

A rapid, isocratic method for phospholipid separation by high-performance liquid chromatography

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Summary A rapid, isocratic method for separating the most prevalent phospholipids by high-performance liquid chromatography is described. Baseline resolution of phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin is achieved in less than 40 min on a silica column. Lipids are injected in 10 μ l of chloroform–diethyl ether 1:2 (v/v) and eluted with a solvent mixture of acetonitrile–methanol–sulfuric acid 100:3:0.05 (v/v/v) at a flow rate of 1 ml/min. Neutral lipids and cardiolipin elute with the solvent front. Chromatography of a radioactive cell lipid extract indicates a recovery of better than 97%. The procedure is sensitive enough to permit the analysis of the main phospholipids present in a monolayer culture containing about 100 μ g of cell protein.—**Kaduce, T. L., K. C. Norton, and A. A. Spector.** A rapid, isocratic method for phospholipid separation by high performance liquid chromatography. *J. Lipid Res.* 1983. **24**: 1398–1403.

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A number of methods for separation of the phospholipids contained in biologic samples by high-performance liquid chromatography (HPLC) are now available (1–9). These procedures employ solvent mixtures that have minimal absorption in the range of 200 nm, enabling detection of the phospholipids spectrophotometrically without derivatization. One solvent mixture that is used often contains acetonitrile, methanol, and water as described by Jungalwala et al. (1–3); the other contains a hexane, methanol, and water gradient as described by Geurts Van Kessel et al. (5). Although the separation that can be obtained by these HPLC methods are superior to those possible by thin-layer chromatography (TLC), they are not widely used in metabolic studies because they are too complex and time-consuming for experiments involving a large number of samples.

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; SPH, sphingomyelin; CL, cardiolipin.

Several modified HPLC methods for phospholipid separation have been introduced recently in an attempt to simplify the procedure. For example, Patton, Fasulo, and Robins (9) have separated all of the rat liver phospholipids in a single run, but this requires 125 min. Chen and Kou (10) also have achieved phospholipid separation in a single elution, but phosphatidylinositol (PI) is not well separated from the neutral lipid that elutes with the solvent front. Moreover, the solvent mixture used by Chen and Kou (10) contains phosphoric acid, increasing the potential for error in the subsequent quantitation of the isolated phospholipids by lipid phosphorus measurements. Yandrasitz, Berry, and Segal (11) have added sulfuric acid to the hexane, methanol, and water system as an ion suppressant. While this allows baseline separation of the most prevalent phospholipids in a single elution, the time required is fairly long because a water gradient is employed and the column must be recycled. Finally, Gross and Sobel (12) have developed a rapid, isocratic method that provides good separation of lysophosphoglycerides from the corresponding phosphoglycerides. This method, however, does not completely separate phosphatidylethanolamine (PE) from phosphatidylserine (PS).

In this communication we describe an isocratic HPLC procedure that completely separates the main phospholipids usually present in animal tissues. The method is rapid, offers several advantages with respect to ease of analysis of the eluted phospholipids, and is sensitive enough to be applicable to experiments with monolayer cell cultures.

METHODS

MATERIALS

HPLC grade glass-distilled acetonitrile, chloroform, and methanol were obtained from MCB Manufacturing Chemists Inc. (Cincinnati, OH). Analytical ACS grade sulfuric acid was purchased from Fisher Scientific Co. (Fair Lawn, NJ), and 12% BF_3 in methanol (w/v) was obtained from Supelco, Inc. (Bellefonte, PA). Phosphatidylcholine (PC), PE, and 1-oleoyl-L- α -lysocleithin (LPC) were obtained from P. L. Biochemicals Inc. (Milwaukee, WI), while PI, PS, and sphingomyelin (SPH) were purchased from Avanti Biochemicals Inc. (Birmingham, AL). [$1\text{-}^{14}\text{C}$]Oleic acid and [3H]arachidonic acid were obtained from New England Nuclear Inc. (Boston, MA) and [$1\text{-}^{14}\text{C}$]dipalmitoyl phosphatidylcholine was purchased from Applied Science Inc. (State College, PA).

HPLC procedures

A Beckman Model 332 liquid chromatographic system was employed, consisting of a Model 110A pump,

and a Model 420 system controller. Lipids were detected using a Beckman 155 variable wavelength detector fitted with a 20- μ l, 1-cm optical path cell. For phospholipid chromatography, the wavelength of the detector was set at 202 nm. Phospholipid separation was achieved with a Beckman 4.6 \times 250 mm column packed with 5 μ m Ultrasphere-Si. A guard column of 4.6 \times 45 mm packed with silica gel was used in conjunction with the analytical column. For routine phospholipid separations, the lipid extract was applied to the column in 10 μ l of chloroform–diethyl ether 1:2 (v/v) at a concentration of about 1 mg/ml, and the solvent system consisted of acetonitrile–methanol–sulfuric acid 100:3:0.05 (v/v/v). The solvent mixture was delivered by the pump at a flow rate of 1 ml/min, which produced a pump pressure of about 1500 PSI. When in continuous use over a period of several days, the column is washed each night with a solvent mixture of acetonitrile–methanol–sulfuric acid 100:3:0.1 (v/v/v) at a flow rate of 0.1 ml/min. Once every 2 months, the entire system is purged with 300 ml of methanol.

Tissue lipids

Bovine aortic endothelial cells, passage 14, were incubated with [1-¹⁴C]oleic acid for 120 min (13). After washing, the cellular lipids were extracted with chloroform–methanol 2:1 (v/v) (14). Phospholipid classes were then separated by either HPLC or TLC on silica gel H plates (Analtech, Inc., Newark, DE) impregnated with 1% boric acid. The TLC solvent system contained chloroform–methanol–ammonium hydroxide–water 120:75:2.6 (v/v) (15). Additional radioisotope incorporation experiments were done with [³H]arachidonic acid to check the adequacy of the HPLC separation. In these experiments an aliquot of each fraction collected from the HPLC column was subjected to TLC in order to determine its radiopurity. Radioactivity was measured using 4 ml of Budget Solve (Research Products Int., Mount Prospect, IL) in a Packard 2420 liquid scintillation spectrometer. A ²²⁶Ra external standard was used in order to monitor and correct for quenching. To measure phospholipid content, the lipid fractions were dried under N₂, digested with perchloric acid for 20 min at 270°C, and assayed for phosphorus (16).

Transesterification and gas–liquid chromatography

The lipids contained in the fractions separated by HPLC were transesterified to form fatty acid methyl esters directly in the eluting solvent mixture. To each fraction 2 ml of 12% BF₃ in methanol was added (17) and the mixture was incubated at 100°C for 2 hr. After the fatty acid methyl esters were removed by extraction with heptane, analysis by gas–liquid chromatography (GLC) was performed with a Hewlett-Packard Model

5700 chromatograph equipped with a 2 mm \times 1.9 m glass column packed with 10% SP2330 on 100/200 mesh Chromosorb W-AW (Supelco, Inc.) (13).

RESULTS

Effects of mobile phase composition

Preliminary studies demonstrated that the main phospholipid classes can be separated on an Ultrasphere-Si column with a mobile phase containing acetonitrile, methanol, and sulfuric acid. Optimum separation was obtained with a 100:3:0.05 (v/v/v) mixture. Even small changes from this composition were found to have a marked effect on the phospholipid retention times and, therefore, the resulting separation. For example, PI eluted with the neutral lipids when the mixture was changed to 100:5:0.1. The retention times of all of the phospholipids increased when the methanol content was reduced, with PI being affected most. When the solvent ratio was 100:1:0.1, PI eluted together with PE.

Changes in the sulfuric acid content of the mobile phase produced little effect on the retention time of PI. As the sulfuric acid content was reduced, however, the retention times of PC, PE, and PS increased, and broadening of all of the absorbance peaks occurred. When sulfuric acid was omitted, PC, PE, and PS did not elute from the column.

Phospholipid separations

Fig. 1 shows the separation of a mixture of PC, PE, PI, PS, LPC, SPH, and cardiolipin (CL) with the most effective mixture of acetonitrile–methanol–sulfuric acid 100:3:0.05 delivered by the pump at a flow rate of 1 ml/min. The first large absorbance peak is due to the presence of chloroform and diethyl ether which elute in the solvent front and absorb at 202 nm. Under these conditions, triacylglycerol, cholesterol, cholesteryl ester, and fatty acid standards eluted with the solvent front. CL also eluted in the solvent front and was not separated from the neutral lipids. Baseline separation was obtained for the other phospholipid classes, the order of elution being PI, PS, PE, PC, LPC, and SPH. A small, unidentified absorbance peak appeared between PE and PC. All components eluted in 40 min.

A separation of the phospholipids extracted from cultures of bovine aortic endothelial cells is shown in **Fig. 2**. The lipids were extracted from a confluent monolayer culture containing 300 μ g of cell protein dissolved in chloroform–diethyl ether 1:2, and applied to the HPLC column. Peaks corresponding to PI, PS, PE, PC, LPC, and SPH were detected by absorbance at 202 nm. A small unidentified absorbance peak was noted between PI and PS, and two small unidentified absorbance

peaks occurred between PE and PC. Smaller amounts of lipid were chromatographed in routine analyses of the endothelial cells, corresponding to 90 to 100 μg of cell protein. Under these conditions, neither LPC nor SPH appeared on the chromatograms. The PC fraction in Fig. 2 appears to include two absorbance peaks. Only a single PC peak was observed, however, in the routine assays where smaller amounts of lipid extract were chromatographed. Furthermore, experiments with radioactive endothelial cell lipid extracts indicated that more than 95% of the radioactivity contained in the entire PC fraction migrated with a PC standard on TLC.

Radioactivity was incorporated into the lipids of bovine aortic endothelial cultures by incubation of the cells for 120 min with $[1-^{14}\text{C}]$ oleic acid. The lipids were extracted from the cells and chromatographed either by this HPLC procedure or by TLC (15). Of the radioactivity applied to the Ultrasphere-Si column, 97.4 ± 2.5 (mean \pm SE, $n = 3$) was recovered in the eluent collected over 40 min. **Table 1** shows the distribution of radioactivity in the various fractions eluted from the HPLC column and, for comparison, the distribution of radioactivity in the fractions separated by TLC. Although there were small differences in several of the fractions, the percentage distributions obtained by the HPLC separation were, in general, quite similar to those obtained by TLC separation.

Additional radioisotope incorporation experiments were done to check the purity of the fractions separated

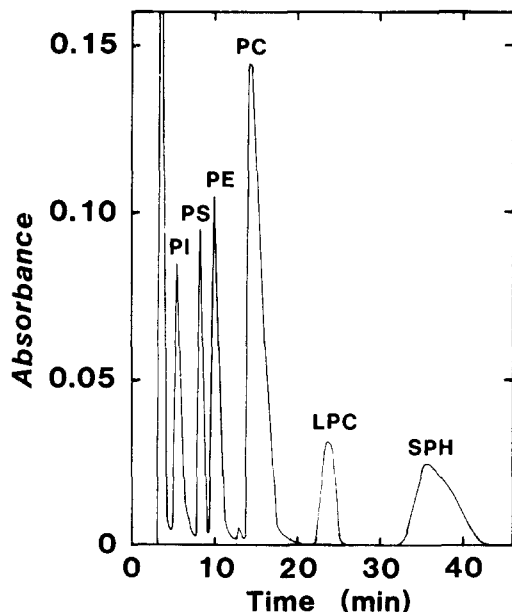


Fig. 1. Separation of a phospholipid standard mixture. The mobile phase consisted of acetonitrile-methanol-sulfuric acid 100:3:0.05 (v/v/v) at a flow rate of 1.0 ml/min. Absorbance was measured at 202 nm

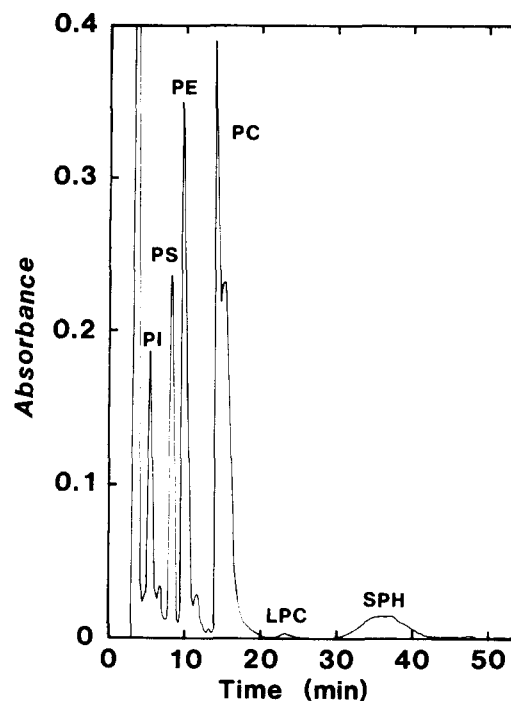


Fig. 2. Separation of bovine aortic endothelial cell lipids. The material chromatographed was extracted from a culture containing 300 μg of cell protein. The conditions were the same as indicated in Fig. 1.

by HPLC. The endothelial cultures were incubated for 16 hr with $[^3\text{H}]$ arachidonic acid. The distribution of radioactivity as determined by HPLC was 37% as PC, 29% PE, 21% PI, 2.2% PS, 7.2% as neutral lipids, and 3.5% in the remaining fractions. Each of the eluted fractions then was subjected to TLC with the corresponding standard added, and the percentage of radioactivity that co-chromatographed with the standard was determined. The recoveries were: PC, $96 \pm 1\%$; PE, $84 \pm 1\%$; PI, $89 \pm 1\%$; PS, $89 \pm 8\%$; and neutral lipids, $93 \pm 2\%$ (mean \pm SE, $n = 3$).

TABLE 1. Comparison of the distribution of lipid radioactivity following separations by HPLC and TLC

Lipid Fraction	Radioactivity	
	HPLC	TLC
	%	
PC	64.0 ± 0.7	60.5 ± 0.6
PE	7.8 ± 0.2	10.9 ± 0.7
PS	3.2 ± 0.2	6.3 ± 0.6
PI	1.7 ± 0.1	1.4 ± 0.2
Neutral lipids	18.9 ± 0.6	17.6 ± 0.5
Others	4.4 ± 0.5	2.9 ± 0.2

Monolayer cultures of confluent bovine aortic endothelial cells were incubated with $[1-^{14}\text{C}]$ oleic acid for 120 min. Each value is the mean \pm SE of the data obtained from three separate cultures.

Transesterification for GLC

GLC analysis of the lipids contained in the solvent mixture of acetonitrile-methanol-sulfuric acid 100:3:0.05 revealed that transesterification can be achieved by directly adding BF_3 -methanol (17), without first drying the sample. An initial experiment was done with [$1\text{-}^{14}\text{C}$]dipalmitoyl phosphatidylcholine dissolved in 5 ml of this solvent mixture in order to determine the time course of transesterification. After 2 ml of 12% BF_3 in methanol was added, the mixture was incubated at 100°C for various times and the percentage conversion of radioactivity into fatty acid methyl esters was determined. There was a 55% conversion in 30 min, 75% in 45 min, 88% in 1 hr, and 94% in 2 hr or 3 hr. Additional studies indicated that there was no appreciable difference in either the percentage or rate of conversion to fatty acid methyl esters when the amount of BF_3 in methanol added to 5 ml of the HPLC elution mixture was between 1 and 3 ml.

Table 2 shows the recovery of the various fatty acids contained in soybean PC following separation by HPLC and methylation in the eluting solvent mixture. The samples separated by HPLC were methylated by adding 2 ml of 12% BF_3 in methanol to 5 ml of the eluting solvent mixture and incubating at 100°C for 2 hr. The fatty acid composition of the PC fraction separated by HPLC was almost identical to that of corresponding aliquots of the soybean PC preparation that were not subjected to HPLC. Further, there was no appreciable change in the fatty acid composition when the PC fraction was stored for 3 days at 4°C in the eluting solvent mixture before it was methylated. Additional studies revealed that a considerable loss of polyunsaturated fatty acids occurred if the eluting solvent was evaporated before the BF_3 -methanol was added. This loss could be prevented if the mixture was neutralized with methanolic NaOH prior to drying (data not shown).

Lipid phosphorus assay

Aliquots of soybean PC containing 16 nmol of lipid phosphorus were injected into the HPLC column and the PC fraction was collected. After the eluting solvent mixture was evaporated under N_2 , the lipid residue was digested with perchloric acid. The recovery of lipid phosphorus was 15.5 ± 0.5 nmol (mean \pm SE, $n = 4$), accounting for 97% of the material injected into the column.

DISCUSSION

This HPLC procedure offers many advantages for the routine separation of the main phospholipids usually contained in animal tissues. Several of these are due to

TABLE 2. Effect of HPLC separation on the fatty acid composition of soybean PC

Fatty Acid	Composition ^a		
	Soybean PC	PC Fraction Collected following HPLC	
		Methylated Immediately in HPLC Eluting Solvent Mixture	Methylated after Storage for 3 Days in HPLC Solvent Mixture
		%	
16:0	12.1 \pm 0.5	12.5 \pm 0.2	13.3 \pm 0.1
18:0	4.0 \pm 0.1	4.1 \pm 0.1	4.6 \pm 0.1
18:1	8.4 \pm 0.1	8.5 \pm 0.1	8.8 \pm 0.1
18:2	67.6 \pm 0.7	66.4 \pm 0.2	65.2 \pm 0.2
18:3	7.8 \pm 0.3	8.0 \pm 0.1	7.3 \pm 0.3
Others	0.1 \pm 0.1	0.5 \pm 0.2	0.8 \pm 0.1

^a Mean \pm SE of three determinations.

the fact that the elution is done under isocratic conditions. Since only one pump is required, the HPLC equipment is less expensive than that needed for gradient separations. In addition, processing of multiple samples is speeded up because the column does not have to be recycled. The isocratic elution also avoids excessive baseline absorbance changes, simplifying interpretation of the chromatogram. These advantages also apply to the isocratic separations for phospholipids developed by Gross and Sobel (12) and Chen and Kou (10). However, the former method does not resolve PS and PE (12), and the latter procedure does not provide a good separation between PI and the neutral lipids that elute in the solvent front. Furthermore, the fact that phosphoric acid, which is contained in the mobile phase of Chen and Kou (10), is not used eliminates a potential source of error in subsequent phospholipid assays. Finally, the very small water content of the solvent system allows preparation of fatty acid methyl esters by transesterification without first drying the eluted phospholipids.


While the present method resolves CL from the other phospholipids, it does not separate CL from any neutral lipids contained in the sample. This should not represent a serious difficulty, however, for CL usually is not a major phospholipid component in biological specimens. Another potential problem may be encountered if the sample contains disaturated phosphoglycerides. Silica columns often separate the disaturated components of a phospholipid class from molecular species containing unsaturated acyl groups (5, 12). Since detection is by ultraviolet absorbance, disaturated phosphoglycerides would not appear on the chromatogram if they eluted separately. Although this must be taken into account in studies with surfactant or liposomes, it is not an important consideration in most metabolic ap-

plications because the phospholipid classes usually contain few disaturated species. For example, Gross and Sobel (12) have shown that at least 95% of the phospholipids contained in rabbit myocardium elute from a silica column in ultraviolet absorbing peaks. In addition, Mahadevappa and Holub (18) have shown that only 1.6% of the total diacyl phospholipid species in human platelets is disaturated.

Several other possible sources of error should be recognized by those contemplating use of this procedure. Since the HPLC system utilizes an ultraviolet absorbance detector, the peak areas obtained from the chromatogram tracing are not a measure of the weight or molar amounts of lipid. To obtain quantitative data, some type of chemical assay of the eluted fractions is required. Careful attention must also be given to the composition of the mobile phase because even small changes in the methanol or sulfuric acid content can have an important effect on the phospholipid separation. Because of this, we prepare the mobile phase freshly each day. Furthermore, lipid samples are injected in a chloroform-diethyl ether mixture in order to avoid introducing an excess of methanol into the system. Finally, the method is not suitable for the recovery of acid-labile phospholipids such as plasmalogens because the mobile phase contains sulfuric acid. Experiments with the plasmalogen form of PE indicate that most of it is converted to the lyso derivative during chromatography and that lyso PE elutes with PC in this system.

Sphingomyelin (SPH) was detected in the standard mixture and when large amounts of endothelial cell lipids were separated. In routine studies where smaller amounts of endothelial cell lipids were analyzed, however, SPH was not detected. Gross and Sobel (12) also did not detect SPH by ultraviolet absorbance in chromatograms of rabbit myocardial lipids. One possibility is that the amount of SPH present in these tissues is too small to detect unless large amounts of lipid are chromatographed because the fatty amide group contains relatively little unsaturation. Alternatively, SPH may be labile in the acidic mobile phase that was employed, so that some SPH remains only when large amounts are present initially. Since SPH recovery was not measured, the possibility that this HPLC procedure is not suitable for use with SPH cannot be excluded.

Even though the present HPLC procedure is rapid, it is still much more time-consuming than a one-dimensional TLC separation in which a number of samples can be analyzed in a single chromatogram. There are, however, several important advantages of this HPLC method over TLC. One is the separation of PI and PS. While PI and PS can be resolved by one-dimensional TLC (19), this often causes a loss of resolution of other

phospholipids. Good separation of all phospholipids is obtained by two-dimensional TLC (20), but only one sample can be run on each chromatogram, only a small amount of lipid can be separated, and positive identification is sometimes difficult in those cases where standards cannot be added to the same chromatogram. Another problem with phospholipid separation by TLC is the possible loss of polyunsaturated fatty acids as a result of staining (21). Since HPLC avoids such problems, a procedure such as the one that we have developed appears to be a preferable approach for some metabolic applications. 

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