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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF EGG YOLK PHOSPHOLIPIDS

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SUMMARY

A rapid and efficient method for the separation of egg yolk phospholipids by normal-phase high-performance liquid chromatography has been developed. The separation is accomplished on an Ultrasil-NH_2 column using hexane, isopropanol, methanol and water mixtures with direct ultraviolet detection at 206 nm. Phospholipids from a methanol extract of fresh lyophilized egg yolks were analyzed providing complete separation of (in order of elution) neutral lipids, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine and phosphatidylethanolamine. Identification of eluting species was accomplished by comparative retention times of standard samples and by thin-layer chromatographic analyses of collected fractions. Furthermore, a mixture of naturally occurring phospholipid standards from bovine and egg sources has been separated by this method. In addition to separating the individual classes of phospholipids, in some instances, separation of molecular species within a class was achieved as in the case of cerebrosides, sphingomyelin and partially with phosphatidylethanolamine.

INTRODUCTION

Egg yolk phospholipids are popularly used as emulsifiers in the manufacture of pharmaceutical emulsions. Column and thin-layer chromatography (TLC) techniques for analysis of phospholipids can be time-consuming and elaborate. An appropriate high-performance liquid chromatography (HPLC) method would be of great practical advantage as an analytical tool as well as for purification or preparation of selective mixtures of phospholipids on a preparative scale.

The application of HPLC as a technique to separate neutral lipids is well documented[1–3]. However, documentation of this technique for the separation of polar lipids, such as phospholipids, is limited. Phospholipids vary in their degree of hydrophobic and hydrophilic nature owing to different fatty acid moieties and phosphate substituents, respectively. The amphipathic character of polar lipids makes HPLC separation challenging.

HPLC methods using silica gel columns to separate a variety of phospholipids have been reported[4–7]. Hax and co-workers reported separating phospholipids ex-

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tracted from suboesophageal ganglia of snail[4], a synthetic mixture of phospholipids[4,5] and phospholipids extracted from erythrocytes[5]. Though complete separation of the four lipid classes of the synthetic mixture was achieved, in the remaining analyses two major lipid classes, phosphatidylcholine and sphingomyelin, were only partially separated. Jungalwala and co-workers[6,7] also utilized silica gel columns, UV detection, and multicomponent solvent systems to separate phospholipids from a variety of sources. Though the methods were effective in separating phosphatidylcholine and sphingomyelin, phosphatidylserine when present co-eluted with phosphatidylethanolamine. In addition to the resultant co-elution of some lipid classes, these methods used water in the mobile phase while using silica gel as the stationary phase. Recently, another HPLC method for separating phospholipids using silica columns and UV detection was reported by Gross and Sobel[12]. Complete separation of phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine was achieved using acetonitrile–methanol–water as the eluent. In our practice the silica gel was quickly deactivated by the water component of the mobile phase and required lengthy regeneration processes.

Another HPLC method developed by Kiuchi *et al.*[8] for separation of phospholipids used an amine packing material with a multicomponent solvent system that included chloroform as well as water as eluent components. With a solvent combination containing chloroform as a major component, UV detection is precluded by the absorption of chloroform in the detection region of phospholipids. Therefore, a flame ionization detector (FID) was utilized. The FID is often incompatible with multicomponent solvent systems that create interferences with the detection of the sample. Additionally, while UV detection is a non-destructive mode of detection, the FID requires destructive analysis of the sample.

This paper demonstrates the utilization of HPLC to provide a rapid and efficient method to separate, isolate and identify components of phospholipids extracted from chicken egg yolk. Direct UV detection and a column packing material resistant to water are used, thereby providing advantages of non-destructive detection and convenient use of water in the mobile phase. TLC analyses are described as applied for analysis of collected fractions to confirm species identification. The application of this method in separating a synthetic mixture of phospholipids into seven major classes of lipids and eleven species in less than 30 min is also demonstrated. The capacity of this assay for application to other types of phospholipids is exemplified by the separation of a mixture of naturally occurring phospholipid standards.

EXPERIMENTAL

Instrumentation

The HPLC apparatus was assembled from commercially available components. The two pumps used were dual-piston reciprocating type, Altex Model 100A (Altex, Berkeley, CA, U.S.A.). An Altex/Hitachi Model 100-30 (Altex) variable-wavelength UV detector set at 206 nm was employed for the detection of the eluting species. The detector was attached to a Hewlett-Packard Model 3380A integrator (Hewlett-Packard, San Diego, CA, U.S.A.) for recording and integrating the peaks corresponding to the eluting compounds.

The column employed for analysis was an Ultrasil-NH_2 (Altex) with a 10-μm packing in a stainless-steel tube (250 × 4.6 mm I.D.). A stainless-steel precolumn (40 × 3.2 mm I.D.) was packed by the incremental dry method with a polar bonded-phase packing (Vydac PBP, Altex), 30–44-μm diameter.

Reagents

Two mobile phases were prepared: solvent A, hexane–isopropanol (5.5:8.0), and solvent B, hexane–isopropanol–methanol–water (5.5:8.0:1.0:1.5). The solvents were degassed by sonication for 15 min after preparation. LiChrosolv HPLC grade hexane, isopropanol and methanol (E. Merck, Darmstadt, G.F.R., purchased from Scientific Products, Irvine, CA, U.S.A.) were used for HPLC analyses. The water used was deionized, distilled and filtered through submicron filters (1.2, 0.45 and 0.22 μm).

Solvents for TLC were analytical reagent grade (Mallinckrodt, St. Louis, MO, U.S.A.) and each was dried over molecular sieve 3A prior to use. TLC was conducted on 20 × 20 cm chromatoplates of 0.25-mm thick silica gel 60 (E. Merck) and KC_{18} chromatoplates, 0.20 mm thick and 20 × 20 cm (Whatman, Clifton, NJ, U.S.A.), for reversed-phase TLC. Reagents for visualization were ninhydrin (E. Merck), molybdenum blue (Applied Science Labs., State College, PA, U.S.A.), Dragendorff's reagent (Applied Science Labs.) and iodine vapor (U.S.P. resublimed crystals, Mallinckrodt).

Standards

Natural polar lipids derived from egg used as standards were phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine. Natural polar lipids derived from bovine used as standards were sphingomyelin, cerebrosides, phosphatidylinositol, phosphatidylserine and lysophosphatidylethanolamine. Fatty acid standards were oleic acid, linoleic acid, linolenic acid and arachidonic acid. Other standards included cholesterol, cholesterol oleate and phosphatidic acid. Phosphatidic acid was purchased from Avanti Biochemicals, Birmingham, AL, U.S.A. All other standards were purchased from Applied Science Labs.

Procedure

To prepare the phospholipid samples, fresh lyophilized egg yolk (50 g) was extracted three times with 100-ml portions of HPLC grade methanol. The filtrate was then concentrated to 25% of the original volume and analyzed by HPLC.

Standards were prepared for HPLC and TLC analysis by evaporating to dryness those supplied in a solvent, dissolving in HPLC grade methanol (*ca.* 10–20 mg/ml) and filtering through a 0.2-μm fluoropore filter (Millipore, Bedford, MA, U.S.A.).

The chromatographic system was programmed for gradient elution using the two mobile phases described. The percentage of solvent B in solvent A was increased from 55% to 100% in a two-stage gradient as shown in Fig. 1. The initial flow-rate was 0.7 ml/min and was increased at 3 min to 0.85 ml/min. The extracted phospholipid (20 μl, *ca.* 3 mg) was injected onto the column and the eluted substances were detected spectrophotometrically using UV detection at 206 nm with a sensitivity of 2.0 a.u.f.s. The analysis was completed in less than 30 min, providing complete separation of five major peaks.

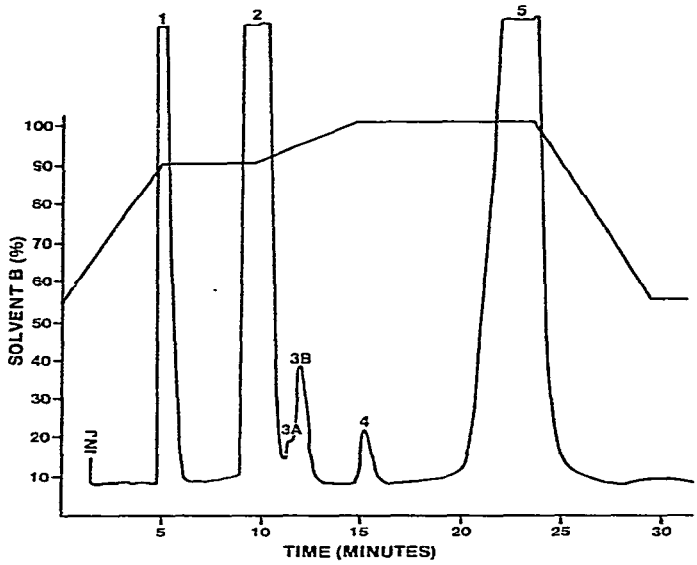

Fig. 1. HPLC separation of methanol-extracted egg yolk phospholipids. Five species: neutral lipids (1), phosphatidylcholine (2), sphingomyelin (3A and 3B), lysophosphatidylcholine (4) and phosphatidylethanolamine (5). Chromatographic conditions: column, Ultrasil-NH_2; eluent, solvent A, hexane–isopropanol (5.5:8.0), solvent B, hexane–isopropanol–methanol–water (5.5:8.0:1.0:1.5); gradient technique used as indicated; flow-rate 0.7 ml/min increased to 0.85 ml/min at 3 min; temperature, ambient; detection, UV absorbance at 206 nm.

RESULTS

Peak identification was accomplished by both comparative HPLC retention times of standards and by TLC of the collected individual fractions. HPLC retention times of neutral lipid standards such as phosphatidic acid, linoleic acid, oleic acid, linolenic acid, arachidonic acid, cholesterol and cholesterol oleate essentially corresponded to the retention time of peak 1, the first eluting peak of the egg yolk phospholipid analysis (Fig. 1). When the material eluting as peak 1 was collected and subjected

TABLE I

IDENTIFICATION OF COMPONENTS IN PEAK 1 BY TLC

Chromatoplates (silica gel 60, 0.25 mm thick, 20 × 20 cm) were spotted with the collected peak 1 material (10 μl) and eluted with diethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2); then in the same direction diethyl ether–hexane (6:94). Iodine vapor was used for development.

Spot	R_F	*Components**
A	0.12	Monoglycerides
B	0.36	Cholesterol
C	0.49	1,2-Diglycerides
D	0.54	1,3-Diglycerides
E	0.67	Triglycerides

* Identification established from R_F values for this solvent system[11]. Cholesterol standard (Applied Science Labs.) and soybean oil for triglycerides standard (Glenco, Glendale, CA, U.S.A.) provided additional data for positive identification of these species.

to TLC analysis, five species were separated and identified as monoglycerides, cholesterol, 1,2- and 1,3-diglycerides, and triglycerides (Table I). Absence of fatty acids was confirmed by reversed-phase TLC analysis eluting with acetonitrile–acetic acid–water (80:10:25). While fatty acid standards had R_F values of 0.31–0.51, the peak 1 material remained at the origin.

The remaining peaks in Fig. 1 were identified as phosphatidylcholine (peak 2), sphingomyelin (peak 3), lysophosphatidylcholine (peak 4) and phosphatidylethanolamine (peak 5) by comparing retention times of standards on HPLC and by TLC analysis of individually collected fractions (Table II). Phosphatidylserine and phosphatidylinositol, when injected as individual standards, did not elute under these assay conditions.

TABLE II

IDENTIFICATION OF REMAINING ELUTING COMPONENTS BY TLC

Chromatoplates: silica gel 60, 0.25 mm thick, 30 × 20 cm.

Peak	*Spot*	R_F*	R_F**	I_2	*Ninhydrin*	*Molybdenum Blue*	*Dragendorff*	*Tentative identification*
2	A	0.0	0.0	+	+	−	−	Unknown***
	B§	0.25	0.6	+	−	+	+	Phosphatidylcholine
3	A	0.14	0.03	+	+	+	+	Sphingomyelin
4	A	0.0	0.0	+	−	−	−	Unknown***
	B§	0.09	0.08	+	−	+	+	Lysophosphatidylcholine
5	A	0.0	0.0	+	−	−	−	Unknown***
	B	0.42	0.21	+	−	+	−	Phosphatidylethanolamine§§
	C§	0.45	0.23	+	−	+	+§§§	

* Solvent system: chloroform–methanol–water (65:25:4).

** Solvent system: ethyl acetate–chloroform–*n*-propanol–methanol–0.25% potassium chloride (25:25:25:10:7) (Applied Science Labs., 1979).

*** The minor unknown spots that remain at the origin may be collected components or oxidation products incurred during workup that are insoluble in the eluent.

§ Indicates major spot.

§§ Two spots of the same R_F value as spots B and C of peak 5 were seen to the same degree in the phosphatidylethanolamine standard.

§§§ The positive choline test is probably due to the unsaturated fatty acids in this component.

To examine better the selectivity of the amine column as well as the capacity of this assay for other phospholipids, combined standards were analyzed. Complete separation of cholesterol, cerebrosides, phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylethanolamine is shown in Fig. 2.

DISCUSSION

The Ultrasil-NH_2 column was effective in the separation of the major components of both methanol-extracted egg yolk phospholipids and a synthetic mixture of natural phospholipid standards. Complete separation of neutral lipids, phospha-

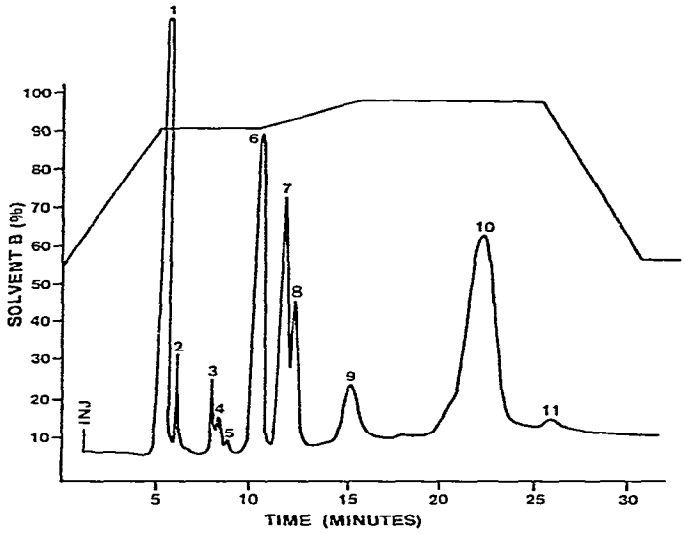

Fig. 2. HPLC separation of a mixture of natural phospholipid standards. Cholesterol (1), cerebroside (2, 3, 4 and 5), phosphatidylcholine (6), sphingomyelin (7 and 8), lyophosphatidylcholine (9), phosphatidylethanolamine (10), lysophosphatidylethanolamine (11). Chromatographic conditions as in Fig. 1.

tidylcholine, sphingomyelin, lysophosphatidylcholine and phosphatidylethanolamine was achieved in the analysis of egg yolk phospholipids (Fig. 1). Further, when present as part of the synthetic mixture, separation of cerebrosides and lysophosphatidylethanolamine was achieved as shown in the analysis of the synthetic mixture of natural phospholipids (Fig. 2).

The major classes of phospholipids were separated. In addition, the capacity to separate the molecular species of a phospholipid class was achieved for cerebrosides, sphingomyelin, and partially with phosphatidylethanolamine. Cerebrosides separated to provide four species (Fig. 2), sphingomyelin separated to provide two species (Figs. 1 and 2), and phosphatidylethanolamine partially separated to provide two species as evidenced by a shoulder on the major peak (Fig. 2).

It has been reported that in addition to cerebrosides (ceramide monohexosides), ceramide di-, tri-, and tetrahexosides also occur in animal tissues[9]; it is assumed that the four peaks separated from bovine cerebrosides may correspond to these species. Similarly it has been reported that sphingomyelin is composed of two species — one containing very long-chain fatty acids and one containing fatty acids of medium chain length[9]. Though uncharacterized, the two peaks of sphingomyelin as separated by this method may represent these two species. Further, the method reported presently allows differentiation of the relative concentrations of each species of sphingomyelin dependent on the source from which they are derived. Recent studies by Do and Ramachandran[10] have indicated that phosphatidylethanolamine from egg yolk contains a species which has an ether linkage instead of an ester linkage at the 1-position of the glycerol backbone. Presumably this could account for the peak and shoulder of phosphatidylethanolamine in the chromatogram.

Comparison of previously reported methods to separate phospholipids with the method reported here reveal significant differences in elution behavior. The HPLC methods for analyzing phospholipids by Hax and co-workers[4,5] and Jungalwala and co-workers[6,7] reported using silica gel with UV detection. Hax and co-workers[4,5] reported separating cholesterol, phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine and lysophosphatidylethanolamine, whereas phosphatidylcholine and sphingomyelin were only partially separated. Jungalwala and co-workers[6,7], using silica gel as the stationary phase and a mixture of acetonitrile, methanol and water as the eluent, reported separating phosphatidylcholine and sphingomyelin, whereas phosphatidylethanolamine and phosphatidylserine co-eluted under these conditions. Phosphatidylserine and phosphatidylethanolamine could be separated when mixtures of dichloromethane, methanol and 15 *M* ammonia were used[7]. The behavior of phosphatidylcholine and sphingomyelin under these conditions was not discussed. The method reported presently using an amine column provided complete separation of phosphatidylcholine and sphingomyelin.

Further, the elution order of phosphatidylcholine and phosphatidylethanolamine differed in analyses using amine columns from analyses using silica gel. Kiuchi *et al.*[8] reported using an amine column to separate dipalmitoyl derivatives of phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine. Phosphatidylcholine and phosphatidylethanolamine were the only phospholipids common to the Kiuchi method and the method reported presently. Both methods employed an amine column for the separation. In both cases, phosphatidylcholine eluted prior to phosphatidylethanolamine. The HPLC methods for analyzing phospholipids reported by Hax and co-workers[4,5] and Jungalwala and co-workers[6,7] used silica gel as the stationary phase. The analyses resulted in the elution of phosphatidylethanolamine prior to phosphatidylcholine.

These results indicate that the amine packing material and silica gel differ in their selectivity in separating phospholipids. Furthermore, peak shape and baseline resolution are better achieved with the amine column, possibly the result of differing particle size, pore size, and theoretical plates of the two packing materials.

The method described presently allows separation of at least seven classes of lipids and a resultant eleven species in a single analysis taking less than 30 min. The utility of analyzing a wide range of phospholipid mixtures which include any or all of these lipid classes has been extended by this method. Additionally, the HPLC method developed here for the separation of phospholipids should provide expanded application of HPLC to other mixtures of amphipathic compounds.

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