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SEPARATION OF PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: ALL MAJOR CLASSES, INCLUDING ETHANOLAMINE AND CHOLINE PLASMALOGENS, AND MOST MINOR CLASSES, INCLUDING LYSOPHOSPHATIDYLETHANOLAMINE

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SUMMARY

High-performance liquid chromatographic methods for the separation and quantitation of phospholipids were developed and shown to give sensitive, reliable measurements of tissue phospholipids, including difficult to-resolve pairs such as choline plasmalogen (plasmenyl choline) and phosphatidylcholine, choline glycerophospholipids and sphingomyelin, phosphatidylinositol and phosphatidylserine, and phosphatidylserine and lysophosphatidyl ethanolamine. Separations of most phospholipids including those mentioned above are more complete than in existing procedures, and require only 40 min per injection. Utilization of the hexane–2-propanol–water system has an advantage over separation techniques that employ acidic solvents in that the plasmalogens are not hydrolyzed and a less degradative environment for labile lipids is provided. Further, a rapid high-performance liquid chromatographic procedure for the separation of intact ethanolamine plasmalogen (plasmenylethanolamine) from phosphatidylethanolamine was developed. Previous procedures have required derivatized samples or acid hydrolysis of the plasmalogen vinyl ether linkage. A slight modification of the primary method (method I) increases the resolution of lysophosphatidylethanolamine from other classes (method II). A third modification (method III) can replace the standard silicic acid column separation of lipids into neutral, glycolipid and phospholipid fractions.

INTRODUCTION

Lipid compositions vary in response to both normal and pathological processes. Lipid alterations cited in the literature for neural tissues alone include changes in phospholipid composition and fatty acyl chain saturation in response to diet [1] and environmental stress [2], loss of specific phospho-

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lipids, cholesterol, anti-oxidants, and increases in free fatty acids, diacylglycerol, and arachidonic acid metabolites (both enzymic and non-enzymic) following trauma [3, 4], and variation of the cholesterol/phospholipid ratio during normal development or by diet [1, 5, 6]. Most of these changes can be correlated with modifications of cell membrane dynamics such as enzymic activity of membrane-bound enzymes (Na^+, K^+-ATPase, acetylcholinesterase [7, 8]), membrane fluidity as measured by fluorescence polarization or spin resonance [9, 10], or more generally as the functional integrity of the tissue [3]. The range of lipid changes that can occur as a result of any given process necessitates examination of as many membrane components as possible in order to detect minor lipid variations that might interact to produce changes in function.

In this regard, we have used high-performance liquid chromatography (HPLC) to develop four separation techniques that can be applied to small physiological samples and that will result in virtually complete separation and quantitation of tissue membrane phospholipids. These separations are based on the *n*-hexane–2-propanol–water system first developed by Geurts Van Kessel et al. [11] and represent considerable improvements in the complete separation of membrane lipids over similar systems currently in use elsewhere [12–20].

The methods discussed in this paper achieve good separation of the more polar lipids, and when used in conjunction with an HPLC method for separating less polar lipids that we have recently developed [21], most tissue lipids can be separated and measured with relatively little expense in time and materials. Additionally, we report here the first complete resolution of underivatized diester from 1-ether-2-ester forms of ethanolamine glycerophospholipids, and partial resolution of these forms of choline glycerophospholipids.

The conditions chosen for three of our methods (I–III) are identical except for such easily varied parameters as flow-rate and solvent gradient profile. Samples that have special separation requirements can be handled quite easily by slight modifications of flow-rate or solvent gradient without the need for an entirely different combination of column and solvents. This flexibility allows enhancement of specific separations, if so desired.

EXPERIMENTAL*

HPLC system

A Beckman Model 324 HPLC system with two Altex 100 A pumps regulated

*Abbreviations: in conformity with the recommendations of the IUPAC-IUB Commission on Nomenclature of Lipids, the following abbreviations are used: phosphatidylethanolamine (the diacyl form) = PtdEtn; phosphatidylcholine = PtdCho; phosphatidylglycerol = PtdGro; sphingomyelin = CerPCho; phosphatidylserine = PtdSer; phosphatidylinositol = PtdIns; phosphatidic acid = PtdH; cardiolipin = Ptd$_2$Gro; lysophosphatidylethanolamine (mixture of 1-lyso, and 2-lyso) = lysoPtdEtn; lysophosphatidylcholine = lysoPtdCho. As there are no official IUPAC-IUB abbreviations for ethanolamine and choline plasmalogens, we have adopted PlasEtn and PlasCho, respectively, as abbreviations for these two phospholipids. Galactosylceramides (cerebrosides) and galactosylceramide sulfates (sulfatides) are designated CerGal and CerGalS, respectively. We are also designating the combined alkylacyl, alkenylacyl, and diacylethanolamineglycerophospholipids as ethanolamine glycerophospholipids (EtnGpl).

by an Altex 420/421 controller was used throughout this study. The injection port was an Altex Model 210. All separations were performed on Dupont Zorbax SIL (25 cm × 4.6 mm I.D., 5–6 μm particle size) columns which were maintained at 34°C with a Jones Chromatography (Columbus, OH, U.S.A.) column heating block. Peak detection was performed at 205 nm with a Perkin-Elmer LC 75 variable-wavelength detector which was connected to a Kipp and Zonen BD 41 chart recorder and to a Nelson analytical intelligent interface, allowing integration of peak areas with a Hewlett-Packard 85 computer and digital storage of chromatograms.

Solvent preparation

Solvents (HPLC grade) were obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.) and were filtered and degassed prior to use. Filtration was performed through Millipore Nylon filters (FH type, 0.5 μm pore size). Four different solvents (A–D) were prepared for the various lipid separations (methods I–IV). Solvents A and B were used for methods I–III, and solvents C and D were used for method IV. Solvent A was hexane–2-propanol (3:2). The water-containing solvent B was made by combining 27.5% ml water (obtained from a Millipore water purification system) with solvent A to a total volume of 500 ml. The two solvents for the separation of PtdEtn and PlasEtn (method IV) are solvent C, hexane–2-propanol (98.8:1.2), and solvent D, 2-propanol–water (93:7). Volumes should be measured individually for each solvent because they are not additive volumetrically. Solvent reservoirs were kept sealed, and the solvents were stirred continuously.

Column care

The column is washed with methanol whenever resolution begins to decrease. The number of injections after which this occurs varies with the type of sample, but is usually noted following 15–30 runs. Approximately 100 ml of filtered methanol are washed through the column, which is then flushed with 100 ml of pure solvent B. If the column is to be used for the PlasEtn–PtdEtn separation, the column is subsequently washed with solvent C until a flat baseline is achieved.

Samples

Bovine brain plasma membrane lipids were prepared as follows. Fresh gray matter homogenate was centrifuged at 12 000 *g* for 30 min and the supernatant saved. A crude plasma membrane fraction was obtained by centrifuging this supernatant at 48 000 *g* for 30 min [22] The pellet was used for extraction of membrane lipids by the method of Hara and Radin [23] using hexane–2-propanol (3:2) in a ratio of 18 ml/g wet weight tissue. The pellet was homogenized in the hexane–2-propanol and filtered through a sintered-glass funnel. After washing the homogenizer and residue three times with hexane–2-propanol, the resulting lipid extract was brought to dryness under a stream of nitrogen. Prior to injection onto the column, all samples were filtered through 0.2-μm pore-size Nylon filters (Rainin, Woburn, MA, U.S.A.), dried under nitrogen, and redissolved in hexane–2-propanol (3:2) for the regular phospholipid separation. For the PlasEtn–PtdEtn separation, the samples were dissolved in hexane–2-propanol (98.8:1.2) before use.

Thin-layer chromatography

Bovine brain phospholipids were separated by the method of Rouser et al. [24] on 20 cm × 20 cm silica gel H plates with 7.5% magnesium acetate (Analtech, Newark, DE, U.S.A.). The solvent for the first dimension was chloroform—methanol—ammonium hydroxide (65:25:5) and for the second dimension was acetone—chloroform—methanol—glacial acetic acid—water (48:36:12:12:6). Purity and identity of PtdEtn, PtdSer, PtdCho, CerPCho, PtdIns, PtdH, Ptd_2Gro, lysoPtdEtn, and lysoPtdCho were demonstrated using the same solvent systems with 12.5 cm × 12.5 cm silica gel H plates with 7.5% magnesium acetate. Separation of the plasmalogen and diacyl forms of ethanolamine glycerophospholipids was confirmed by thin-layer chromatography (TLC), using the second dimension of the method of Horrocks and Sun [25], i.e. PlasEtn and PtdEtn were spotted in separate lanes, exposed for 90 s to hydrochloric acid fumes to hydrolyze the plasmalogen vinyl ether linkage and chromatographed in a solvent consisting of chloroform—methanol—acetone—acetic acid—0.1 *M* ammonium acetate (53:21:21:1.3:3.8). PlasEtn and PtdEtn samples were chromatographed in this system without prior acid treatment to confirm that both were ethanolamine glycerophospholipids. This procedure was repeated for the PlasCho and PtdCho peaks. Lipid phosphorus was assayed by a modification of the method of Rouser et al. [24].

Standards

The identities of the various phospholipids were confirmed not only by two-dimensional TLC (above), but by injection of commercial or prepared standards. PtdIns, PtdGro, PtdEtn, CerPCho, CerGal, and CerGalS were obtained from Supelco. PtdCho and PtdSer were purchased from Avanti, and PtdH, Ptd_2Gro, and lysoPtdCho were purchased from Sigma. LysoPtdEtn was prepared by spotting ethanolamine glycerophospholipids on silica gel G plates, exposing the plates to HCl fumes for 90 s, and running the plates in the second dimension solvent of the method of Horrocks and Sun [25]. The lysoPtdEtn spot was scraped off and the lipid extracted from the gel. A mixture of 1-alkenyl-2-lysoGroPEtn and 1-acyl-2-lysoGroPEtn was also used to confirm the identity of lysoPtdEtn. The platelet activating factor was from Calbiochem.

HPLC phospholipid separation (methods I and II)

A flow-rate of 1.5 ml/min and a column temperature of 34°C were maintained throughout the separations. The initial solvent temperature also affected separation. Therefore, solvents were maintained at 23°C throughout. The initial solvents A—B (50:50) with 2.75% water. For method I, at 9 min a gradient from 50% B to 78% B with 4.3% water was executed over a 5-min period, at 23 min solvent B was then increased to 100% with 5.5% water over a 2-min period, and at 27 min the solvents were returned to a 50:50 ratio over a 2-min period. The separation was complete and the column re-equilibrated in about 37 min, allowing a new sample to be injected every 40 min. A modification (method II) of this procedure allowed complete separation of lysoPtdEtn from PtdSer in addition to the other phospholipids. For this separation, the initial starting solvent composition (A—B, 50:50) was maintained for 25 min,

at which time solvent B was increased to 78% over 5 min. At 33 min solvent B was increased to 100% over 1 min, and solvents were returned to 50% B over a 3-min period at 36 min. A sample could be injected every 50 min.

Three-fraction lipid separation (method III)

Our short HPLC run to obtain neutral lipid, glycolipid, and phospholipid fractions used a flow-rate of 2.0 ml/min, a column temperature of 34°C, and initial solvents of A—B (50:50). Solvent B was increased to 100% over 4 min immediately after injection. At 12 min, solvents were returned to their initial conditions over 3 min.

Ethanolamine plasmalogen—phosphatidylethanolamine separation (method IV)

This separation used a solvent composition of C—D (84:16). The flow-rate was 1.5 ml/min, and the column was maintained at 34°C throughout. After every five to ten separations, the column was flushed with 100% solvent D for about 20 min. This was sufficient to keep the column clean and conditioned without the need to flush the column with methanol.

HPLC of derivatized EGP

We used the derivatization and HPLC separation procedure of Nakagawa and Horrocks [26] to determine whether 1-alkyl-2-acylGroPEtn co-chromatographed with the plasmalogen fraction from our HPLC method. The PlasEtn and PtdEtn fractions were collected from HPLC method IV, dried under nitrogen, and dissolved in 3 ml diethyl ether. This was mixed with 1.0 ml Tris · HCl buffer (0.1 *M*, pH 7.4) and 1—3 mg phospholipase C (*Bacillus thuringiensis* IMA 12077), and stirred at room temperature for 16 h. The ether layer was removed, combined with three washes of the buffer phase, dried, and combined with 0.5 ml acetic anhydride and 0.1 ml pyridine. This was stirred for 3 h at room temperature, dried, and redissolved in hexane for injection onto a μPorasil column (30 cm × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.). The eluting solvent was cyclopentane—hexane—methyl *tert.*-butyl ether—acetic acid (73:24:3.0:0.03) at a flow-rate of 2.0 ml/min and a column temperature of 36°C.

RESULTS

We have compared the percent distribution of phospholipids and recovery of samples separated by TLC and by our HPLC method. The distribution of phospholipids from bovine brain plasma membrane was similar whether separated by TLC or by HPLC (Table I), but the HPLC method gave smaller sampling errors and higher recoveries. In the past, we have occasionally had problems with TLC separations of PtdSer, PtdIns, and CerPCho. This loss of resolution did not occur with HPLC (Fig. 1).

Much larger samples were needed for the TLC separation than for the HPLC method (400 nmol versus 180 nmol lipid phosphorus) because of greater difficulty in visualizing minor lipid components (such as PtdH) on TLC. Detection of phospholipids eluting from the HPLC silica gel column was limited by the sensitivity of the detector. However, all major phospholipids were quite

TABLE I

COMPOSITION OF PHOSPHOLIPIDS AS DETERMINED BY TWO-DIMENSIONAL TLC AND HPLC

Lipids were extracted from a bovine brain plasma membrane fraction and separated by TLC or by HPLC. Values are mean ± S.D. and are normalized to 100% recovery of lipid phosphorus. The high value for lysophosphatidylethanolamine on TLC may be indicative of hydrolysis of ethanolamine plasmalogen to the lyso compound while on the TLC plate.

Phospholipid	Recovered lipid phosphorus (%)	
	TLC (n = 4)	HPLC (n = 4)
Phosphatidylcholine	35.8 ± 1.3	35.9 ± 1.1
Ethanolamine glycerophospholipids	28.9 ± 2.2	28.4 ± 0.9
Phosphatidylserine	10.2 ± 1.0	11.9 ± 0.9
Sphingomyelin	1.8 ± 1.7	4.9 ± 0.1
Phosphatidylinositol	3.5 ± 1.9	5.5 ± 0.2
Phosphatidic acid	0.5 ± 0.4	1.1 ± 0.3
Cardiolipin	2.0 ± 2.7	2.2 ± 0.4
Lysocardiolipin	3.7 ± 3.1	1.6 ± 0.2
Lysophosphatidylethanolamine	7.2 ± 1.1	1.6 ± 0.1
Lysophosphatidylcholine	N.D.*	2.0 ± 0.5
Origin**	6.6 ± 0.5	4.9 ± 0.3
Recovery	95.6 ± 2.9	97.9 ± 3.7

*N.D. = not detectable.
**For the HPLC method, this value represents unknown, polar compounds eluting after lysophosphatidylcholine.

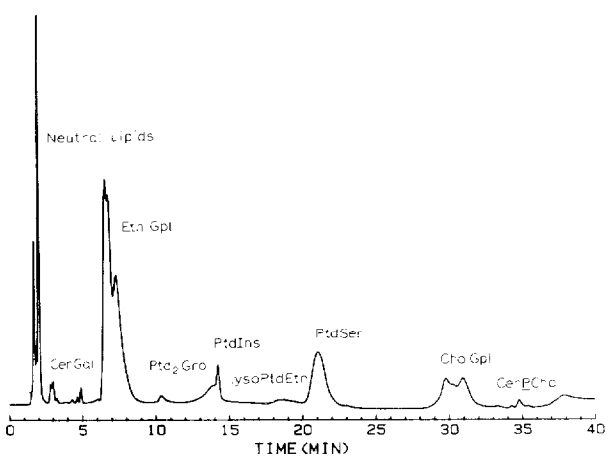

Fig. 1. Chromatogram illustrating the elution order of bovine brain plasma membrane lipids with HPLC method I.

apparent in samples containing less than 50 nmol lipid phosphorus. Chemical assay of such small samples only gave values for the larger components due to the limits of sensitivity of the phosphorus assay of Rouser et al. [24].

Samples that did not overload the column (roughly less than 600 nmol lipid phosphorus) could be resolved into the following components using method I: EtnGpl, PtdGro, Ptd_2Gro, PtdIns, lysoPtdEtn, PtdIns, lysocardiolipin, PlasCho,

PtdCho, CerPCho, platelet activating factor, and lysoPtdCho (Fig. 1). PtdH was not a sharply defined peak and may contaminate lysoPtdEtn and PtdSer if present in large amounts. We found reasonable values for this phospholipid if the region between PtdIns and lysoPtdEtn was collected. TLC of this region and of lysoPtdEtn and PtdSer showed that most PtdH eluted prior to lysoPtdEtn and was only a minor contaminant of the lysoPtdEtn or PtdSer fractions. Separations of lysoPtdEtn from PtdSer and PlasCho from PtdCho were not complete in our normal method, but our modification (method II) did resolve lysoPtdEtn from PtdSer (Fig. 2). TLC of PlasCho and PtdCho obtained with method I showed a relatively small amount of cross-contamination between the two fractions, although there clearly is some overlap.

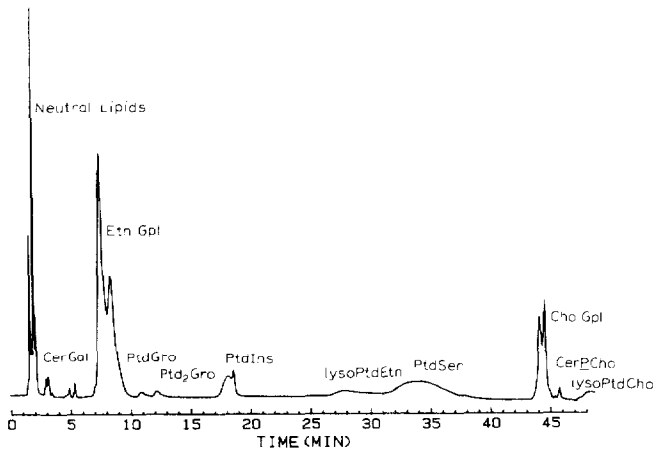

Fig. 2. Chromatogram illustrating the increased separation of lysophosphatidylethanolamine (lysoPtdEtn) and phosphatidylserine (PtdSer) with method II.

The 40-min separation was appropriate for most tissue samples that we have processed (brain and/or spinal cord from cat, rat, mouse, man, and cow, bovine skeletal and porcine heart muscle, murine sciatic nerve, human blood, rat liver, and chick egg yolk). Samples containing especially large amounts of lysoPtdEtn may benefit from a modified procedure we developed that is adapted to separating lysoPtdEtn and PtdSer, which may not separate completely in our regular separation. The longer time of 50 min is the only prohibition against using this modified separation protocol for all phospholipid separations.

The short three-fraction (3-F) lipid separation was developed as an adjunct to an HPLC method we devised for separating neutral lipids (Fig. 3). Although a neutral lipid fraction is provided by our standard phospholipid procedure, the latter required 40 min per run. Gravity silicic acid columns are fast because multiple columns can be run concurrently, but with the standard chloroform and methanol separations, lipids are exposed to the degradative environment of the column for a relatively long period of time, and the columns require large amounts of solvent. Our 3-F procedure required 17 min for separation of the sample into neutral, glyco-, and phospholipids, and 3 min more for column re-equilibration. In addition, pure ethanolamine glycerophospholipids could be collected and saved for separation with method IV (Fig. 4).

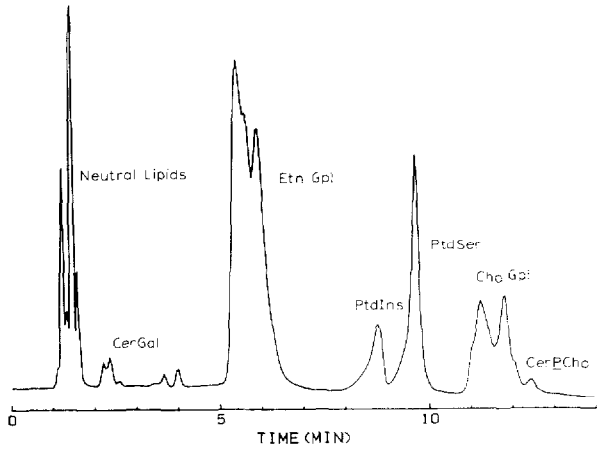

Fig. 3. Chromatogram obtained with method III giving rapid separation of neutral, glycolipid, and phospholipid fractions. The ethanolamine glycerophospholipid fraction was collected from this run, and ethanolamine plasmalogen (PlasEtn) and phosphatidylethanolamine (PtdEtn) were separated by method IV, giving the elution profile shown in Fig. 4.

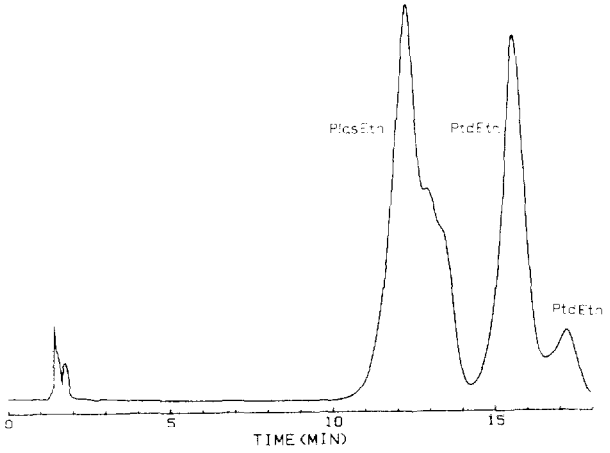

Fig. 4. Ethanolamine glycerophospholipids were collected from method III and rechromatographed using method IV to give ethanolamine plasmalogen (PlasEtn) and phosphatidylethanolamine (PtdEtn) fractions.

Method IV, for the separation of intact PlasEtn from PtdEtn, resolves these two types completely. In addition, alkylacyl GroPEtn, the direct metabolic precursor of PlasEtn, was partially resolved from PlasEtn and fully resolved from PtdEtn (Fig. 4). This was determined using the procedure of Nakagawa and Horrocks [26] to separate dephosphorylated, acetylated derivatives of ethanolamine glycerophospholipids into three components based upon the type of bond present in the 1-position (alkenyl, alkyl, and acyl) (Fig. 5). Most methods currently in use for quantitating levels of PlasEtn in biological samples use acid treatment to cleave the vinyl ether bond [24] producing a lysophospholipid which can then be separated from the acid-stable forms of ethanolamine glycerophospholipid (diacyl and alkylacyl GroPEtn). As a result, the total amount of ether-linked ethanolamine glycerophospolipid is underestimated with these methods.

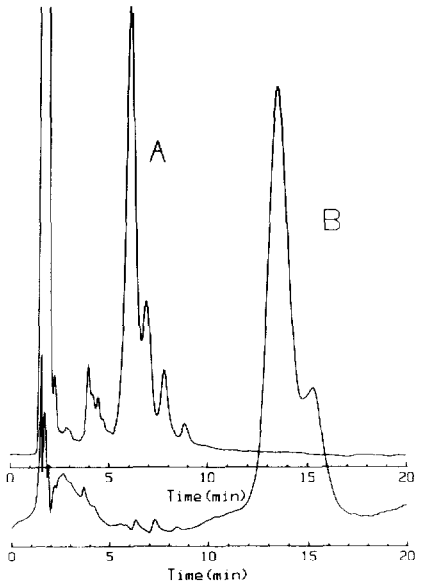

Fig. 5. Purity of the fractions obtained with method IV. (A) Ethanolamine plasmalogen; (B) phosphatidylethanolamine (diacyl GroPEtn) fraction.

DISCUSSION

The sensitive, non-degradative nature of HPLC makes it an ideal method for the separation of small samples and labile or easily oxidized compounds. Our work with membrane lipids from small samples of neural tissue induced us to convert from TLC to HPLC because of these advantages. Although a significant body of knowledge exists regarding separation of phospholipids and other membrane lipid types on thin-layer silica gel plates, commonly used TLC solvent systems are not of general use for silica-based HPLC columns because of their lack of transparency in the low ultraviolet region. The widespread use of UV absorption in the region of 200 nm as a means of detecting lipids eluted from HPLC limits the solvent systems that may be used to those that are transparent at this wavelength. Other means of detection such as moving wire detectors [22] and differential refractometry [27] have been used with non-UV transparent solvents. These methods suffer from lack of sensitivity thus requiring relatively large sample size. Two types of UV-transparent systems have been developed for the separation of membrane lipids on silica columns. These are the acetonitrile–methanol–water system of Jungalwala et al. [28], and the hexane–2-propanol–water system of Geurts Van Kessel et al. [11], both initially used for phospholipid separations. The acetonitrile-based system achieves a rapid separation of PtdCho and CerPCho, but other phospholipids are not resolved, thereby making this system of limited general utility. The original hexane–2-propanol–water system used gradient elution to separate several classes of phospholipids. This method has since been modified by several laboratories to improve resolution of phospholipid classes [12–20] and to separate a number of neutral lipids including mono- and diacylglycerol, vitamin E, and cholesterol [29]. The methods described here greatly increase

the number of membrane lipid classes which can be completely separated using gradient elution with varying proportions of hexane–2-propanol—water.

Phospholipid separation by HPLC is extremely sensitive, allowing samples containing only 100 nmol total lipid phosphorus to be reproducibly separated and accurately quantitated. In addition, unsaturated lipids, labile functional groups, and positional isomers appear to be preserved fat better by HPLC than TLC; samples that were reinjected several times on HPLC showed no breakdown after repeated exposure to the column, whereas standards that were purified by two-dimensional TLC showed fairly significant breakdown when chromatographed by HPLC. We have included a comparison of the percentage distribution of phospholipids obtained from TLC and HPLC for bovine brain plasma membrane lipids showing better recovery, smaller sampling errors, and, most significantly, a much smaller value for lysoPtdEtn (signifying less breakdown) for the HPLC method. A modification of the above procedure gives a rapid (17 min) but less complete separation that is especially suitable for fractionating lipid samples into neutral, glycolipids and phospholipid portions, thus taking the place of the standard silicic acid column procedure, but with two advantages over the silicic acid method: (i) the lipids are exposed to the potentially degradative silicic acid for a shorter time and (ii) the expenditures of time and solvents are less. Further, this short procedure allows the collection of ethanolamine glycerophospholipids which can then be separated into PlasEtn and PtdEtn using method IV described in this paper.

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