CHROM. 5860

ANALYSIS OF PHOSPHOLIPIDS BY THIN- LAYER CHROMATOGRAPHY AND X-RAY EMISSION SPECTROMETRY

L. R. CHAPMAN
The Procter & Gamble Co., Miami Valley Laboratories, P.O. Box 39175, Cincinnati, Ohio 45239 (U.S.A.)

(Received October 20th, 1971)

SUMMARY

The use of X-ray emission spectrometry for *in situ* quantitative analysis of phospholipids after separation by two-dimensional thin-layer chromatography is described. With this procedure, microgram quantities of phospholipids can be analyzed. The precision is comparable to that of other methods for analyses of phospholipids after thin-layer chromatography but time-consuming recovery steps are eliminated.

INTRODUCTION

Chromatographic methods are widely used for the isolation and determination of individual phospholipids. With the limited quantity of sample available from biological tissues, thin-layer chromatography (TLC) has become especially useful. Development with a single solvent system[1,2], multiple development in one dimension[3], and development in two dimensions[1,4–8] have recently been applied in methods for phospholipid analysis. Quantitation of the separated phospholipids is often difficult. The separated components can be recovered from the adsorbent layer and analyzed spectrophotometrically[7,8]. Alternately, the components can be visualized by spraying with an appropriate reagent or with sulfuric acid followed by charring of the organic material and the resultant spots scanned with a densitometer[9,10]. Reviews of the various qualitative and quantitative techniques have been published[11,12].

There are problems involved with both these quantitation steps. Elution from the adsorbent layer and subsequent analysis is arduous. Complete elution and hydrolysis as well as quantitative color development are essential. Densitometry is an excellent technique for quantitation of one-dimensional chromatograms but extremely difficult to apply to two-dimensional separations, which are generally required for the complete separation of phospholipids in biological samples.

The use of X-ray emission spectrometry (XES) to quantitate TLC has been reported[13]. Combined TLC–XES was shown to be applicable to the *in situ* determina-

tion of a variety of materials, including phospholipids. All TLC separations, however, were one-dimensional.

This paper describes a method for the separation and quantitative analysis of phospholipids using two-dimensional TLC and XES analysis (*in situ*) of each component for phosphorus content. The method has been applied to the analysis of rat liver and testis for the following phospholipids: phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and sphingomyelin.

METHODS

Preparation of phospholipids

Phospholipid standards obtained from Supelco, Inc. were used for calibration of the X-ray spectrometer. The phospholipid fractions of pooled liver and testis lipids from three 2-month old rats (Sprague-Dawley) were isolated by chromatography on Florisil[14].

For chromatography a weighed quantity of phospholipid, either biological sample or standard mixture, is dissolved in sufficient chloroform–methanol (2:1, v/v) to yield a final concentration of 50–100 μg/μl.

All samples were stored under nitrogen at 0°.

Thin-layer chromatography

TLC was carried out on 20 × 20 cm silica gel coated polyethyleneterephthalate sheets (Eastman Chromagram 6061). Reagent grade solvents were used without prior distillation.

Approximately 1 cm of the adsorbent layer was removed from all four sides of the sheet to minimize contamination of the layer during handling. Using a microsyringe (Hamilton No. 701, 10-μl capacity) the sample (200–900 μg for standards, 300–400 μg for samples) was applied in a single small spot in the lower left corner, 3 cm from each edge. The sheet was irrigated in the first dimension for 2 h using a solvent system consisting of chloroform–methanol–ammonium hydroxide (140:50:7). After air-drying for 2½ h, the sheet was irrigated in the second dimension (90° to the first) for 2 h with a solvent system consisting of chloroform–methanol–acetone–acetic acid–water (10:2:4:2:1). After all traces of acetic acid were removed by air-drying, the phospholipid zones were visualized by exposure to iodine vapor for 2–3 min.

Preparation for XES analysis

A clear polyethyleneterephthalate sheet was placed over the chromatogram and the outlines of the phospholipid zones were traced on this overlay. Using this tracing as a guide the portions of the chromatogram containing phospholipid components were cut into 5/8 in. × 5/8 in. sections. Several such sections were required to encompass the larger spots. These sections were then analyzed individually for phosphorus content.

X-ray emission spectrometry

Quantitative analysis for phosphorus was performed with a Norelco PW-1212 spectrometer under a helium atmosphere. Chromium K_α incident radiation (60 kV, 24 mA), a germanium analyzing crystal and a proportional flow counter were used.

Counts were accumulated for 40 sec. The XES analysis was carried out essentially as described by LIBBY[13] with a few modifications. The 5/8 in. square sections of the chromatogram were attached to 25-mm diameter circles of Whatman 40 filter paper with a drop of rubber cement and placed in the spectrometer. As noted, counting of emitted radiation was carried out under a helium atmosphere rather than under vacuum. As no "blank" sections (*i.e.*, areas not exposed to sample during chromatography) of the TLC sheet were available to use for background count correction, the emission at 2.0° on either side of the phosphorus angle (141.06° 2Θ) was counted on four sections from each chromatogram. These counts were then averaged and used for background correction.

Calibration curves

Three mixtures of the phospholipid standards containing approximately 50, 100, and 200 μg, respectively, of each phospholipid were chromatographed and analyzed by XES as described above. Curves for μg phospholipid *vs.* net counts (gross counts less average background count) were prepared for each phospholipid of interest.

RESULTS AND DISCUSSION

The TLC separation of approximately 100 μg of each of the five phospholipids is shown in Fig. 1. Several commercially available and laboratory prepared silica gel

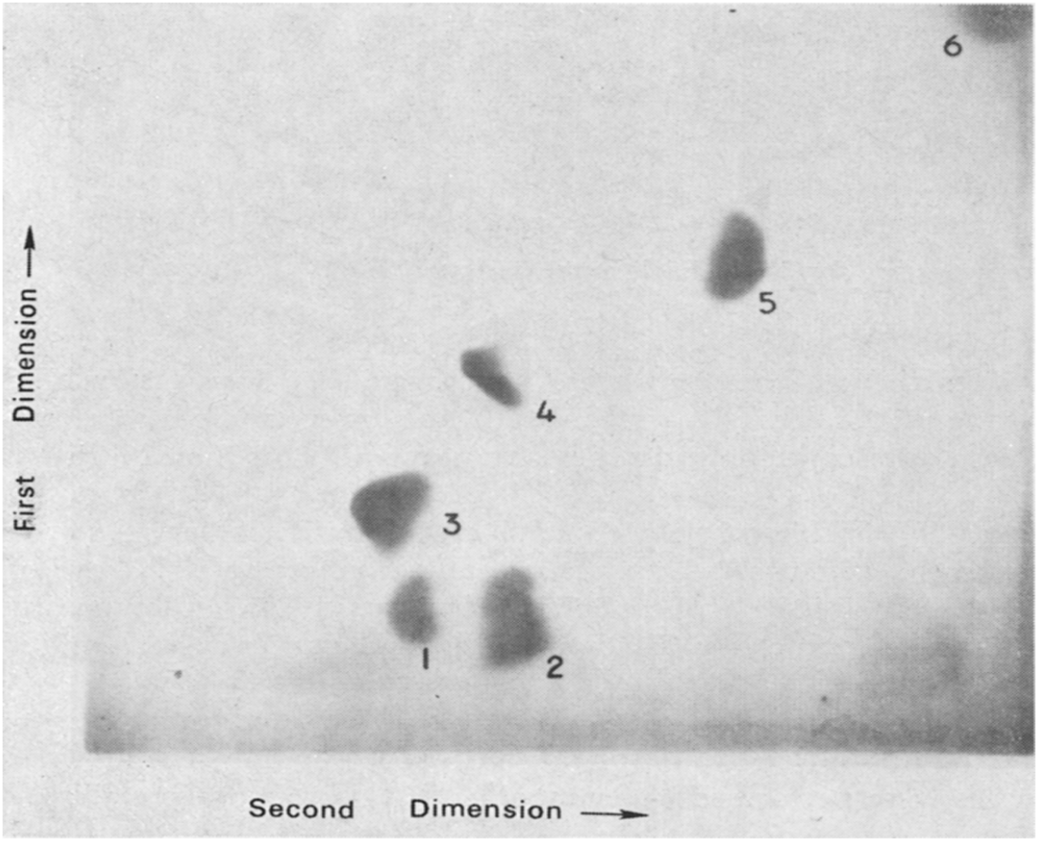

Fig. 1. Separation of phospholipid standard mixture containing approximately 100 μg of (1) phosphatidyl inositol; (2) phosphatidyl serine; (3) sphingomyelin; (4) phosphatidyl choline; (5) phosphatidyl ethanolamine and (6) neutral lipid.

coated polyethyleneterephthalate sheets were investigated for use in TLC. Also, a number of solvent systems were evaluated. The specific combination of adsorbent and solvent systems reported here gave the most consistent performance in terms of reproducible separation and spot size as well as minimal spreading or tailing of the various spots. In an attempt to minimize the adsorption of emitted phosphorus X-rays by the silica matrix, and thus increase sensitivity, the use of other adsorbent layers such as alumina and cellulose was investigated. Unfortunately, satisfactory separation of the phospholipids could not be achieved.

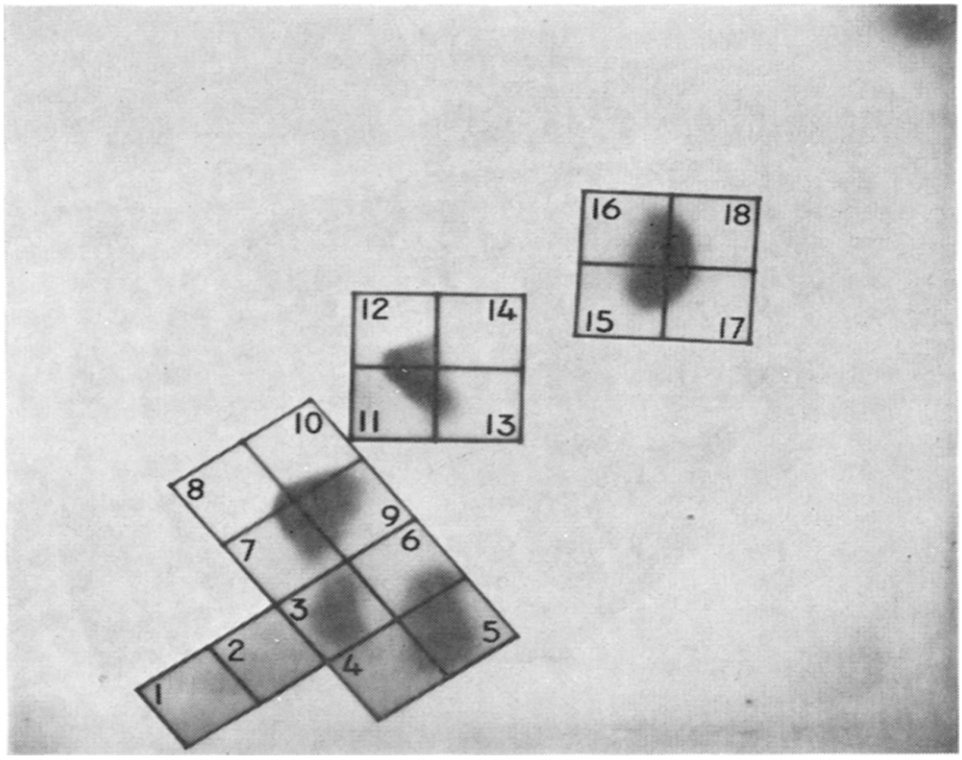

Fig. 2. Sectioning of chromatogram for XES. Each numbered section cut out and analyzed by XES. Numbers correspond to data in Table I.

Fig. 2 demonstrates the sectioning of a chromatogram (see Fig. 1) for XES analysis. The compositing of the XES data resulting from this sectioning is demonstrated in Table I. (These data correspond to the ~100 μg points on the calibration curves.)

The XES analysis of a phosphorus solute in a silica gel matrix is very sensitive to matrix adsorption and the extent of adsorption of emitted phosphorus X-rays by the silica gel layer is different for each phospholipid, perhaps due to differences in penetration[13] or spot shape. Therefore, individual calibration curves were required for each phospholipid (Fig. 3).

Because of variations introduced by XES counting statistics and minor day-to-day changes in the setting up and operating of the XES spectrometer, the analyses of standards and samples were performed the same day. If this was not possible, at least one point of each calibration curve was checked each day and if agreement with the previous data was not satisfactory ($\pm 5\%$ relative), XES analysis of all curve points was repeated.

TABLE I

ANALYSES OF CHROMATOGRAM SECTIONS

See Fig. 2.

Phospholipid	*μg spotted*	*Section No.*	*Net XES counts*	*Total counts*
Phosphatidyl inositol	93.0	1 & 2	—	
		3	740	740
Phosphatidyl serine	94.0	4	81	
		5	423	
		6	260	764
Sphingomyelin	106.6	7	312	
		8	93	
		9	309	
		10	172	886
Phosphatidyl choline	81.4	11	519	
		12	195	
		13	107	
		14	17	838
Phosphatidyl ethanolamine	108.6	15	140	
		16	95	
		17	337	
		18	377	949

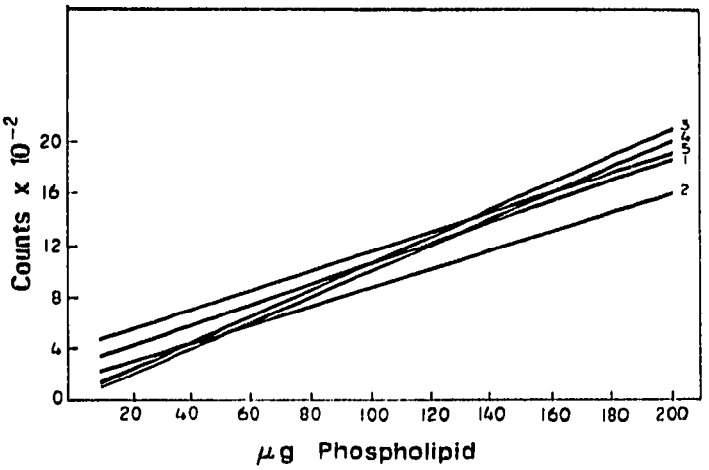

Fig. 3. XES calibration curves. Numbers correspond to Fig. 1.

Figs. 4 and 5 show the separation of phospholipids isolated from rat liver and testis, respectively. No attempt was made to identify any components other than the five phospholipids of interest. Phospholipid compositions determined by XES analyses of these chromatograms are in satisfactory agreement with literature data obtained by TLC and spectrophotometric phosphorus analysis (Tables II and III). The precision on replicate samples is comparable[1,3].

Preparation for XES and the actual analysis required approximately 2 h per chromatogram (5 components). Problems with incomplete recovery from the adsorbent, ashing and color development were eliminated.

A modification of this procedure has been used in this laboratory to monitor Florisil column separations of phospholipids. An aliquot of each eluant fraction is

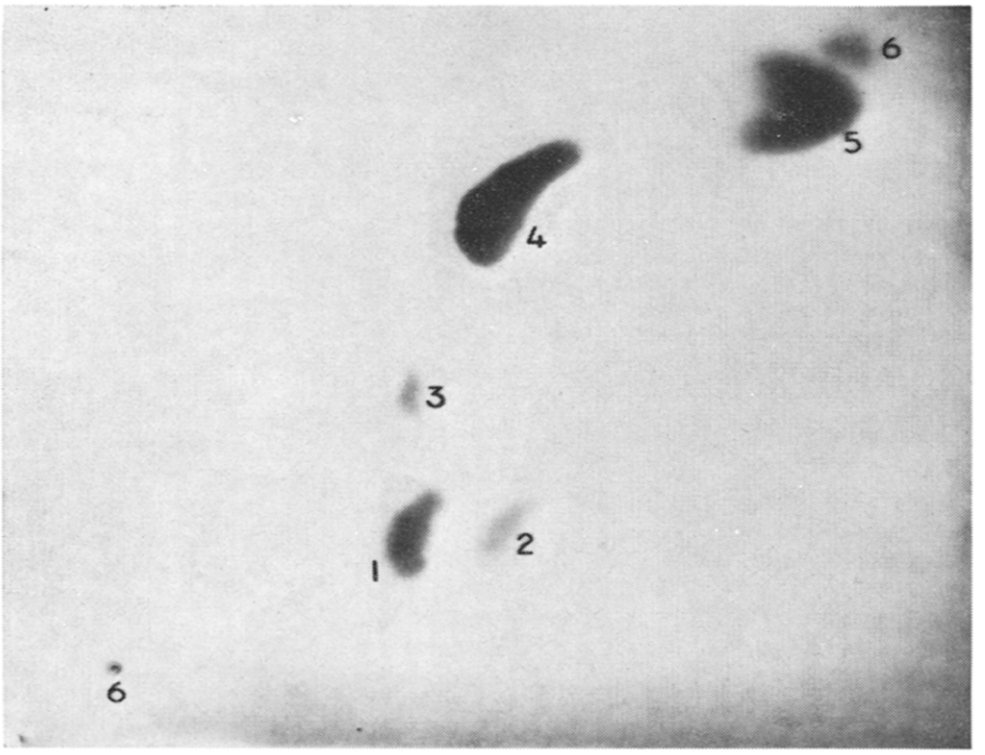

Fig. 4. Separation of 413 μg of rat liver phospholipids. (1) to (5) correspond to Fig. 1; (6) unknown. (XES analysis in Table II.)

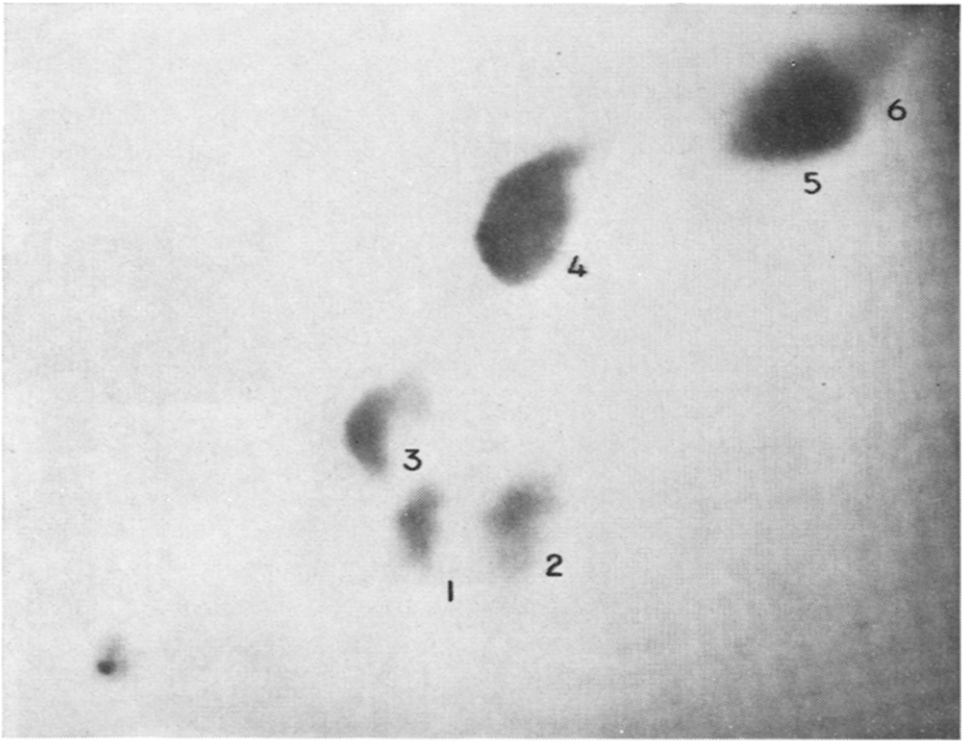

Fig. 5. Separation of 350 μg of rat testis phospholipids. (1) to (5) correspond to Fig. 1; (6) unknown. (XES analysis in Table III.)

spotted on a polyethylene disc and analyzed by XES. The phosphorus content is determined by comparison to a calibration curve prepared in the same manner.

TABLE II

PHOSPHOLIPID COMPOSITION OF RAT LIVER

Phospholipid	*TLC/XES*[a]	*Ref. 1*[b]	*Ref. 3*[b]
Phosphatidyl inositol	10.9 ± 0.9	8.8	6.5
Phosphatidyl serine	3.1 ± 0.3	3.0	4.3
Sphingomyelin	4.0 ± 0.9	1.8	4.1
Phosphatidyl choline	53.3 ± 2.5	55.0	52.9
Phosphatidyl ethanolamine	25.7 ± 2.5	25.3	27.1
Others	4.2[c]	6.0	5.2

[a] Mean of four determinations ± average deviation; expressed as per cent of total phospholipid found.
[b] Expressed as per cent of lipid phosphorus.
[c] Unknown, calculated as phosphatidyl ethanolamine.

TABLE III

PHOSPHOLIPID COMPOSITION OF RAT TESTIS

Phospholipid	*TLC/XES*[a]	*Ref. 15*[b]
Phosphatidyl inositol	7.6 ± 1.4	13.4
Phosphatidyl serine	7.1 ± 0.6	
Sphingomyelin	8.3 ± 1.0	9.6
Phosphatidyl choline	42.3 ± 2.5	44.0
Phosphatidyl ethanolamine	31.0 ± 2.7	28.8
Others	2.6[c]	tr

[a] Mean of four determinations ± average deviation; expressed as per cent of total phospholipid recovered.
[b] Expressed as per cent of lipid phosphorus.
[c] Unknown calculated as phosphatidyl ethanolamine.

ACKNOWLEDGEMENTS

The contributions and assistance of W. B. GEHLING are gratefully acknowledged. The phospholipids from rat liver and testis were isolated by E. J. HOLLENBACH.

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