Quantitative analysis of phospholipids and phospholipid fatty acids from silica gel thin-layer chromatograms

FRANK PARKER and NORMAN F. PETERSON

Division of Dermatology, Department of Medicine, University of Washington School of Medicine, Seattle, Washington

SUMMARY An improved procedure for the quantitative assay of phospholipids separated by TLC is described, in which a specially washed Silica Gel H and a newly designed chromatography unit are employed.

Analysis of phospholipid phosphorus and phospholipid fatty acids was accomplished in the presence of silica gel scraped directly from the chromatoplates. Recovery of phosphorus ranged from 96 to 100%.

The washing of the Silica Gel H with chloroform–methanol–formic acid resulted in extremely low phosphorus blanks on the silica gel, and also removed impurities which were found to cause various peaks on GLC.

KEY WORDS thin-layer chromatography · phospholipids · phosphorus determination · fatty acid composition · saturation chamber · impurities · Silica Gel H · 2′,7′-dichlorofluorescein

QUANTITATIVE ANALYSIS of phospholipids by TLC can be accomplished by the elution of the phospholipids from the silica gel (1, 2) or by direct digestion of the lipids in the presence of the silica gel (3–7). The latter approach has the advantage that it involves fewer steps. In both procedures, the colorimetric measurement of phospholipid phosphorus has resulted in significant optical density readings for blanks (2, 3, 6, 7). It has also been reported that the presence of silica gel inhibits development of the full phosphorus color (5).

In addition to analysis of phospholipid distribution, some attention has been directed to the gas–liquid chromatographic analysis of phospholipid fatty acids following TLC (8). It has been shown that TLC itself does not alter fatty acids (8–10). However, unless care is taken to eliminate impurities from silica gel and from the reagents used for locating phospholipids, these impurities are extracted with the fatty acid methyl esters and will be observed as spurious peaks or as increments in existing peaks on gas–liquid chromatograms.

The present communication describes a rapid and accurate micromethod for the determination of the percentage composition of phospholipids and their component fatty acids. Chromatography is performed on specially washed Silica Gel H in a saturation chamber. The washing of the silica gel has greatly reduced the silica gel blanks in the phosphorus determination and removed impurities which gave rise to various peaks on gas–liquid chromatograms.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

MATERIAL AND METHODS

Materials

Reagents. The adsorbent for TLC was Silica Gel H.[1] The solvents used for TLC and silica gel washing were reagent grade (Baker and Adamson; Allied Chemical Corp., Morristown, N.J.) and all except acetic acid were redistilled before use. Solvents for the preparation of fatty acid methyl esters were checked for gas–liquid chromatographic purity before use. 2′,7′-Dichlorofluorescein ("Chromatospray", Research Specialties Co., Richmond, Calif.), 0.2% in methanol, was diluted with an equal volume of water and washed three times with an equal volume of redistilled hexane, and the aqueous phase was then diluted to 0.04% with redistilled methanol.

[1] Brinkmann Instruments, Inc., Great Neck, L.I., N.Y.

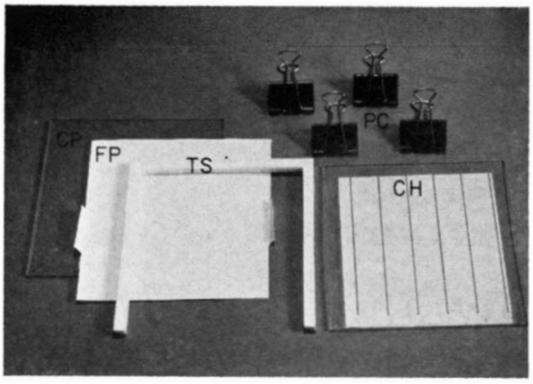

FIG. 1. Components of the saturation chamber. From left to right: cover plate (*CP*), filter paper (*FP*) cut to same size as cover plate, teflon spacer (*TS*), paper clamps (*PC*), and chromatoplate (*CH*) with a margin of adsorbent removed from the top and sides. Boundaries of lanes 3-cm wide are scored on the chromatoplate 1.5 cm from the bottom to the top of the adsorbent. In assembling the chamber, the teflon spacer is placed on the scraped margin of the chromatoplate and the cover plate with filter paper over it is placed against the teflon spacer. Paper clamps hold the unit together.

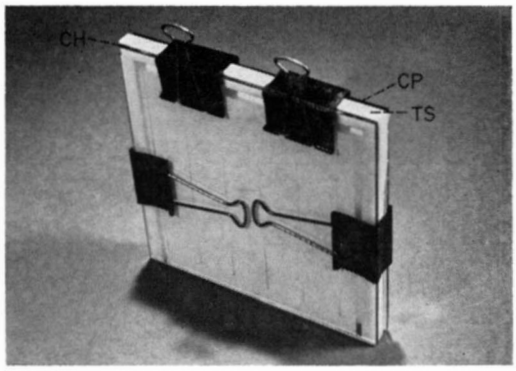

FIG. 2. Saturation chamber in assembled position. The Silica Gel H on chromatoplate (*CH*) faces the filter paper-lined cover plate (*CP*), but is separated from it by the teflon spacer (*TS*).

Standards and Tissue Lipids. All reference phospholipids except phosphatidyl inositol were obtained through the kindness of Dr. D. J. Hanahan, Department of Biochemistry, University of Washington. Wheat sprout phosphatidyl inositol was generously supplied by Dr. M. Fauré, Pasteur Institute, Paris.

Fatty acid methyl ester standards were obtained from the Hormel Foundation, Austin, Minn.

Lipids were extracted from 30 g of liver pooled from two adult, male albino rats with methanol–chloroform 1:2 (v/v) and purified by the method of Folch, Lees, and Sloane Stanley (11). These were to serve as a model mixture of phospholipids.

Methods

Preparation of Silica Gel H. The adsorbent, 250 g of Silica Gel H, was placed in a porcelain Buchner filter funnel (Coors No. 4) fitted with two disks of Whatman No. 2 filter paper. The funnel was placed on a 4 liter filter flask, and 2 liters of methanol–chloroform–formic acid 2:1:1 (v/v/v) was poured slowly over the silica gel, followed by 1 liter of glass-distilled water. Suction was continued until water no longer dripped through the funnel. The washed silica gel was spread in a pyrex tray lined with aluminum foil and dried at 110° for 48 hr. Any large silica gel lumps were broken up after the first 24 hr of drying.

Preparation of Plates. Washed silica gel, 30 g, was placed in a Waring Blendor with 58 ml of glass-distilled water. The resultant slurry was mixed for 5 min at medium speed.

This slurry was sufficient to coat five 20 × 20 cm plates with a 0.2 mm layer of the silica gel using the Desaga apparatus with adjustable applicator.[1] The plates were air-dried for 1 hr and activated for 1 hr at 110° just before use.

Chromatographic Procedures. Phospholipids were applied with a microliter syringe. In experiments to determine recovery of phospholipid phosphorus, known amounts of phospholipid (1.85–7.17 μg phosphorus) were applied as narrow streaks on 3-cm wide lanes, 1.5 cm from the bottom of the plate. Quadruplicate aliquots of the same amount of phospholipids were placed in 20-ml test tubes for later determination of the total phosphorus. When the fatty acids of phospholipids were to be analyzed, 45 μg of phosphorus was streaked on a lane 8 cm wide.

The plates were subjected to ascending chromatography in a "saturation chamber" somewhat similar to that described by Davies (12), or available commercially as the "S-tank."[1] They were prepared by scraping a 1.5 cm margin of adsorbent from the sides and top of the plate to which the lipid was applied. A 1-cm thick, three-sided teflon spacer (20 × 20 × 1.25 cm) was placed on the scraped margin (see Figs. 1 and 2). A 20 × 20 cm glass cover plate faced with a similarly sized piece of Whatman No. 1 filter paper was placed against the teflon spacer with the filter paper between the cover plate and teflon frame. Four paper clamps (Swingline Binder Clip No. 110) were used to hold the saturation chamber securely together.

The saturation chamber was placed in a closed glass developing tank (Brinkmann Instruments, Inc., 27 × 29 × 10 cm) which contained 184 ml of chloroform–methanol–acetic acid–water, 25:15:4:2 (1). This solvent was allowed to rise to within 0.5 cm of the top of the adsorbent. Average running time was 1.5–2 hr.

Detection of Spots. For the analysis of phospholipid phosphorus, the plate was exposed to iodine vapor and the spots were immediately outlined with the point of a needle. For the detection of phosphatidyl ethanolamine and serine in rat liver, ninhydrin spray was used. Other phospholipids of rat liver were identified by simultaneous

chromatography of reference phospholipids, and comparison with previously published data using the same solvent system (1).

For the detection of phospholipids whose fatty acids were to be analyzed by GLC, the plate was placed under ultraviolet light in a darkened room and sprayed with the specially washed 0.04% 2′,7′-dichlorofluorescein (see Materials) until the lipid just became clearly distinguishable. The observed bands were then outlined with a needle.

Analysis of Phospholipid Phosphorus. After the iodine had evaporated from the plate, each outlined spot was scraped directly into a 20 ml pyrex test tube. Adjacent areas of blank silica gel corresponding in size and position to the areas containing phospholipid were also scraped into test tubes. Concentrated sulfuric acid, 0.5 ml, was added with a calibrated syringe to all tubes, including tubes to contain reagent blanks and inorganic phosphorus standards.

The tubes were placed in a heating block (Research Specialties tube heater) and digested for 3 hr at 250°. After 2 hr of digestion, the tubes were gently swirled to break up clumps of silica gel.

Following digestion, the tubes were removed from the heating block and allowed to cool, and 2–3 drops of 30% hydrogen peroxide were added. The tubes were then returned to the heating block and digested for an hour at 160°.

Phospholipid phosphorus was determined by a modification of the method of Bartlett (13). To all the tubes containing silica gel, the reagent blanks, and "total phospholipid" aliquot, 9.1 ml of 0.26% ammonium molybdate solution (made by mixing 6 parts of 0.4% ammonium molybdate with 3.1 parts of distilled water) were added. To the inorganic phosphorus standards, inorganic phosphorus (2.5 μg of P per ml in aqueous solution) plus enough water to give a volume of 3.6 ml (including the 0.5 ml of sulfuric acid) was added, followed by 6 ml of 0.4% ammonium molybdate. All tubes were then mixed on a Vortex mixer, 0.4 ml of 1-amino-2-naphthol-4-sulfonic acid (ANSA) reagent added to each, and mixed again. The tubes were placed in a boiling water bath for 10 min, removed, and mixed again. The tubes containing silica gel were centrifuged at 300 × *g* for 40 min. Colorimetric determinations were performed on a Zeiss spectrophotometer (Model PMQ II) at a wavelength of 820 mμ using glass cells with a 1 cm light path. Samples were transferred to the glass cells with the aid of disposable capillary pipettes and were read against distilled water.

Analysis of Phospholipid Fatty Acids. At the conclusion of each TLC run, the position of each phospholipid was determined by spraying with fluorescein; the area of each phospholipid streak was measured, and then the samples were scraped directly into 20-ml reflux tubes. Adjacent areas of blank silica gel corresponding in size and position to the areas containing phospholipid were also sprayed with fluorescein and scraped into reflux tubes.

Methanolysis was accomplished as described by Ways, Reed, and Hanahan (14) except that the methyl esters were extracted three times with 2 ml of redistilled hexane. The hexane extract was washed twice with 15 ml of water, dried under nitrogen, redissolved in a suitable volume of hexane, and analyzed on a Barber-Colman, Model 5000, GLC apparatus equipped with a hydrogen flame detector. Glass columns, 6 ft × 5 mm i.d., were packed with 19 g of 12% ethylene glycol succinate polyester on 60–80 mesh Chromosorb W (Applied Science Laboratories, State College, Pa.). A column temperature of 190° was maintained with a nitrogen carrier gas flow of 175 ml/min at the outlet. The retention times of methyl stearate and methyl oleate were 7.26 and 8.58 min respectively.

Peaks were identified by comparison with standards and with the aid of logarithmic plots of relative retention time versus chain lengths, or degrees of unsaturation. Peak areas were calculated by multiplication of peak height by width at half-height.

RESULTS

Table 1 shows the recovery of reference phospholipids applied singly to thin-layer plates. Quadruplicate aliquots of each phospholipid were placed directly in phosphorus tubes and onto thin-layer plates. The reference phospholipids were then scraped directly back into test tubes without chromatography. Phosphorus determina-

TABLE 1 RECOVERY OF REFERENCE PHOSPHOLIPIDS FROM THIN-LAYER PLATES (NO CHROMATOGRAPHIC DEVELOPMENT)

Phospholipid Standard	Aliquot Not Spotted	Aliquot Spotted and Scraped	% Recovered
	μg P*	μg P*	
Phosphatidyl ethanolamine	4.25 ± 0.02	4.08 ± 0.05	96 ± 1
Phosphatidyl serine	2.90 ± 0.02	2.90 ± 0.09	100 ± 3
Phosphatidyl inositol	4.07 ± 0.03	3.94 ± 0.06	97 ± 1
Phosphatidyl choline	2.19 ± 0.03	2.10 ± 0.05	96 ± 2
Sphingomyelin	1.85 ± 0.02	1.79 ± 0.02	97 ± 1
Lysophosphatidyl choline	3.34 ± 0.03	3.25 ± 0.02	97 ± 1

Phosphorus determinations were performed on identical aliquots of reference phospholipids placed directly in phosphorus tubes ("aliquot not spotted") and on chromatoplates which were then scraped directly, without chromatography, into tubes for P determination ("aliquot spotted and scraped").

* Means and standard deviations of four determinations.

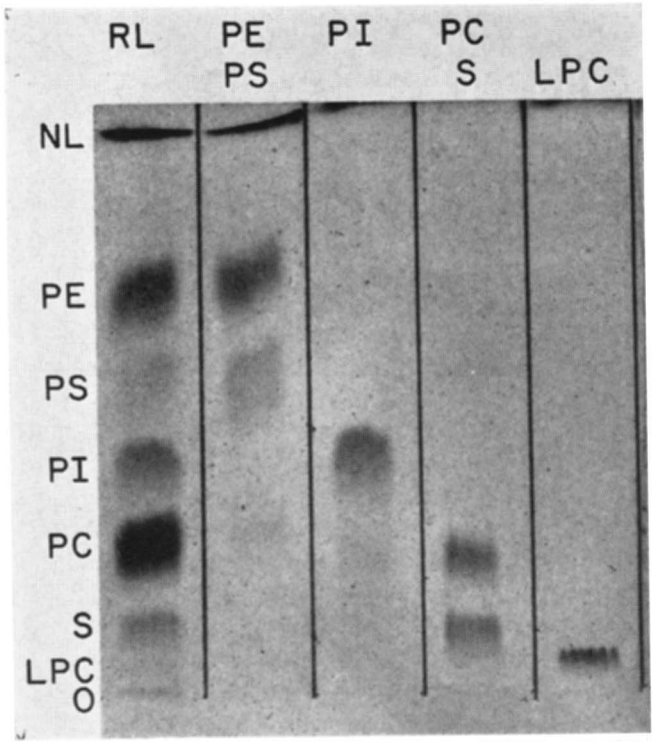

FIG. 3. Chromatographic separation of rat liver phospholipids (*RL*) (far left lane) and reference phospholipids. Neutral lipid at the solvent front (*NL*); phosphatidyl ethanolamine (*PE*); phosphatidyl serine (*PS*); phosphatidyl inositol (*PI*); phosphatidyl choline (*PC*); sphingomyelin (*S*); lysophosphatidyl choline (*LPC*); origin (*O*). A total of 7.17 μg of rat liver phospholipid phosphorus was applied as a thin streak. Detection method for this picture was with concentrated sulfuric acid spray.

tions were performed, and 96–100% recovery was obtained for each phospholipid studied.

Figure 3 shows the separation of rat liver and reference phospholipids after ascending chromatography in the saturation chamber. This separation is similar to that reported by Skipski, Peterson, and Barclay (1).

Table 2 shows the results of nine analyses of rat liver phospholipids. The percentage of each phospholipid is similar to that reported by Skipski et al. (1). Table 2 also shows the areas and optical densities of equivalent silica gel blanks scraped from adjacent lanes of the same plates. Each squared centimeter of blank Silica Gel H (plates 0.2 mm thick) weighed 7 mg and gave an optical density reading corresponding to 0.004 μg of phosphorus. One microgram of phosphorus has an optical density of 0.090. Equivalent amounts of unwashed Silica Gel H gave blank readings which were five times greater than for the washed gel.

The effects of TLC and fluorescein spraying on the fatty acid patterns of three reference phospholipids are shown in Table 3. Neither of these procedures altered the patterns of fatty acids compared with those of the same phospholipids which were methylated directly.

GLC of the fluorescein-sprayed blank areas of adsorbent, of size comparable to those of the reference phospholipids, revealed only a minute peak with the same retention time as methyl palmitate. One microgram of methyl palmitate gave a peak area of 515 mm^2 while the impurity in the blank gave an area of 5 mm^2 at the same attenuation setting.

The recovery of phospholipid fatty acids on GLC following TLC was not precisely determined, but identical amounts of the phospholipid standards methanolyzed directly and after TLC gave almost identical GLC peak areas.

DISCUSSION

The analysis of phospholipid distribution was accurately accomplished in the presence of silica gel. In early phases of our studies, it appeared that phospholipid was more difficult to digest when it was adsorbed to the silica gel. Procedures for the digestion of phospholipids commonly utilize either perchloric or sulfuric acid. Sulfuric acid was chosen, because it does not evaporate at the high temperature (250°) employed, and when it was used in conjunction with hydrogen peroxide complete oxidation occurred. Since 0.5 ml of sulfuric acid was required for

TABLE 2 RECOVERY OF RAT LIVER PHOSPHOLIPIDS EXPRESSED AS PERCENTAGES OF TOTAL PHOSPHORUS

Phospholipids	Average Recovery of Phosphorus*	Adjacent Equivalent Silica Gel Blanks	
		Area Scraped	Optical Densities†
	%	cm^2	
Solvent front (everything above P.E.)	5.1 ± 0.14	12.0	0.007 (0.004–0.009)
Phosphatidyl ethanolamine	22.8 ± 0.44	7.5	0.003 (0.002–0.004)
Phosphatidyl serine	3.7 ± 0.13	6.0	0.002 (0.001–0.003)
Phosphatidyl inositol	8.0 ± 0.60	6.0	0.003 (0.003–0.004)
Phosphatidyl choline	49.9 ± 1.2	7.5	0.004 (0.003–0.004)
Sphingomyelin	4.1 ± 0.22	4.2	0.001 (0.000–0.002)
Lysophosphatidyl choline	1.0 ± 0.15	1.5	0.001 (0.000–0.002)
Origin (everything below lysolecithin)	1.5 ± 0.22	1.5	0.000
Total recovery	96.1 ± 2.02		

A total of 7.17 μg of rat liver phospholipid phosphorus was applied in a volume of 30 μl to each of three lanes on three different chromatoplates.

* Mean and standard deviations of three different chromatographic runs, each done in triplicate.

† Mean and range of optical density reading of five adjacent blank areas, each corresponding in size and position to the areas containing phospholipid.

TABLE 3 EFFECTS OF TLC AND FLUORESCEIN SPRAY ON PHOSPHOLIPID FATTY ACID PATTERNS

Fatty Acid	Phosphatidyl Choline				Sphingomyelin				Lysophosphatidyl Choline			
			C				C				C	
	A	B	1	2	A	B	1	2	A	B	1	2
16:0	28.3 ± 0.5	28.6 ± 0.7	29.4	28.3	17.5 ± 1	16.7 ± 0.7	16.0	17.5	62.3 ± 0.5	62.8 ± 0.4	62.0	61.2
16:1					tr.	tr.	tr.	tr.				
17:0					0.8 ± 0.08	0.8 ± 0.07	0.8	0.8				
18:0	14.2 ± 0.3	13.7 ± 0.2	14.9	14.8	10.2 ± 0.4	10.2 ± 0.4	10.7	10.4	34.1 ± 0.4	33.7 ± 0.5	34.5	34.9
18:1	17.1 ± 0.2	16.8 ± 0.3	16.9	16.4	tr.	tr.	tr.	tr.	3.6 ± 0.1	3.5 ± 0.1	3.5	3.9
18:2	34.9 ± 0.9	34.2 ± 0.2	33.3	33.7								
19:0					0.8 ± 0.2	0.9 ± 0.2	0.7	0.6				
20:0					12.9 ± 0.9	12.3 ± 0.3	12.6	12.1				
20:4	6.0 ± 0.3	5.7 ± 0.1	5.5	6.7								
21:0					1.5 ± 0.4	1.4 ± 0.7	1.1	1.4				
22:0					15.5 ± 0.2	15.7 ± 0.1	16.2	15.7				
23:0					3.1 ± 0.9	3.5 ± 0.8	3.1	2.8				
24:0					13.2 ± 0.5	13.2 ± 0.1	12.7	12.6				
24:1					20.6 ± 0.7	21.1 ± 0.1	20.8	20.8				
24:2 or 25:0					5.1 ± 0.5	3.9 ± 0.3	5.0	4.5				

The methyl esters of three phospholipid standards, expressed as peak area per cent, are shown before TLC (A), and after chromatography when applied singly (B), or as a mixture of the three standards (C). Thin-layer chromatography with a mixture of the three standards was done in duplicate and these results are given in columns 1 and 2.

complete wetting of the largest silica gel spots, a final reagent volume of 10 ml was necessary in order to keep within the normality range for maximum color development of the Bartlett method (13).

The very low phosphorus optical density readings from blank areas of silica gel were a consequence of the washing procedure, during which considerable amounts of orange-colored material were removed and a five-fold decrease in the phosphorus optical density reading was obtained. The present method reports silica gel phosphorus blanks eight (3) to twenty (2, 6) times lower than those given in the literature. This washing procedure also greatly reduced the number and magnitude of GLC peaks representing contaminants which were otherwise extracted with fatty acid methyl esters.

Because the iodination of double bonds results in the partial loss of polyunsaturated fatty acids (10), phospholipids whose fatty acid composition is to be determined cannot be located by means of iodine vapor. For this reason 2′,7′-dichlorofluorescein was employed. However, impurities in the fluorescein were observed as peaks on gas–liquid chromatograms. These were reduced to inconsequential proportions by hexane washing of the fluorescein, and spraying the developed chromatoplates with a minimum amount of dilute fluorescein in a darkened room under ultraviolet light.

The separations reported in this communication using washed Silica Gel H and a saturation chamber are comparable to those reported by Skipski et al. (1). With a multicomponent solvent, such as used in this system, reproducible separations appear to be dependent upon complete saturation of the atmosphere in the chromatographic tank (15). This condition is conveniently obtained when a saturation chamber of small volume is used. The application of lipid samples to the chromatoplates in a narrow streak rather than a spot also promoted better separation.

It may be necessary to alter the solvent system when attempting to obtain optimal separation of phospholipids from other tissue sources. To date, excellent separations of rabbit skin phospholipids and human red cell membrane phospholipids have also been accomplished with only minor alterations in the amount of chloroform used in the solvent system.

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