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ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF PHOSPHOGLYCERIDES AND LYSOPHOSPHOGLYCERIDES

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SUMMARY

An isocratic, rapid method for separation of phospho- and lysophospholipids by high-performance liquid chromatography employing acetonitrile, methanol and water as the mobile phase is described. Separation is achieved on a silica based cation-exchange column with individual components monitored directly by UV absorbance at 203 nm. In solutions of phospho- and lysophospholipid standards and in extracts of rabbit myocardium, recovery of individual components exceeds 95%. With the exception of phosphatidyl serine and phosphatidyl ethanolamine that could not be resolved other major phospho- and lysophospholipid constituents found in extracts of cell membranes are separated completely within 40 min. Retention time for selected moieties can be shortened even further with flow programming.

INTRODUCTION

Because of the profound influence of subtle changes, such as an altered content of lysophospholipids, in the lipid environment of membrane bound enzymes and receptors on their biological activities, quantification of membrane phospholipid constituents has become increasingly useful. One approach, high-performance liquid chromatography (HPLC), offers the potential advantages of speed, reproducibility, and the avoidance of exposure of samples to strongly acidic or basic conditions encountered with most two-dimensional thin-layer chromatography (TLC) systems that frequently induce undesirable covalent modifications of labile phospholipids[1]. Although HPLC has been employed for phospholipid separations previously, adequate resolution of multiple compounds has generally required gradient elution[2,3] or a mobile phase that precludes UV detection[4]. Furthermore, HPLC has not been utilized extensively or validated for separations of lysophosphoglycerides.

Most membrane lipids contain either a primary or quarternary amino functional group that is positively charged at neutral pH. Recently, Twitchett *et al.*[5] found that amines were strongly retained by a microparticulate cation-exchange column and that their elution could be accomplished readily with the use of an organic mobile

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phase. We have developed a procedure for phospholipid separations relying on these characteristics of the cation-exchange column to permit an isocratic, quantitative separation of phospho- and lysophosphoglycerides suitable for rapid analysis of tissue extracts. Because of our interest in pathophysiological consequences of myocardial lysophosphoglycerides, the present procedure was evaluated with extracts of rabbit heart muscle.

MATERIALS AND METHODS

HPLC was performed with a Waters Assoc. liquid chromatographic system comprising a UK 1600 injector, Model 6000A Pump, Model 450 variable wavelength UV detector, and a printer–plotter integrator (PPI). The UV monitor contained an 8-μl flow cell, and UV absorbance was monitored at 203 nm. Full scale deflection was set at 0.4 a.u.f.s. A Whatman PXS 10/25 SCX column (25 cm × 4.6 mm I.D.) packed with 10 μm silica with covalently bound benzene sulfonate (SCX) residues was employed, and a Whatman guard column, 7 cm × 2.1 mm I.D., packed with 10–40 μm SCX pellicular media was placed directly in front of the cation-exchange column.

Solvents

Acetonitrile, methanol, and water (all HPLC grade) were obtained from P. J. Colbert, St. Louis, MO, U.S.A. All solvents had a UV cutoff < 210 nm. Immediately prior to use, solvents were filtered through a 0.45-μm Millipore filter and degassed.

Lipids

Lipid standards —bovine liver phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), lysophosphatidyl ethanolamine (LPE), soybean lysophosphatidyl choline (LPC), and bovine brain sphyngomyelin (SPH) and phosphatidyl serine (PS)— were obtained from Sigma (St. Louis, MO, U.S.A.). Radioactive labeled standards —dipalmitoyl PC, dipalmitoyl PE, and palmitoyl LPC— were obtained from New England Nuclear Corporation (Boston, MA, U.S.A.).

Lipid extraction, TLC and quantification

Rabbit myocardial phospholipids were extracted in acidified butanol[6] or chloroform–methanol[7] as indicated. Phospholipid constituents separated by the HPLC procedure developed were characterized as described previously[6] by two-dimensional TLC on silica OF plates —15 μm silica gel with an organic, fluorescent (OF) binder— obtained from Analabs (North Haven, CT, U.S.A.) in the following solvent systems: first dimension, chloroform–methanol–NH_4OH–pyridine (130:58:15:4); and second dimension, chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10). Phosphate was assayed by the Bartlett procedure as modified by Dittmer and Wells[8], and radioactivity determined by scintillation spectrometry.

RESULTS

For the HPLC system, selected combinations of acetonitrile, methanol, isopropanol, hexane, and water were evaluated. A solvent containing acetonitrile, methanol and water (400:100:34) was found to be most successful. This mobile phase

allowed isocratic elution of PE, PC, LPE, LPC, and SPH well within 40 min. Representative chromatograms are shown in Figs. 1 and 2.

The recovery of radioactivity and of phosphates in PC, PE, LPC, LPE, and SPH in samples applied to the column was consistently greater than 95%. [Quantitation of radioactivity was reproducible within 2% (S.D.) and peak areas computed by the PPI were reproducible within 3% (S.D.)]. In both acidified butanol and chloroform–methanol extracts of myocardial tissue, two-dimensional TLC analysis of fractions obtained by HPLC corresponding to individual HPLC peaks demonstrated only one component detectable by either iodine staining or sulphuric acid spraying and charring. Free fatty acid, cardiolipin and phosphatidyl inositol were eluted with the solvent front.

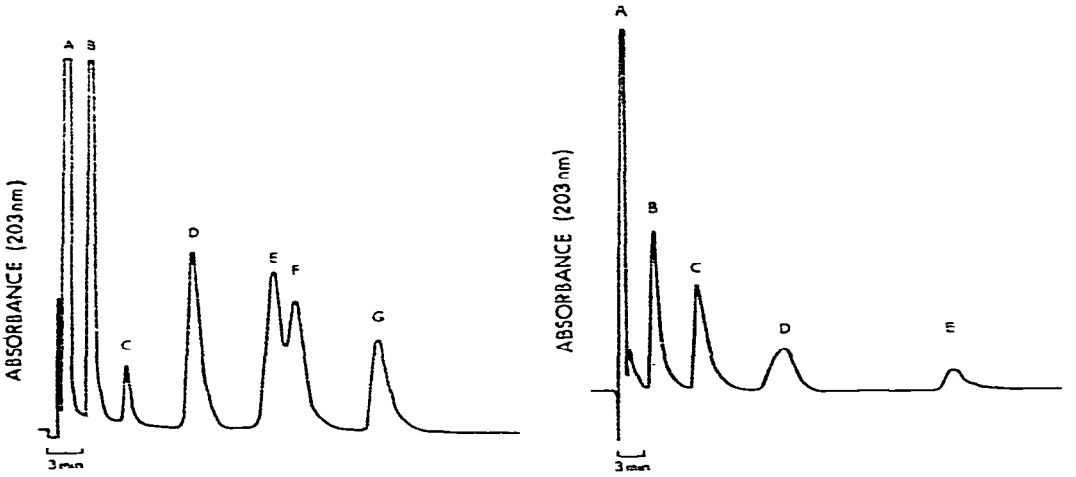

Fig. 1. Chromatogram of phospholipid standards in chloroform–methanol (2:1), 20-μl injection volume. Conditions: 2.5 ml/min flow-rate; sample content, 75 nmoles of lipid phosphorous; mobile phase, acetonitrile–methanol–water (400:100:34); UV detection at 203 nm. Regions of the chromatogram identified include the following: A = solvent front; B = PE; C = LPE; D = PC; E and F = SPH; G = LPC.

Fig. 2. Chromatogram of acidified butanol extract of rabbit myocardium containing 30 nmoles of lipid phosphorous. Conditions are the same as those indicated in the legend to Fig. 1. Regions of the chromatogram identified include: A = solvent front; B = PE; C = LPE; D = PC, E = LPC. Although no SPH peak is identified by UV detection, SPH was present in the region of the chromatogram corresponding to the peak obtained with authenticated standards, based on analysis of phosphorous. The lack of its expression in this chromatogram probably reflects the lack of unsaturated centers in the acyl groups in the biological material.

Because the content of PS is low in myocardial extracts and because others have detected contamination of PE by PS under conditions somewhat analogous to those used in the HPLC procedure developed, additional separations were performed with tissue extracts supplemented by a large excess of bovine liver PS. Based on two-dimensional TLC analysis of HPLC fractions, we found that some PS was eluted prior to PE, some with PE, and some for as long as 1 min after PE in a broad peak. LPE was not contaminated with PS. Despite utilization of numerous combinations of solvents, PS and PE could not be resolved.

Varying concentrations of acetonitrile and water changed retention times as illustrated in Tables I and II. Although the sequence of constituents eluted was not changed, the retention times of phospholipids were altered markedly by changes in composition of the solvent, with an increasing percentage of water resulting in briefer retention. A gradient elution (data not shown) with linearly increasing water content

TABLE I

RETENTION TIMES OF STANDARDS

All solvent systems contained 6.5% water, acetonitrile as indicated, and methanol; retention times are expressed in min; flow-rate was 2.5 ml/min; sample volumes were 20 μl.

Acetonitrile (%)	*PE*	*LPE*	*PC*	*LPC*
80	3.64	7.53	14.50	35.86
70	2.51	4.56	8.55	19.18
60	1.98	3.13	5.83	12.10

TABLE II

RETENTION TIMES OF STANDARDS

All solvent systems contained 7.8% water, acetonitrile as indicated, and methanol; retention times are expressed in min; flow-rate was 2.5 ml/min; sample volumes were 20 μl.

Acetonitrile (%)	*PE*	*LPE*	*PC*	*LPC*
90	4.85	11.71	19.85	41.65
80	2.96	5.71	10.28	24.66
70	2.21	3.73	6.65	14.46
60	1.85	2.76	4.88	9.78

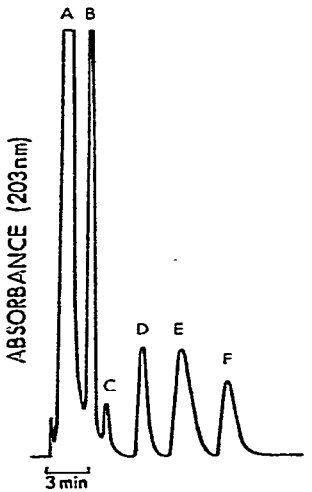

Fig. 3. A Chromatogram of standards analyzed under conditions of flow programming. Conditions are the same as those indicated in the legend to Fig. 1, with the exception of the flow-rate which was initially 2 ml/min and which was increased after 4.5 min to 4.0 ml/min. Regions identifiable on the chromatogram include: A = solvent front; B = PE; C = LPE; D = PC; E = SPH; F = LPC. The mobile phase comprised acetonitrile–methanol–water (300:150:35).

from 5.5 to 8.5% resulted in separation of PE, LPE, PC, LPC, and SPH within 20 min. However, because of our desire to obtain repeated analyses of samples for lysophospholipids in a minimal amount of time, without the need for re-equilibration of columns between, flow programming rather than gradient elution was utilized to decrease the retention time of LPC to less than 15 min as shown in Fig. 3.

DISCUSSION

UV absorption of phospholipids is due largely to the $\pi \rightarrow \pi^*$ transition (λ_{max}, 165 nm) of long chain olefins present in the C-2 fatty acid side chains of most naturally occurring phosphoglycerides[9]. Although detection based on UV absorption is convenient, several limitations are inherent to this approach: (1) UV detection requires that the mobile phase has an UV cutoff smaller than 210 nm. Isocratic elution is preferable to gradient elution because it avoids excessive deviation of baseline absorbance at this wavelength. (2) The area under a given absorbance peak does not reflect the molar amount of phospholipid eluted but rather the quantity of unsaturated centers present in constituents within the fraction[9]; and (3) a disaturated phospholipid species may precede or follow a peak containing unsaturated acyl groups detected by UV monitoring.

In the separation described here, all mobile phase components had a UV cutoff <210 nm. Because the constituents are separated in a volatile mobile phase, quantification of fractions after concentration, by scintillation spectrometry and/or phosphate assay is facilitated. In addition, the isocratic technique utilized avoids marked baseline absorbance change favoring accurate integration of peak areas and identification of peak borders.

If quantitative analysis of phospholipid components in a mixture with constituents of known unsaturation is desired, area integration provides an accurate method of analysis[9]. If, however, the degree of unsaturation is unknown, concurrent analysis of phosphate is necessary. The ratio of peak area to phosphate depends on the average number of unsaturated centers per mole of phosphate which we will refer to as an unsaturation index. Analysis of changes in the unsaturation index accompanying physiological or pathophysiological processes may reflect alterations in membrane fluidity due to changes in fatty acid unsaturation.

It has been shown previously with silica based columns that the retention time of individual phospholipids varies with the fatty acyl constituents[10]. Thus, UV transparent phospholipids (*i.e.* disaturated phospholipid species) could be eluted in fractions other than those containing their UV opaque counterparts. We have attempted to exclude errors attributable to this possibility by quantifying radioactivity in dipalmitoyl PE, dipalmitoyl PC, and palmitoyl LPC. In each case more than 95% of the radioactivity in the sample was recovered within the fraction exhibiting a UV peak with the corresponding unsaturated phospholipid.

Most membrane lipids contain either a primary or quarternary amino functional group which leads to retention by the cation-exchange column employed. Phospholipids that do not contain an amino functional group but which appear to be relatively polar on silica based HPLC systems (*e.g.* phosphatidyl inositol) are eluted in the void volume. Thus, the bi-modal nature of the separation employed (charge and polarity) is evident, reflected by retention of charged functional groups by the ionic

stationary phase and further separation achieved by partitioning on the basis of polarity facilitated by the use of a highly organic mobile phase.

We have recently reported the contamination of nominally synthetic lysophosphoglycerides from commercial sources with phospholipase A_2 (PLA_2) (ref. 11). The HPLC system described provides a convenient means of purification of such contaminated material. With a solvent system comprising acetonitrile–methanol–water (300:100:70) LPC can be purified promptly, free from detectable PLA_2 activity, with 95% recovery based on phosphate analysis (Fig. 4). With a mobile phase comprising acetonitrile–methanol–water (400:100:34) comparable recovery of similarly purified LPE can be accomplished readily. As much as 1 mg of palmitoyl LPC can be applied to the HPLC column in one injection (20-μl volume) with retention of peak geometry. As little as 0.3 nmoles of naturally occurring PE or PC can be detected easily (UV absorbance 0.2 a.u.f.s.).

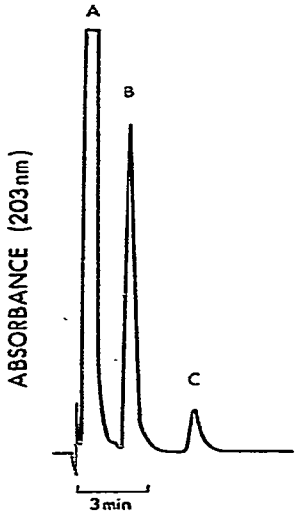

Fig. 4. The effects of a polar mobile phase on retention time. Conditions are the same as those indicated in the legend to Fig. 1, except for the composition of the mobile phase, which was acetonitrile–methanol–water (300:100:70).

The HPLC system developed and characterized in this study appears to be well suited to rapid and efficient separation of most phospholipid constituents present in biological membranes. The technique is particularly well suited to the quantitative analysis of material in extracts of tissues and is readily adaptable for the additional analysis of phosphate and radioactivity often required. Although PE may be contaminated by small amounts of PS present in such extracts, the separation of lysophosphoglycerides, PC, and SPH appears to be complete and rapid. The use of isocratic elution allows repeated separations without column re-equilibration. Retention times for specific moieties of interest such as LPC can be shortened even further with flow programming.

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