Journal of Chromatography, 227 (1982) 25—31
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1073

IMPROVED PROCEDURE FOR THE SEPARATION OF PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 29th, 1981; revised manuscript received August 19th, 1981)

SUMMARY

We describe a rapid and efficient high-performance liquid chromatography procedure for the separation of phospholipids. The separation is accomplished on a microparticulate silica gel column using isocratic elution and UV detection at 203 nm. The solvent mixture is acetonitrile—methanol—85% phosphoric acid(130:5:1.5, v/v). Complete separation is achieved within 30 min of phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidyldimethylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine and sphingomyelin. The method is suitable for the analysis of phospholipids in tissue extracts.

INTRODUCTION

Currently the use of high-performance liquid chromatography (HPLC) for the analysis of phospholipid constituents present in biological membranes is still limited. A major obstacle to the development of a satisfactory method for this application has been the problem of detection. Refractive index and flame ionization detection methods are insensitive [1]. UV detection is sensitive and non-destructive, but the 200-nm range of phospholipid absorbance limits the choice of eluting solvents to those which do not absorb in that region. The solvent systems described in previous reports [2—5] provide the separation of only a few phospholipids. These methods are inadequate for the analysis of phospholipids in tissue extracts. It is well recognized that in the separation of phospholipids by silica gel thin-layer plates the presence of either acids or bases in chloroform—methanol—water solvent mixtures greatly improves the resolution. By analogy with thin-layer chromatography (TLC) we have developed an HPLC procedure using a silica gel column and a solvent mixture of ace-

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tonitrile—methanol—85% phosphoric acid. With this procedure all the major phospholipid components in tissue lipid extracts can be separated in a single run.

EXPERIMENTAL

Materials

Soybean phosphatidylinositol, bovine brain phosphatidylserine and lysophosphatidylserine, egg yolk phosphatidylethanolamine and lysophosphatidylethanolamine, egg yolk phosphatidylcholine, bovine liver lysophosphatidylcholine, egg yolk sphingomyelin, dipalmitoyl phosphatidic acid and dipalmitoyl phosphatidylglycerol were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine were obtained from Gibco (Grand Island, NY, U.S.A.). Acetonitrile and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Phosphoric acid, 85%, was of analytical grade from Mallinckrodt (St. Louis, MO, U.S.A.). [^{14}C-Methyl]phosphatidylcholine was obtained from New England Nuclear (Boston, MA, U.S.A.).

Tissue lipid extracts

Sprague-Dawley male rats weighing 150 g were used. They had access to the diet up to the time of sacrifice. Immediately after decapitation, heads and livers were placed in liquid nitrogen. Erythrocytes and serum were obtained from a healthy human donor. A 1-g amount of rat tissue, 1 ml of erythrocytes or 1 ml of serum was homogenized in 30 ml of chloroform—methanol (2:1, v/v). After filtration the lipid extract was separated into two phases according to the procedure of Folch et al. [6]. The lower phase was dried under nitrogen and redissolved in chloroform before HPLC analysis.

Chromatographic conditions

We used a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of a Model 6000 solvent delivery system, a Model U6K injector, a Model 450 variable-wavelength detector and a strip chart recorder. The chromatographic column was a 30 cm × 4 mm I.D. prepacked stainless-steel Micro-Pak SI-10 column (Varian Assoc., Palo Alto, CA, U.S.A.), which contained silica gel, particle size 10 μm. The acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v) solvent was delivered to the column at a flow-rate of 1 ml/min at a pressure of approximately 34 bar (500 p.s.i.) at room temperature (21°C). The detection was at 203 nm. The reference cell contained air. Phospholipid standards and tissue lipid extracts were dissolved in chloroform. Sample volumes and the recorder response are indicated in figure legends. Each day after the analysis the column was washed successively with 30 ml each of methanol—water (1:1, v/v), methanol and dichloromethane before storing it overnight in *n*-hexane.

RESULTS

Several solvent systems, made up of acetonitrile—methanol—85% phosphoric acid in various proportions, were tested for their ability to separate mixtures of phospholipid standards. An isocratic mobile phase containing acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v) was found to be successful in separating the six major phospholipid components present in tissue extracts: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SPH). Fig. 1 shows a representative chromatogram. Most of the minor phospholipids, except for phosphatidyldimethylethanolamine (PDME) and lysophosphatidylethanolamine (LPE), co-eluted with other phospholipids and could not be resolved. Cardiolipin and neutral lipids were eluted with the solvent front. Phosphatidic acid (PA) and phosphatidylglycerol (PG) had the same retention time and were partially co-eluted with PC. Their distinctive

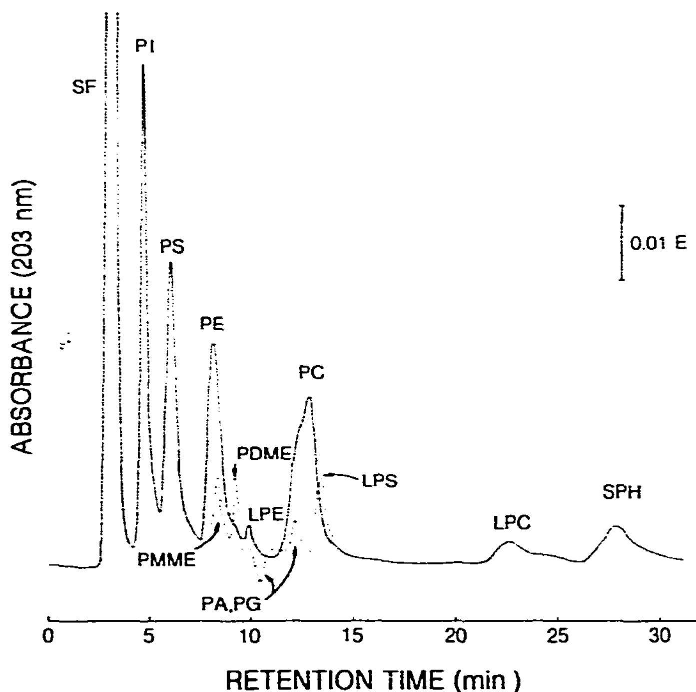

Fig. 1. Chromatogram of phospholipid standards. The amount injected was 1.5 µl of chloroform containing 0.5 µg each of PS, PE and PC, 2.5 µg each of PI and SPH, and 5 µg each of LPC and LPE. Retention times of minor phospholipid classes were determined by separate injections and are indicated by dotted lines. Chromatographic conditions: flow-rate, 1 ml/min; mobile phase, acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v); UV detection at 203 nm; recorder response 0.1 a.u.f.s.; and ambient temperature. Peaks: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; SF, solvent front; and SPH, sphingomyelin.

pattern, characterized by having both a trough and a peak, was also observed by Kiuchi et al. [1] using a flame ionization detector. The mechanism for this pattern is not known. The identity of peaks on the chromatogram was established by injecting into the chromatograph the individual phospholipid standard as well as mixtures of standards. The recovery of phospholipid applied to the column was determined with [^{14}C]-phosphatidylcholine (approximately 9000 dpm per injection), and was found to be greater than 95%.

Before sample analysis the silica gel column was stored in *n*-hexane (see Experimental). Upon changing to a new solvent mixture, sufficient time was allowed for the column to become re-equilibrated. Fig. 2 illustrates that the resolution of phospholipids improved as the time of pumping the solvent through the column increased. If a complete separation of LPC and SPH is desired, we recommend that the column be equilibrated for more than 5 h.

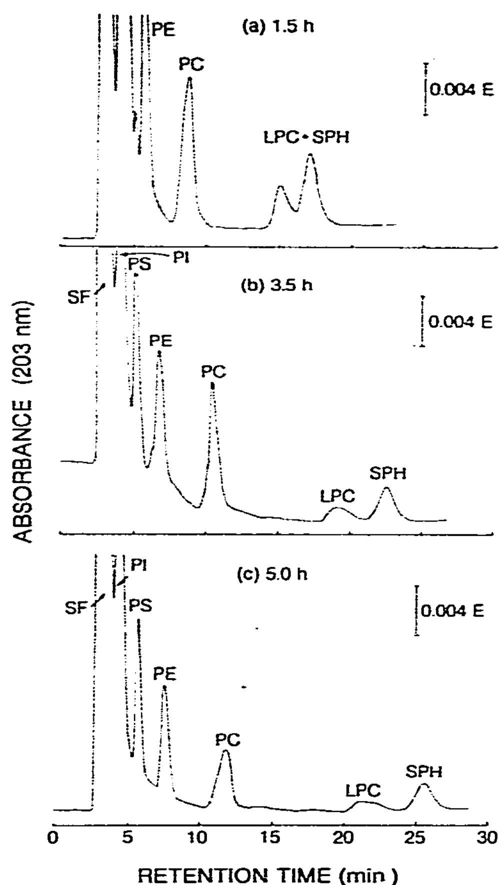

Fig. 2. Influence of equilibration time on the separation of phospholipids. The column absorbant was originally stored in *n*-hexane. The solvent, acetonitrile—methanol—85% phosphoric acid (130:5:1.5), was pumped through the column for (a) 1.5 h, (b) 3.5 h, or (c) 5 h before the injection of samples. Sample volumes were 7 μl and recorder response 0.04 a.u.f.s. Other conditions were the same as in the legend to Fig. 1.

Aliquots of lipid extracts from rat brain, rat liver, human erythrocytes and human serum were injected directly into the chromatograph for analysis. In rat liver (Fig. 3a, b) and brain (Fig. 3c, d) PI, PS, PE and PC peaks were readily detected. The peak of SPH was detectable only when a larger aliquot was injected or when the sensitivity of the recorder was increased. PS, PE, PC, LPC, and SPH were major constituents in erythrocytes (Fig. 3e), while PI was not detectable under the analytical condition. It is noteworthy that the Folch procedure is not well suited for the extraction of erythrocyte lipids [7]. Despite the use of crude Folch extracts, the HPLC method revealed phospholipid peaks free of interferences by other materials. In the serum (Fig. 3f) PC was the largest component. PE, LPC and SPH were present, although in much smaller amounts than PC. PI and PS were not detectable. These patterns are consistent with the published results on the quantitative analysis of rat liver [8], rat brain [9], human erythrocytes [7] and human serum [10].

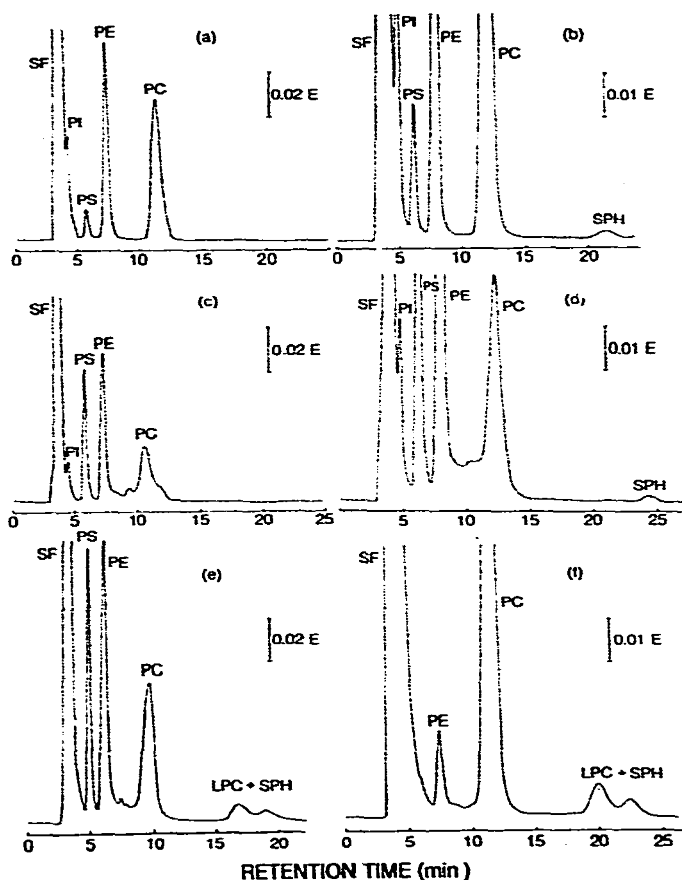

Fig. 3. HPLC analysis of lipid extracts. Lipids were extracted from tissues as described under Experimental. (a) Rat liver, 3.8 μg lipid P; sensitivity, 0.2 a.u.f.s.; (b) rat liver, 7.0 μg lipid P; sensitivity, 0.1 a.u.f.s.; (c) rat brain, 3.6 μg lipid P; sensitivity, 0.2 a.u.f.s.; (d) rat brain, 5.6 μg lipid P; sensitivity, 0.1 a.u.f.s.; (e) human erythrocytes, 1.0 μg lipid P; sensitivity 0.2 a.u.f.s.; and (f) human serum, 2.0 μg lipid P; sensitivity, 0.1 a.u.f.s. Other conditions were the same as in the legend to Fig. 1. Lipid P is phosphorus in lipid extracts.

DISCUSSION

The uniqueness of the method described in this report is the solvent mixture of acetonitrile—methanol—85% phosphoric acid (130:5:1.5, v/v). Compared with the method of Jungalwala et al. [2], which used a silica gel column and a solvent mixture of acetonitrile—methanol—water (65:21:14, v/v), the presence of phosphoric acid greatly improves the resolution. It permits the separation of all the major membrane phospholipid constituents in a single run without using gradient elution. Jungalwala's method is effective in separating PC, SPH, and LPC. However, PS co-elutes with PE. PI is not resolved. The methods reported by other investigators also have various limitations for the analysis of phospholipids in tissue extracts. With a silica-based cation-exchange column and an isocratic mobile phase of acetonitrile—methanol—water, Gross and Sobel [4] were able to separate PC, LPC, LPE and SPH. But, they failed to resolve PS and PE. The method of Geurts van Kessel et al. [3] employed a silica gel column and gradient elution using hexane—isopropanol—water mixtures. They reported separating cholesterol, PA, PE, PI, PS, LPC and LPE, whereas PC and SPH were only partially resolved. Hanson et al. [5] utilizing a silica-based anion-exchange column and gradient elution separated the lipid extract from egg yolk into neutral lipid, PC, SPH, LPC and PE fractions. PI and PS could not be eluted.

UV absorption by lipids at the 200-nm region is due largely to the presence of double bonds [2]. The absorption by other functional groups, such as ester carbonyl and amino, also occurs, but it is small in extent. Being sensitive, convenient and non-destructive, UV detection is ideal for monitoring the separation of lipids by HPLC. However, it is complicated to use UV detection for lipid quantitation, because the area under a given absorbance peak reflects the number of double bonds rather than the number of molecules. Previous investigators [2] suggested two methods for the quantitation of phospholipid fractions. First, the quantity is calculated from the apparent molecular extinction coefficients (ϵ] of the material analyzed and the peak area. The apparent ϵ is determined by performing HPLC of a representative sample, the concentration of which is already known, and measuring the UV response. Second, the effluents from specific peaks are collected and quantified by independent methods. It should be noted that our solvent mixture contains phosphoric acid and may interfere with phospholipid quantitation, since most chemical methods employ acid digestion to liberate phosphorus from phospholipid, which is then measured. It is necessary to use only those methods which do not involve acid digestion [11,12]. Another disadvantage of our method is that the use of acidic solvents may lead to a degradation of plasmalogens.

Jungalwala et al. [13], and we [14] previously used a different approach for the quantitative analysis of phospholipids that contain primary amino groups, i.e., PE, LPE, PS and LPS. Before HPLC analysis these phospholipids are converted into either UV-absorbing biphenylcarbonyl derivatives [13] or fluorescent dansyl derivatives [14]. In these instances, the peak area reflects the amount of phospholipid eluted.

In biochemical research it is a common procedure to measure the radioactivity in the phospholipid fraction following the administration of isotope-labeled lipid precursors to cell cultures. Examples are the measurements of PI turnover

and methylation of PE. These are crucial events occurring on the cell surface in the stimulus—receptor interactions [15, 16]. Being efficient in separating PI, PDME and PC, the HPLC procedure described in this report is well suited for this application. Compared to conventional TLC methods, the HPLC method should be less laborious and more accurate.

ACKNOWLEDGEMENT

This investigation was supported by the Veterans Administration.

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