	B	C	110.2	99.2	98.2
2	B	C	A	103.2	95.3	106.2
3	C	A	B	96.3	107.2	100.2

ing mixture a Latin square of 3 × 3 terms was used[14]. The three variables studied and their levels and designations are set out in Table I. The combinations of variables actually used can be seen in Table II.

From the statistical evaluation it is evident that only the influence of the levels of spraying reagent composition is significant. The best values were obtained under the following conditions: concentration of spraying mixture 50 % w/w, temperature 180°, time of mineralization 60 min.

Estimation of phosphate

The inorganic orthophosphate obtained after direct mineralization was determined quantitatively using HAHN AND LUCKHAUS' reagent. The black spots from the mineralized plates were scraped off and quantitatively transferred to the centrifuge tubes. 4 ml of distilled water and 1 ml of HAHN AND LUCKHAUS' reagent were added to the tubes. After mixing, the tubes were placed in a boiling water bath for 30 min, during which time the blue colour was developed. After cooling the tubes were centrifuged for 10 min at 3000 r.p.m. to precipitate the silicic acid. Several areas of the same dimensions at different levels were taken as controls. Immediately after centrifugation the optical density of the blue supernatants were measured spectrophotometrically at 700 nm on a CF – 4 single beam spectrophotometer, Optica, Milan, using glass cells with a 1 cm light path. The spectral curve of the blue complex in the range of 300–900 nm is given in Fig. 1.

RESULTS AND DISCUSSION

We analysed samples containing known amounts (0.155, 0.310, and 0.465 μmoles of P_i) of lecithin, lysolecithin and phosphatidylethanolamine, by the method described. The values obtained and the calibration curves are presented in Table III

and Fig. 2. A sample of impure natural lecithin containing some other phospholipids was also analyzed. The results obtained are presented in Table IV. Recovery of phospholipids was 95.3 ± 12.9%. We also studied the reproducibility of our method on a sample of pure lecithin. Results are summarized in Table V. Standard deviations of I, II, III, *i.e.* 0.155, 0.310 and 0.465 μmoles P_i are 13.80, 4.51, and 6.01, respectively.

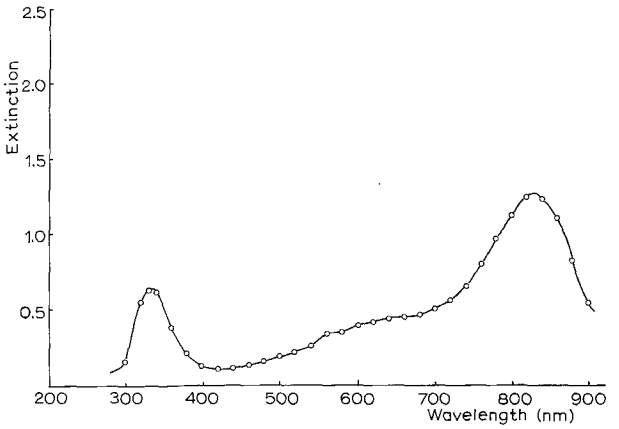

Fig. 1. Spectrum of the blue complex.

TABLE III

VALUES OF P_i OBTAINED BY ANALYSING KNOWN AMOUNTS OF STANDARD PHOSPHOLIPIDS

Every result represents a mean of 10 determinations. I, II, III and IV means 0.000, 0.155, 0.310 and 0.465 μmoles P_i, respectively.

Phospholipid	*Extinction at 700 nm*			
	I	*II*	*III*	*IV*
Lecithin	0.064	0.268	0.466	0.699
Lysolecithin	0.068	0.276	0.465	0.673
Phosphatidylethanolamine	0.060	0.274	0.445	0.707

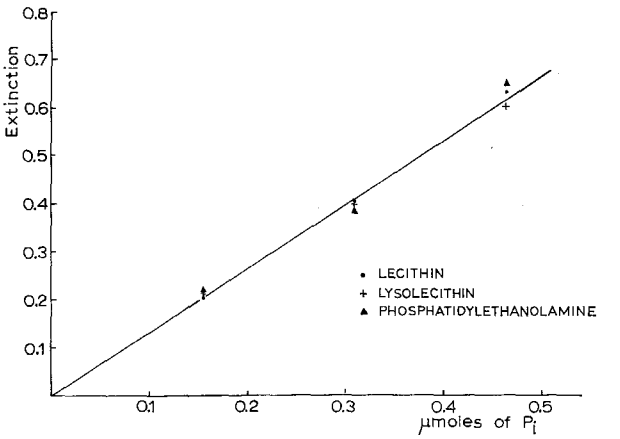

Fig. 2. Calibration curve for lecithin, lysolecithin and phosphatidylethanolamine.

TABLE IV

VALUES OBTAINED BY ANALYSIS OF A PHOSPHOLIPID MIXTURE

(Amount of phospholipids 0.775 μmoles P_i, conditions of TLC as described under MATERIALS AND METHODS.)

No. of estimation	*Micromoles P_i*				
	PE	*PC*	*SP*	*LysoPC*	*Start*
1	0.074	0.577	0.051	0.034	0.010
2	0.068	0.505	0.047	0.026	0.009
3	0.094	0.553	0.071	0.040	0.010
4	0.078	0.579	0.042	0.081	0.000
5	0.087	0.497	0.042	0.043	0.018
6	0.082	0.633	0.028	0.072	0.000
% w/w (average)	10.8	75.3	6.3	6.6	1.0

TABLE V

THE REPRODUCIBILITY OF OUR METHOD

I = 0.155, II = 0.310, III = 0.465 μmoles P_i.

No. of estimation	*Percentages P_i (w/w)*		
	I	*II*	*III*
1	67.3	86.8	90.0
2	76.0	89.0	93.7
3	86.7	89.4	95.6
4	93.5	89.6	96.7
5	94.3	90.2	97.7
6	97.5	95.0	102.5
7	101.5	96.5	104.7
8	103.2	96.7	105.4
9	112.2	98.2	105.6
10	115.5	99.3	107.8
	$\bar{x}$ = 94.77	$\bar{x}$ = 93.07	$\bar{x}$ = 99.97

The differences between experimental and theoretical values are not statistically significant.

As the next step we compared our method with the method described by SKIPSKI *et al.*[2]. These authors repeatedly eluted separated phospholipids from a thin layer chromatogram. The elution was performed with 3 ml and 2 ml of chloroform–methanol–acetic acid–water (25:15:4:2, v/v), 2 ml of methanol and 2 ml of methanol–acetic acid–water (94:1:5, v/v). Finally the eluent was evaporated and the quantity of P_i was determined as in our method. Using their elution procedure we analysed a sample of lecithin in order to obtain a calibration curve. The extinctions for quantities of 0.000, 0.155, 0.310 and 0.465 μmoles of P_i were 0.032, 0.128, 0.287 and 0.507, respectively. The values represent the mean of three determinations.

We also analysed the reproducibility of the method of SKIPSKI *et al.*[2] and obtained the results presented in Table VI.

TABLE VI

THE REPRODUCIBILITY OF THE METHOD DESCRIBED BY SKIPSKI, PETERSON AND BARCLAY[2]
I = 0.155, II = 0.310, III = 0.465 μmoles P_i.

No. of estimation	*Percentages P_i (w/w)*		
	I	*II*	*III*
1	43.8	32.0	53.9
2	47.7	48.3	56.0
3	61.9	53.0	58.1
4	62.6	63.5	62.6
5	63.2	67.7	64.1
6	68.4	68.2	64.6
7	80.7	68.6	66.4
8	84.5	77.0	72.2
9	89.0	77.7	74.0
10	101.6	82.9	87.0
	$\bar{x}$ = 70.34	$\bar{x}$ = 63.89	$\bar{x}$ = 65.89

Standard deviations of I, II, and III, *i.e.* of the results obtained for the concentrations of 0.155, 0.310 and 0.465 μmoles P_i were 18.37, 15.48 and 9.83, respectively. From statistical values it could be seen that the analysis of phospholipids according to our method gives more correct results than elution method described by SKIPSKI *et al.*[2].

To compare our method with other methods often used in the quantitative determination of phospholipids separated by thin layer chromatography[15–19], we mineralized the phospholipids after scraping off the spots visualized by iodine vapour. This mineralization was performed with 70 % perchloric acid at 200° for 2 h. After digestion the determination of inorganic phosphate was accomplished using the method described and the reagent of HAHN AND LUCKHAUS. The extinctions obtained with a sample of lecithin according to this method were 0.139, 0.378, 0.494 and 0.663 for quantities of 0.000, 0.155, 0.310 and 0.465 μmoles P_i, respectively. The values of the extinctions represent the mean of three determinations.

It is evident that the extinctions obtained fro the same sample and quantity of lecithin by our method and this one are very close (*cf.* extinctions given in Table III). The only difference is a rather high blank value obtained by the latter method. The further advantage of our method is the omission of the detection of the phospholipids separated on the thin layer plates, which means a shortening of the complete procedure.

REFERENCES

1 O. RENKONEN AND P. VARO, in G. V. MARINETTI (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Marcel Dekker, New York, 1967, p. 83.
2 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *Biochem. J.*, 90 (1964) 374.
3 D. ABRAMSON AND M. BLECHER, *J. Lipid Res.*, 5 (1964) 628.
4 W. D. SKIDMORE AND C. ENTENMAN, *J. Lipid Res.*, 3 (1962) 471.
5 N. M. NESKOVIC, *Arch. Inst. Pasteur Tunis*, 43 (1966) 513.
6 M. L. BLANK, J. A. SCHMIT AND O. S. PRIVETT, *J. Am. Oil Chemists' Soc.*, 41 (1964) 371.
7 D. N. RHODES AND C. H. LEA, *Biochem. J.*, 65 (1957) 526.
8 C. LONG AND I. F. PENNY, *Biochem. J.*, 65 (1957) 382.
9 C. LONG, R. ODAVIĆ AND E. J. SARGENT, *Biochem. J.*, 102 (1967) 216.

10 B. SHAPIRO, *Biochem. J.*, 53 (1953) 663.
11 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
12 C. LONG, *Biochem. J.*, 37 (1943) 215.
13 F. L. HAHN AND R. LUCKHAUS, *Z. Anal. Chem.*, 149 (1956) 172.
14 O. L. DAVIES, *Design and Analysis of Industrial Experiments*, Oliver and Boyd, London, 1954, p. 169.
15 J. C. CHRISTIAN, S. JAKOVCIC AND D. J. HSIA, *J. Lab. Clin. Med.*, 64 (1964) 756.
16 B. M. PHILLIPS AND N. ROBINSON, *Clin. Chim. Acta*, 8 (1963) 832.
17 W. M. DOIZAKI AND L. ZIEVE, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 91.
18 O. VIKROT, *Acta Med. Scand.*, 175 (1964) 443.
19 N. ROBINSON AND B. M. PHILLIPS, *Clin. Chim. Acta*, 8 (1963) 385.