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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS WITH UV DETECTION: OPTIMIZATION OF SEPARATIONS ON SILICA

JOHN R. YANDRASITZ*, GERARD BERRY and STANTON SEGAL

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, 34th & Civic Center Boulevard, Philadelphia, PA 19104, and the Departments of Pediatrics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA (U.S.A.)*

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SUMMARY

Chromatography of phospholipids was performed on silica columns with detection by absorbance at 205 nm using mixtures of hexane—isopropanol—water in which the role of water and isopropanol in elution was investigated. One system was developed which provided adequate separation of most major phospholipid species. However, lipids with several ionizable groups were not well separated and gave multiple broad peaks. A second system was developed utilizing sulfuric acid for ion suppression. The behavior of phospholipids in this system was found to be dependent on the presence of quaternary ammonium, amino, or hydroxyl groups. Except for plasmalogen, phospholipids were recovered intact. This system was optimized to provide baseline resolution of essentially all phospholipid species commonly found in mammalian tissues.

INTRODUCTION

Development of systems for high-performance liquid chromatography (HPLC) of phospholipids has been rather slow considering the wealth of data available on thin-layer chromatography (TLC) of these compounds. However, almost all TLC solvent systems for phospholipids are incompatible with the most commonly used detection system of ultraviolet (UV) absorbance because of the presence of chloroform or acetic acid. Therefore, direct adaptation of TLC systems to HPLC of phospholipids is somewhat difficult. Chloroform based solvent systems have been used with a moving wire detector for chromatography of phospholipids [1, 2]. Phospholipids from actively metabolizing cells have been analyzed in a chloroform-based system by counting incorporated ^{32}P in the eluent [3]. Photometric detection of amino-con-

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taining phospholipids has been accomplished after derivatization of these groups [4].

Phospholipids do not have a specific absorbance, but may be detected by monitoring the absorbance of double bonds in their fatty acid moieties. Two systems have been developed using solvents that are transparent near 200 nm. Jungawala et al. [5] used an acetonitrile—methanol—water system on a silica column to achieve a rapid and complete separation of phosphatidylcholine and sphingomyelin. However, other phospholipids were not resolved. Several classes of phospholipids were separated on silica by Hax and Geurts van Kessel [6] using gradient elution with hexane—isopropanol—water.

This last system, combining non-destructive UV detection with the separation of several lipid classes appeared to be the most promising for our needs. We therefore sought to optimize this system and expand its usefulness to minor species of phospholipids. We report here our investigations on the effects of solvent composition and especially pH on phospholipid separation and describe a system which appears capable of giving baseline resolution of most phospholipid species.

EXPERIMENTAL

The chromatograph used was a Varian Model 5020 equipped with three solvent inlet valves, a temperature-controlled column-mounting block, and an air-actuated Valco loop injector using the 50-μl loop. A variable-wavelength (Vari-Chrom) detector was operated at 205 nm with an 8-nm bandpass. Columns were 30 cm × 4 mm I.D. packed with 5 μm silica. Packed columns were obtained from Varian (Palo Alto, CA, U.S.A.) (Micro-Pak Si-5) or from Alltech (Arlington Heights, IL, U.S.A.) (Spherisorb S-5W). A 4 cm × 4 mm I.D. guard column packed with Vydac adsorbent was used throughout. All separations were performed at 28°C.

Solvents were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Sulfuric acid (Ultrex grade) was from J.T. Baker (Phillipsburg, NJ, U.S.A.). Cholesterol (Chol), phosphatidic acid (PhA), phosphatidylglycerol (PhG), diphosphatidylglycerol (DPhG) and polyphosphoinositides (DPI, TPI) were from Sigma (St. Louis, MO, U.S.A.). Bovine liver phosphatidylinositol (PhI) and plasmalogen were from Applied Science Labs. (State College, PA, U.S.A.). Other lipids phosphatidylserine (PhS), phosphatidylethanolamine (PhE), lysophosphatidylethanolamine (ly-PhE), phosphatidylcholine (PhC), sphingomyelin (SM), and cerebrosides (Cer.) were from PL Biochemicals (Milwaukee, WI, U.S.A.).

Tissue lipids were extracted by homogenizing approximately 1 g fresh blotted tissue with 3 ml methanol followed by the addition of 3 ml of chloroform. After centrifugation the pellet was re-extracted with 4 ml chloroform—methanol—hydrochloric acid (100:100:1). The combined extracts were adjusted to a chloroform—methanol ratio of 2:1 and partitioned with 0.2 volumes of 0.1 *N* hydrochloric acid. The lower phase was collected and the upper phase re-extracted with chloroform—methanol (85:15). The combined lower phases were run over a silica Sep-Pak (Waters Assoc., Milford, MA, U.S.A.) to remove non-lipid contaminants. This was washed with 5 ml methanol. Lipid extracts

were evaporated with dry nitrogen and re-dissolved in methylene chloride—methanol (2:1). Myelin from rat cerebra was collected from a five-step Ficoll gradient [7].

Solvent mixtures for HPLC were prepared by stirring in a closed container for several minutes with a PTFE stirring bar, and then degassed by application of a vacuum. Corrected retention time $t' = t_x - t_0$, where t_0 is the elution time for an unretained compound. Capacity factor $k' = t'/t_0$. Resolution $R(A/B) = t'_A - t'_B / \frac{1}{2}(w_A + w_B)$ where w is the peak width at baseline.

Lipids were identified by use of standards and by collection of peaks followed by TLC. Peaks were collected in 0.1—0.2 ml pyridine, evaporated with nitrogen, and partitioned with 2 ml methylene chloride—methanol (2:1) and 0.4 ml 50 m*M* hydrochloric acid. TLC was performed on 0.25-mm silica gel HL plates from Analtech (Newark, DE, U.S.A.) with chloroform—methanol—ammonia (65:25:5) or butanol—acetic acid—water (6:1:1). Phospholipids were detected with the spray reagent described by Vaskovsky et al. [8].

RESULTS AND DISCUSSION

In our initial work we experimented with the system described by Hax and Geurts van Kessel [6]. We found, as described by these authors, that water was the important solvent component in eluting phospholipids. Attempts to achieve elution by increasing isopropanol and decreasing water failed. We also found that decreasing the gradient rate did not improve resolution because peak broadening surpassed the increase in retention times. In order to sharpen peaks with a slower gradient we tried adding ammonium chloride as a counter ion. This was somewhat successful in particular for PhS and PhI. Retention times were only slightly affected, while peak broadening was reduced. In the remaining experiments on this system, 0.1 *M* NH_4Cl was substituted for water. The ammonium ion could also be provided by ammonium sulfate, avoiding the corrosive effects of chloride ion.

Effect of hexane—isopropanol ratio in a neutral system

In Table I, we present the results of experiments in which the hexane—isopropanol ratio was varied in solvents with a fixed aqueous content. For all the lipids tested increasing solvent isopropanol content did not accelerate elution as would be predicted for a simple adsorption mechanism. Rather, at a given solvent aqueous content, phospholipids eluted more rapidly and as sharper peaks as the isopropanol was decreased. This effect was least marked for PhG and PhI in which a phosphodiester is the only ionizable group, and greatest for the choline-containing phospholipids. The retention of PhS and DPI, both with two ionizable groups in addition to the phosphodiester, increased to a greater extent with increasing isopropanol than that of PhE with only one group. The data suggest that isopropanol may act by enhancing the interaction of ionized groups with the silica surface.

These findings were used to produce the chromatogram shown in Fig. 1. A small amount of tetrahydrofuran was added to the B solvent to offset a decreasing baseline. The aqueous component increased from 3 to 10% while

TABLE I

VARIATION OF CAPACITY FACTOR k' WITH HEXANE–ISOPROPANOL RATIO IN A NEUTRAL SYSTEM

(A) Column, Micro-Pak Si-5, flow-rate, 1.2 ml/min; solvent, 3.5% 0.1 *M* NH_4Cl, hexane as indicated, isopropanol to 100%. (B) Conditions as above except 7% 0.1 *M* NH_4Cl.

	Hexane (%)	k'_{PhG}	k'_{PhE}	k'_{PhI}	k'_{PhS}
A	30	4.8	7.1	8.4	30
	42	4.8	5.9	8.7	19
	48.25	4.6	5.4	8.2	15.7
	54	4.0	4.3	7.3	11.2
	Hexane (%)	k'_{PhC}	k'_{SM}		k'_{DPI}
B	38.4	11.7, 12.6	14.6, 17.7		16.8
	43.2	7.3, 8.0	9.0, 11.4		15.0
	46	3.6, 4.0	4.4, 5.6		6.8
	48	2.3 (single)	3.0, 3.4		4.1

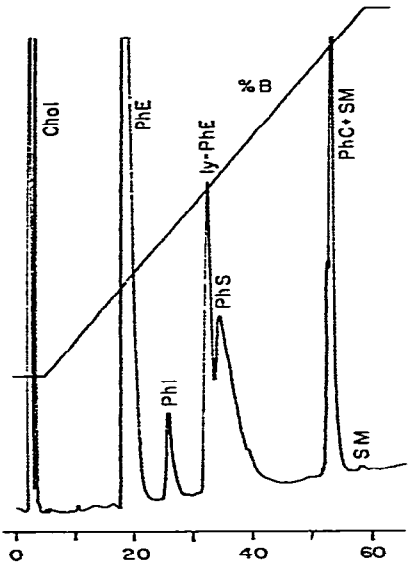


Fig. 1. HPLC of lipid extract from rat brain — neutral system. Column, Micro-Pak Si-5; solvents: A, hexane–isopropanol (50:50); B, hexane–isopropanol–tetrahydrofuran–0.1 *M* NH_4Cl (40:45:5:10); flow-rate, 1.0 ml/min, 30% B for 0 to 5 min increasing to 100% B at 60 min.

hexane and isopropanol remain relatively constant. This system is similar to that reported by Hax and Geurts van Kessel [6] except for the higher solvent hexane content and the use of ammonium chloride. The order of elution of compounds is also similar to that reported for LiChrosorb SI-60 [6] except for PhA.

By use of standards or by collection of the eluent followed by TLC, we

found PhA to elute as several peaks in a broad region between 35 and 45 min. Similar behavior was found for DPhG and for the polyphosphoinositides. This, coupled with the fact that these are minor components of tissue phospholipids, makes these compounds virtually undetectable by UV absorbance in chromatograms such as Fig. 1. This system might be improved somewhat by further increasing the solvent hexane content while decreasing water and isopropanol.

Use of sulfuric acid for ion suppression

While a neutral system provided adequate resolution of the major phospholipid species, it proved only marginal for PhS and unusable for other species with several ionizable groups. The broad multiple peaks obtained for these compounds were judged to arise from the presence of multiple ionized forms, since carboxyl and secondary phosphate ionizations have pK's in the same region as the solvent pH. We therefore tried to achieve suppression of these ionizations. Sulfuric acid was chosen for its UV transparency, acid strength, and lack of interference with assays for phosphate on collected lipids.

With the addition of small amounts of sulfuric acid, the elution of neutral or acidic phospholipids is greatly enhanced. Phospholipids may be divided into three types according to their behavior. First are choline-containing phospholipids which retain the behavior noted in Table I. Water is essential for elution of these species and isopropanol retards them. Second are the phospholipids containing only phosphate and hydroxyl groups which behave in a normal adsorption mode: isopropanol accelerates elution and water in small quantities, affects peak shape more than retention time. Phosphate apparently does not participate greatly in adsorption, and elution order is roughly according to the number of hydroxyl groups. Geometry of these hydroxyl groups also plays a role, as TPI with two hydroxyls fixed axial and equatorial on the inositol ring elutes before PhG. The third group with intermediate properties consists of amino-containing phospholipids. The amino group appears more effective in adsorption than the hydroxyl; this is also seen in the greater retention of cerebroside over monogalactosyldiglyceride. For these lipids, both water and isopropanol, acting in opposite fashion, affect retention.

Effect of solvent water content on elution and resolution

Because of the effect of water on retention of amino-containing phospholipids, resolution and even elution order depend upon the ratio of isopropanol to water. This is illustrated in Table II which shows the effects of a series of different solvents used to establish our final system. With a high ratio of isopropanol to water PhI elutes between PhS and PhE. As water is reduced, the separation of PhI from PhS is improved while that between PhI and PhE is impaired. This occurs primarily because of the greater effect of water on PhE and PhS relative to PhI. The effect of sulfuric acid concentration is seen with the two solvents containing 2% water. While more sulfuric acid is required with solvents of higher water content to achieve ion suppression, once this level is achieved additional sulfuric acid may sharpen peaks but has little effect on retention time.

TABLE II

DEVELOPMENT OF GRADIENT SYSTEM

Sample, lipid extract from rat liver; column, Micro-Pak Si-5; solvent A, hexane—isopropanol—sulfuric acid (97:3:0.02), flow-rate, 1.5 ml/min, 2% B 0 to 5 min increasing linearly to 50% B at 50 min.

Solvent B hexane—isopropanol—water—sulfuric acid	Corrected retention time (min)			Resolution	
	PhS	PhI	PhE	PhI/PhS	PhE/PhI
48:48:4:0.5	38.0	39.1	45.0	0.50	2.13
49:48:3:0.5	37.0	38.6	44.6	0.87	2.73
49:49:2:0.4	33.8	36.1	40.2	1.52	2.34
49:49:2:0.2	33.4	35.6	40.0	1.16	1.57

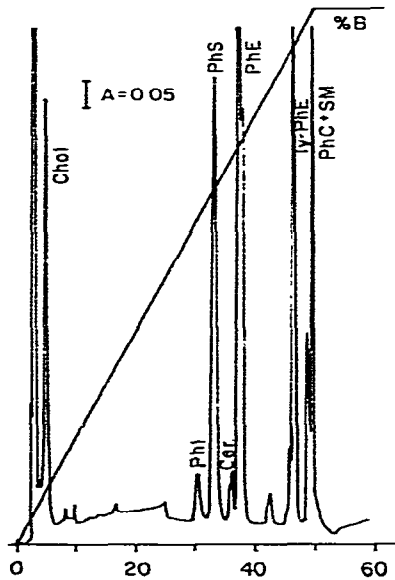


Fig. 2. HPLC of lipid extract from rat brain — acid system with high water. Column, Micro-Pak Si-5; solvents, hexane—isopropanol—water—sulfuric acid, A (95:5:0:0.02), B (60:37.5:2.5:0.1); flow-rate, 1.2 ml/min, 0 to 100% B at 50 min. Note elution of PhI before PhS.

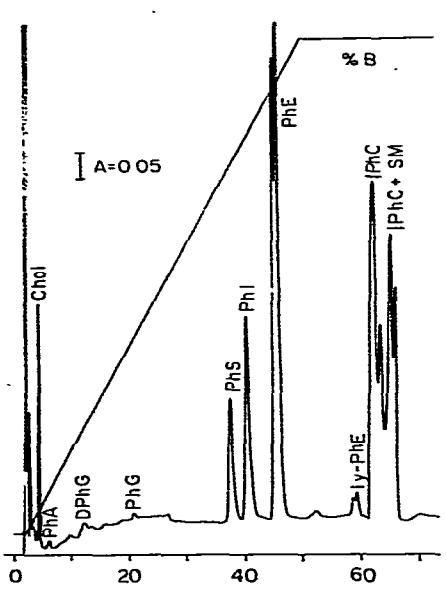
At even higher ratios of water to isopropanol PhS will actually elute after PhI. This is shown in Fig. 2 which depicts separation of rat brain lipids using a system where the amount of water is near saturation for this isopropanol content. This results in several solvent artifacts in the first half of the chromatogram. This sytem was not found to be stable, and the resolution between PhI and PhS degraded within a few weeks on irregular silica columns and could not be achieved at all on Spherisorb. In addition, the exact conditions for this separation varied widely from column to column, resulting in a large expenditure of time for optimization.

In contrast, a system with low water content gives essentially the same separations on various silica columns including Spherisorb. This system may

be rapidly optimized for a new column using the principles discussed above for a particular application. The A solvent, 3% isopropanol in hexane with 0.02% sulfuric acid was chosen for consistent resolution of PhA from the solvent front. The peak shape of PhA may be improved by addition of B solvent. Then a fresh lipid sample may be chromatographed using a slow gradient with a series of B solvents containing varying amounts of water and sulfuric acid in approximately 1:1 hexane—isopropanol as described in Table II.

Behavior of phospholipid species

Applications of this system to lipids from several tissues are shown in Figs. 3—5. The resolution of PhS, PhI, and PhE is best seen in a lipid sample from a tissue such as liver which is deficient in glycolipids (Fig. 3). In whole brain (Fig. 4) and especially in myelin (Fig. 5) cerebrosides and sulfatides (Cer.) overlap the retention times of PhI and PhE. Most of the SM is found in the last peak of these chromatograms but a portion elutes as a broader pre-peak along with PhC. Complete separation of these compounds may be achieved by stopping the gradient with 90% B at 45 min. This however increases the analysis time by about 10 min.



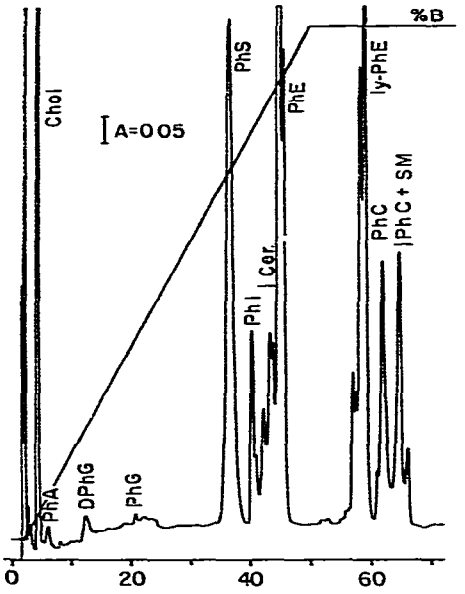


Fig. 3. HPLC of lipid extract from rat liver — acid system with low water. Column, Micro-Pak Si-5; solvents, hexane—isopropanol—water—sulfuric acid, A (97:3:0:0.02), B (75:24:0.9:0.1); flow-rate, 1.5 ml/min, 4% B for 2 min then increasing linearly to 100% B at 50 min.

Fig. 4. HPLC of lipid extract from rat brain — acid system with low water. Conditions as in Fig. 3.

The multiple peaks seen for PhE and PhC are probably related to fatty acid species, since their relative abundances are consistently related to the tissue of origin. A different phenomenon is seen for DPhG. Part of this lipid is not retained by the column and elutes with the solvent. This portion, which may

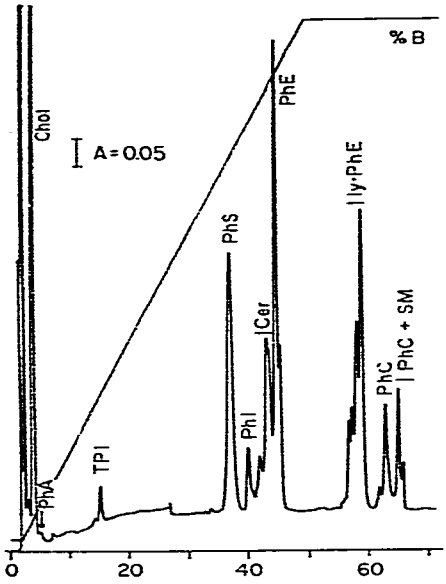


Fig. 5. HPLC of lipid extract from isolated rat cerebral myelin. Conditions as in Fig. 3.

be most of this species, increases with the total load of lipid and the volume and polarity of the sample solvent. This problem is most pronounced when DPhG is present as a free acid.

Stability of phospholipids

The large amounts of ly-PhE seen in samples from brain (Figs. 4 and 5) are also seen in heart but not liver (Fig. 3). This tissue specificity [9], as well as the high UV absorbance indicate that this is 1-lyso PhE derived from plasmalogen. While some plasmalogen is degraded by our extraction procedure (see Fig. 1), most of the degradation of the acid sensitive vinyl ether bond occurs on the column. This degradation may be complete, since commercially prepared plasmalogen, which gave no detectable ly-PhE on TLC, ran as 80—90% ly-PhE on the HPLC system. Except for plasmalogen all other phospholipids are recovered intact. Peaks collected in a small amount of pyridine may be re-chromatographed by HPLC or TLC.

Column regeneration

The HPLC system now in use (Figs. 3—5) appears stable after two months. However, several precautions are taken for column condition. The two most important are the use of a guard column which is changed regularly and a specific regeneration scheme used between runs. Pure isopropanol is connected to the third solvent inlet and a 30-min program is run. This consists of 20 column volumes of 50% isopropanol and solvent A, changing to pure A over 20 volumes, and a final 20 volumes with the starting solvent composition. This scheme is also used at the start of the day after 20 volumes of B solvent. The column is conditioned overnight by 100 ml of isopropanol and 100 ml of hexane. Eventually column performance does degrade for phospholipids and probably other ionic compounds, but not for the aromatic com-

TABLE III

EFFECT OF COLUMN CONDITION ON CAPACITY AND RESOLUTION

Sample and conditions as in Fig. 3.

Column	Corrected retention time (min)			Resolution PhI/PhS
	PhE	PhS	PhI	
New, 2 months	44.4	36.1	39.0	2.06
Old, 6 months	41.4	33.2	36.5	0.96
Reconditioned	41.8	33.1	36.5	1.32

pounds found in text mixtures. Resolution between PhI and PhS is lost first due mainly to peak broadening. Column performance may be partially restored by a reconditioning process. The column in isopropanol is purged by pumping 100 ml each of 0.1% sulfuric acid in water, isopropanol, methylene chloride, and hexane. Results of this operation are shown in Table III. While the column is not restored to its original condition, complete resolution of PhS and PhI is regained. This is mainly due to a sharpening of these peaks rather than a change in retention times.

Low-wavelength UV absorbance offers a sensitive means for detection of most phospholipids. The chromatograms shown in Figs. 3—5 were run at low detector sensitivity and represent lipids from 5 to 10 mg wet weight of tissue. However, the absorbance recorded arises primarily from double bonds in fatty acid moieties [5], and molar extinction coefficients will vary with fatty acid composition which may change with tissue type and metabolic state. Further, since most unsaturated fatty acids are found at the 2-position of glycerol, 2-lysophospholipids may be virtually transparent at 205 nm. Direct quantitation might be more reliable with a moving wire detector [1, 2] or by post-column reactions of phosphate [10]. If quantitation of phospholipids is routinely performed by phosphate assays on isolated lipids HPLC offers an easier and more complete method for collection than scraping TLC plates.

Only plasmalogen appears to be adversely affected by HPLC in acid solvents. This might be an advantage in certain circumstances since the degradation appears to be complete. For other lipids collection from the HPLC eluent avoids the oxidation of fatty acids which may occur on TLC plates with drying. This method may prove excellent for the preparative isolation of individual phospholipids. In our hands HPLC provides better and more consistent resolution of minor phospholipid species than two-dimensional TLC. These may be isolated by HPLC more easily than by TLC since the former will tolerate a much greater sample load without loss of resolution.

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NOTE ADDED IN PROOF

The lack of retention of DPhG (most notable in Fig. 3) may be avoided simply by dissolving the phospholipid sample in the starting solvent. With this procedure, DPhG is eluted before PhA.

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