A large-scale purification of phosphatidylethanolamine, lysophosphatidylethanolamine, and phosphatidylcholine by high performance liquid chromatography: a partial resolution of molecular species

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Abstract Egg yolk phospholipids, on a 10 g scale, were resolved by high-performance liquid chromatography on an 8-m silica column with elution by a stepwise chloroform–methanol gradient into homogeneous phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and lysophosphatidylethanolamine fractions. Within these fractions, partial resolution on the basis of fatty acyl side chain composition was achieved.

Supplementary key words preparative chromatography · egg yolk phospholipids

High-performance liquid chromatography of lipids has great potential that has only partly been realized. To date, high-performance liquid chromatography has been used for analytical or small-scale preparative (a fraction of a milligram to a few milligrams) separations (1, 2). However, judicious use of this technique is potentially of great value in preparative separations of phospholipid classes on the scale of several grams. It is the objective of this paper to investigate this technique by carrying out familiar separations (3), namely that of egg yolk phospholipids on silica column packing, with use of a high-pressure pump and columns. We have achieved separation of the egg phospholipid components, phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylethanolamine, and phosphatidylcholine.

Recent research, directed towards the elucidation of structure–function relations in biological membranes, has demonstrated the important relationship between membrane function and membrane fluidity (4). The latter property is related to the chain length and the degree of unsaturation and substitution of the acyl side chains of the component phospholipid molecules (5). Information concerning the fluidity characteristics of molecular species of different classes of phospholipids has been primarily confined to synthetic phospholipids with homogeneous acyl side chains. This is primarily due to the difficulty in synthesizing specific molecular species with heterogeneous acyl side chains. An alternative approach is to attempt to fractionate natural phospholipids with respect to degree of unsaturation and in large enough quantities to be employed in a range of physical and functional studies. To this end, we have achieved a preliminary subfractionation of the molecular species of the egg yolk phospholipids on the basis of chain length and degree of unsaturation.

MATERIALS AND METHODS

High-performance liquid chromatography

Separation of lipid components was achieved by means of a silicic acid packing (Biosil HA minus 325 mesh, Bio-Rad Laboratories, Richmond, CA) of 20–40 μm particles in stainless steel columns under pressure. Columns were constructed from type 316 stainless steel tubing with Lichroma finish ordered to our specifications from Handy and Harmon Tube

Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography.

Company (Norristown, PA). A small test column was made from 0.2 in OD × 0.150 in ID Lichroma finish tubing cut to 1 m with a column volume of 12.6 ml; the large column was made from 0.500 in OD × 0.397 in ID tubing cut to 1 m lengths. Eight such meter-length preparative columns were connected in series. An individual length of preparative column had an internal volume of 78.5 ml per segment, 628 ml for the whole system. The fittings and bed support for the test column were Swagelok (Crawford Valve and Fitting, Cleveland, OH) 1/4 in to 1/16 in reducing unions (SS-400-6-1) with an inset 2 μm snubber frit. The fittings and bed support for the preparative columns were Swagelok 1/2 in to 1/16 in reducing unions (SS-810-6-1) with an inset 5 μm snubber frit. Connections between the columns were through standard type 316 stainless steel 1/16 in OD × 1/32 in ID tubing purchased from Tube Sales (Atlanta, GA). The columns were dry packed by adding small amounts of silica while vibrating and rotating the column. Packing of a 1-m column was done in about 45 min. After running a large amount of solvent through the column at high pressure, the fittings were removed and it was observed that there was negligible settling of the bed; this shows that the simple packing procedure suffices for preparative columns of particles in this size range.

Solvent delivery was accomplished by means of an alternating piston pump (Waters Associates, Milford, MA, Model 6000) with flow rate adjustable from 0.1 ml/min to 9.9 ml/min and a pressure limit of 6000 psi. An air-operated six port loop injection valve (ACV-6-UHPa-7000-C20) from Valco Instruments (Houston, TX) was used to deposit the sample at the head of the column. The sample was put into the sample loop using a Glenco Syringe adaptor (Glenco Scientific, Houston, TX) and the injection was triggered pneumatically using a Humphrey Tynamite 4-way air valve (062-4E1, Humphrey Products, Kalamazoo, MI).

Column operation

For this investigation a sample loop of 10 ml volume was used. Crude phospholipids prepared from hen egg yolks comprised the sample (6). Preliminary work, done on the small column, consisted of loading approximately 100 mg and 300 mg on separate occasions and varying the solvent conditions. This was done to examine the effects of sample size elution conditions as well as to find reasonable elution polarities for the individual lipid components. Initial work was done with glass-distilled solvents (from Burdick and Jackson, Muskegon, MI). However, reagent grade solvents filtered through a 0.2 μm Millipore filter were shown to be satisfactory and were used in the large-scale separation. Percentage figures for solvent mixtures refer to percent total volume of methanol added to chloroform.

Elution was done with a step gradient of chloroform–methanol mixtures. Appearance of particular components was monitored by spotting aliquots of fractions on silica TLC (MN Polygram, Silica G without gypsum) plates and staining with ninhydrin or cuprous acetate. Fractions were spotted and run against standards on silica TLC plates for absolute identification (chloroform–methanol–water 65:25:4 (v/v) for phosphatidylcholine, lysophosphatidylethanolamine, and phosphatidylethanolamine; and chloroform–methanol–water 9:7:2 (v/v) for phosphatidylinositol) and assessment of purity.

The large-scale separation used a sample of 10 g of crude egg yolk phospholipids. The sample loop was filled and the phospholipid deposited on the head of the column in chloroform. This process was repeated until the entire 10 g, estimated on the basis of a phosphate analysis, was deposited. This was the same level of loading that had been employed on the small column. Approximately 500–600 ml of chloroform were run through the column to elute the carotenoids. Starting polarity was 10% methanol and conditions of polarity were adjusted according to the appearance or disappearance of particular components in the eluant fractions. The flow rate was maintained at a constant 5 ml/min and fractions of 15 ml were collected. Altogether 844 such fractions were collected, ending at a polarity of 50% methanol. Approximately one additional liter was collected in five flasks using 75–80% methanol. All fractions were covered with nitrogen and stored in the freezer at −20°C.

Fraction analysis

The basic chromatographic profile from the large-scale separation is shown in **Fig. 1**, a graph of total phospholipid concentration vs. fraction number, derived from a phosphate analysis (6) on every 5th or 10th fraction. Additional information was obtained by determining total amino phospholipid concentration in each of these fractions.

Amino group analysis

The usefulness of TNBS as a specific reagent for the colorimetric analysis of amino groups of amino acids and proteins was first described by Satake (7). Siakotos (8) later modified the procedure for water-insoluble compounds by use of an organic reaction medium and demonstrated reactivity of phosphatidylethanolamine and phosphatidylserine. The method

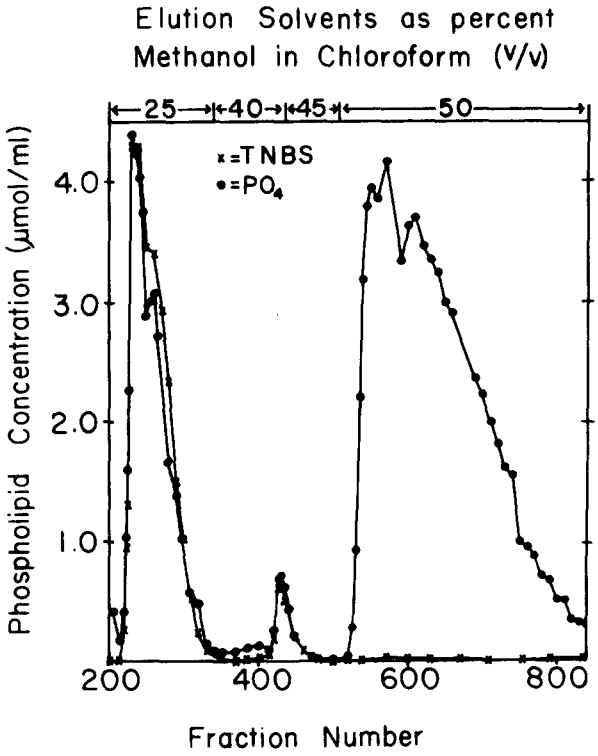

Fig. 1. HPLC profile of the elution of egg phospholipids from a large-scale preparative column, as determined by both phosphate and amino group analysis. Crude egg phospholipids, 10 g, were loaded onto a 0.397 in ID × 315 in column formed from eight equivalent-length sections. The column was eluted with a chloroform–methanol step gradient in 15-ml fractions, at a flow rate of 5 ml/min. The column packing consisted of Biosil HA minus 325 mesh (Bio-Rad Laboratories).

described here utilizes a detergent, Triton X-100, as a solubilizing medium for phospholipids. Complete reactivity of both phosphatidylethanolamine and phosphatidylserine is observed in the detergent labeling system as evidenced by TLC of the reaction mixture.

Amino-containing phospholipid (no more than 0.25 μmol of amino groups) was dispersed in 0.8 ml of 0.4% Triton X-100 in 0.2 M $NaHCO_3$, pH 8.5. A 20-μl aliquot of a 1.5% TNBS in aqueous solution was added and the sample was mixed and allowed to incubate in the dark for 30 min at room temperature. After the incubation period, 0.4 ml of 0.5% Triton X-100 in 1.5 N HCl was added to the sample, followed by mixing and storage in the dark. Absorbances should be measured within an hour of acidification. The absorbance was read at 410 nm, at which wavelength one obtains 1.13 absorbance units per 0.25 μmol of amino groups. The absorbance was linear to at least 1.5 absorbance units. The TNBS solution was made up fresh each week and stored in a brown bottle at 4°C.

Gas–liquid chromatography

Fractions within the peak of an individual phospholipid class were selected for acyl side chain analysis by GLC. Both preparation of the methyl esters of the acyl side chains of the phospholipids in these fractions and the chromatography were as previously described (9), with the exception that the column was held at an initial temperature of 160°C for 15 min prior to the rise to a final temperature of 210°C at the rate of 6°/min. Peak areas were used to determine percent composition of the individual side chain species in the selected fractions.

RESULTS

Calculation of theoretical plates (theoretical plates = $16\,V^2W^{-2}$ where V is elution volume and W is peak width) is often used as one criterion for the resolving power of a column (10). While this criterion has some drawbacks in evaluating a step gradient elution of the type employed in this study, it still is valuable for comparison with other separation methods. Both the phosphatidylethanolamine and the phosphatidylcholine are mixes of heterogeneous diacyl species. Only in the case of lysophosphatidylethanolamine is the fatty acid analysis a direct identification of molecular species. Therefore, only lysophosphatidylethanolamine serves as an adequate estimate of theoretical plates. The lysophosphatidylethanolamine elution profile corresponded to 6,500 theoretical plates; this is much better than TLC separations which are in the range of one hundred to a few hundred theoretical plates. With precisely the same column and packing, it is possible to achieve higher resolving power by lighter loading. In our case, baseline separation of the phospholipid classes was obtained at a 10 g loading. Perhaps an even greater loading at the expense of resolving power could be used without destroying the separation.

Using the 100 mg loading of egg phospholipid on the small column resulted in late appearance of the phospholipid components relative to a simple gravity column on silicic acid. Phosphatidylethanolamine, the first component, did not appear until elution polarity reached 40% methanol, while phosphatidylcholine did not appear until 70 or 80% methanol. The heavier loading of 300 mg caused an earlier elution of the phospholipids, with some loss of resolution. Phosphatidylethanolamine appeared after approximately 30 ml at 20% methanol and phosphatidylcholine at 50–60% methanol. The separation was good enough to serve as a basis for scaling up to the large preparative column. Fig. 1 shows that

the peak positions in the large-scale preparation agree with the preliminary work. Phosphatidylethanolamine appeared at 25%, lysophosphatidylethanolamine at 40%, and phosphatidylcholine at 50% methanol; complete separation of the phospholipid components was achieved. Phosphatidylinositol eluted as a small peak just before lysophosphatidylethanolamine.

An analysis of each phospholipid class, with the exception of phosphatidylinositol, was made by plotting the percent composition of each of the individual acyl side chains against the fraction number. The early fractions of phosphatidylethanolamine were enriched in polyunsaturated acyl side chains while the later fractions were composed of the more saturated acyl side chains. It is interesting that while the composition of polyunsaturates was relatively high in fractions 225 through 250 and dropped almost to zero in the later fractions, the percent total of unsaturated acyl side chains remained approximately constant across the phosphatidylethanolamine peak, thus indicating a nearly one to one ratio of saturated to unsaturated acyl side chains per molecule. Although the level of resolution did not allow an absolute identification of molecular species, it is clear that the 18:0, 20:4, and 22:6 acyl side chains showed parallel fall off with increasing fraction numbers, while 16:0, 18:1, and 18:2 showed a parallel rise with increasing fraction number (**Fig. 2**). This implies a preferential pairing of the 18:0 with the longer polyunsaturated 20:4 and 22:6 while the 16:0 pairs preferentially with the 18:1 and 18:2.

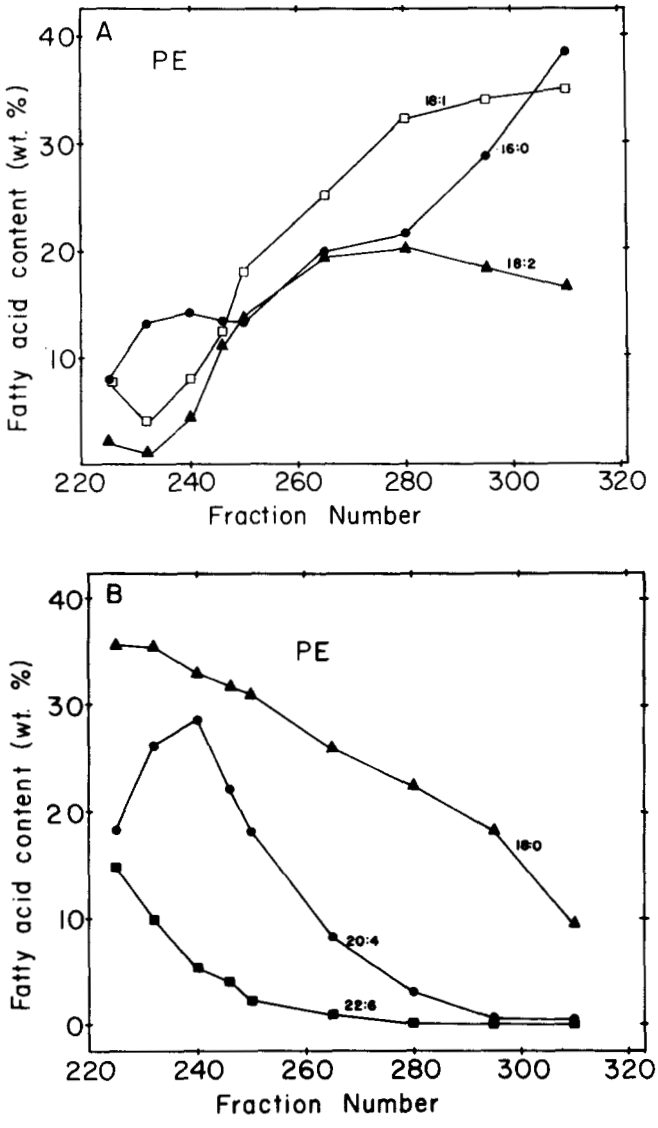

Fig. 2. Acyl side chain composition of fractions across the phosphatidylethanolamine (PE) elution peak. (*A*) Acyl side chains that increase in composition with increasing fraction number. (*B*) Acyl side chains that decrease in composition with increasing fraction number.

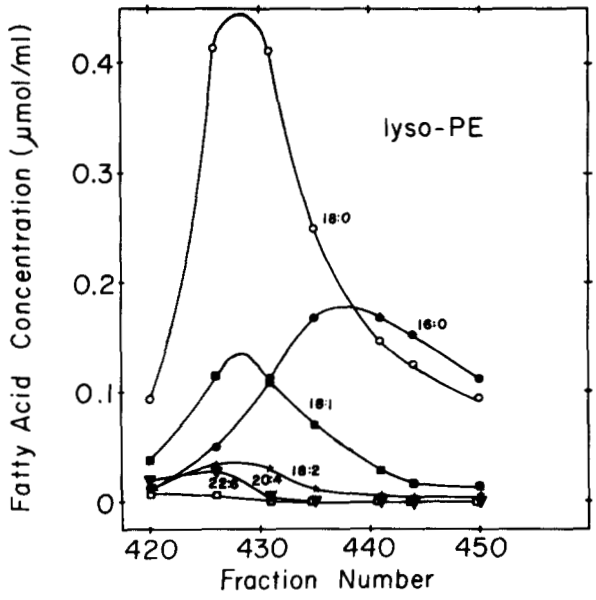

Fig. 3. Acyl side chain composition across the lysophosphatidylethanolamine (lysoPE) elution peak.

Only 20% of the acyl side chains of lysophosphatidylethanolamine were unsaturated (**Fig. 3**), whereas 50–55% of the side chains in phosphatidylethanolamine are unsaturated. The presence of the unsaturated fatty acids could reflect either a mixture of position-1 and position-2 unsaturated isomers of phosphatidylethanolamine or the presence of some percentage of lysophosphatidylethanolamine derived from diunsaturated species.

Phosphatidylcholine, although generally less unsaturated than phosphatidylethanolamine, showed the same trend as phosphatidylethanolamine with respect to containing a relatively high concentration of polyunsaturated acyl side chains in the early fractions, while the later fractions contained more of

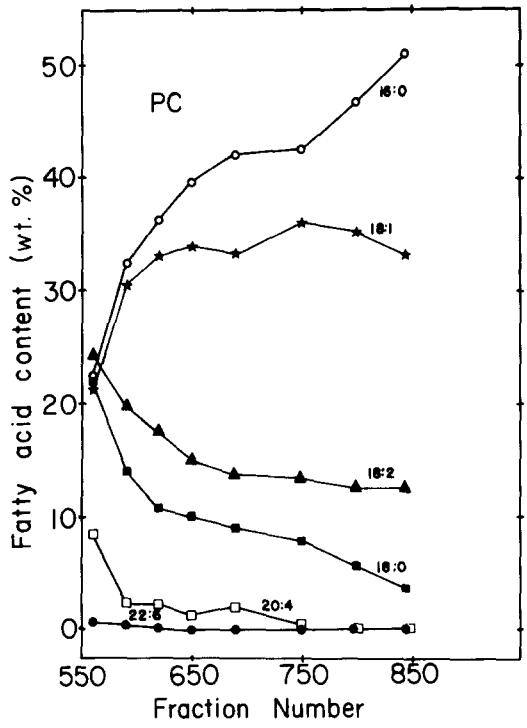

Fig. 4. Acyl side chain composition of fractions across the phosphatidylcholine (PC) elution peak.

the mono- and diunsaturated and saturated acyl side chains (**Fig. 4**). The association of 20:4 and 20:6 with 18:0 in the early fractions and of 16:0 with the 18:1 and 18:2 in the latter fractions is similar to that which was observed with phosphatidylethanolamine. The percent of unsaturated side chains remained close to 50% over most of the fractions, rising to 55% in the later fractions.

DISCUSSION

The strategy of approach in liquid chromatography varies considerably with the type of compound to be separated and with the purpose of separation. HPLC has been used primarily as an analytical technique and, in such separations, the most important product is information per unit time, in terms of theoretical plates per unit time at a resolution adequate to separate the components of interest. These separations are usually carried out on small particle (5–10 μm) pellicular (silica shell on an inert glass bead) packings.

For preparative separations the considerations are somewhat different. The small particle packings that are best in a small volume analytical column are too expensive and difficult to pack to be practical in a 500 ml preparative column. We have chosen to use a packing at the small particle size range of what has been previously used in low pressure chromatography of phospholipids, and to take advantage of the capabilities of high pressure pumps and columns by lengthening our system to 26 ft, thereby increasing the resolution. In doing so we have been able to obtain separations significantly better than analytical TLC and at a loading of 10 g.

One approach to preparative separation would be to initially employ a lower resolution separation to obtain partially purified components, then to further resolve these into pure constituents at a lower loading. In our separation, we carried out chemical analyses and TLC on every few fractions to evaluate the separation. For this reason the off-column procedures took much longer than the column separations themselves. Therefore, we have found it most practical to use baseline separations in a single pass over the column. The use of TLC, which has lower resolution than HPLC with respect to lysophosphatidylethanolamine and phosphatidylcholine, as a criterion for performance may at first seem questionable. However, high loading on the TLC plates, coupled with the chemical specificity of the sprays employed, allows a good evaluation of chemical purity of each phospholipid class.

The results obtained here demonstrate the practicality of employing HPLC as a large-scale method of purification of egg phospholipids. A baseline separation was obtained between lysophosphatidyl ethanolamine and phosphatidylcholine. No attempt was made to obtain purified sphingomyelin from the crude egg phospholipid mixture. In addition to the good resolution of individual phospholipid classes, a considerable degree of resolution within each class was obtained with respect to acyl side chain composition. As seen in Figs. 2–4, the leading fractions of each phospholipid peak were very high in polyunsaturated acyl side chains, while the later fractions were almost devoid of these side chains. Although resolution to the molecular level was not obtained in this study, the parallel trends observed for specific acyl side chains across a phospholipid peak were consistent with the actual pairings observed for molecular species in egg yolk phosphatidylcholine (11).

HPLC possesses the potential of yielding even higher resolution by running fractions from the preparative column on a smaller column capable of even higher resolution than the preparative column,

with the goal of obtaining resolution at the molecular level within a phospholipid class. This would be desirable from several points of view. Most importantly it would greatly simplify the analysis of positional distribution of acyl side chains in the phospholipids of various tissues; this process currently involves enzymatic formation of diglycerides, acetylation of the 3-hydroxyl group, and analysis by GLC and is therefore destructive of the original phospholipids (12).

The virtue of obtaining separation at the molecular level within phospholipid classes would be the ability to prepare relatively large amounts of the individual molecular species that could be used in studies aimed at obtaining information concerning both the physical properties of these species and their relation to membrane function. Several homogeneous, disubstituted phospholipid species are available commercially. However, the difficulty of preparing asymmetrically substituted phospholipid species has resulted in only a very restricted number of these types of compounds being commercially available and then at very high prices. The ability to resolve molecular species of phospholipids in a preparative procedure would make these pure, natural phospholipid components available for general use in membrane research and greatly extend the useful range of application of HPLC.

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