

# Rapid separation of gram quantities of phospholipids from biological membranes by preparative high performance liquid chromatography

John S. Ellingson and Robert L. Zimmerman

Department of Pathology, Jefferson Medical College of  
Thomas Jefferson University, Philadelphia, PA 19107

**Summary** We present a detailed description of a rapid preparative high performance liquid chromatography (HPLC) procedure for isolation of large scale quantities (a minimum of 200 mg) of rat liver microsomal and mitochondrial phospholipid classes in 40 min on a 25 × 2.5 cm preparative HPLC column of 7- $\mu$  silica gel particles using a linear gradient of hexane-isopropanol-water mixtures. A minimum of 1.5 g of phospholipids can be quantitatively separated per day into diphosphatidylglycerol (cardiolipin), phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, and a phosphatidylcholine-sphingomyelin fraction. The procedure uses no salts, buffers, acids, or bases, and the isolated phospholipids are suitable for preparing model membranes. — **Ellingson, J. S., and R. L. Zimmerman.** Rapid separation of gram quantities of phospholipids from biological membranes by preparative high performance liquid chromatography. *J. Lipid Res.* 1987. **28**: 1016–1018.

**Supplementary key words** microsomes • mitochondria • liposomes • phosphatidylcholine • phosphatidylethanolamine • phosphatidylinositol • phosphatidylserine • sphingomyelin • diphosphatidylglycerol (cardiolipin)

A considerable number of analytical HPLC procedures for separating phospholipids have been described (for a partial list see ref 1.) which could possibly be scaled up to a preparative level. We needed a rapid HPLC procedure that could conveniently separate gram quantities of phospholipids extracted from different types of membranes isolated from ethanol-fed rats. Our purpose was to prepare model membranes by recombining known amounts of microsomal or mitochondrial phospholipids from ethanol-fed or untreated rats and observe whether the recombined lipid bilayer membranes would have the same response to ethanol as the intact membranes from which they were extracted. We wanted to avoid using elution solvents containing phosphate, because they would complicate the quantitation of lipid phosphate in the isolated fractions, and we did not want to use acid-containing elution solvents, as acids would hydrolyze plasmalogens (1).

We modified the procedure of Geurts Van Kessel et al. (2) and developed a preparative procedure to isolate gram quantities of the extracted membrane phospholipids. These

procedures should be useful to other investigators who need to isolate membrane phospholipid classes in sufficient quantities to examine their chemical and physical properties or make model membranes.

## METHODS

### Preparative HPLC

Up to 200 mg of phospholipids was separated on a 250 × 25 mm Merck Hibar RT preparative column packed with 7- $\mu$  LiChrosorb-Si-60 silica gel (E. M. Science, Cherry Hill, NJ) using the following gradient procedure. The initial solvent mixture of hexane-2-propanol-water 6:8:0.75 (by vol) (solvent A) was run at ambient temperature for 7 min at a flow rate of 18 ml/min, and then a linear gradient was run for 10 min to achieve the final solvent mixture of hexane-2-propanol-water 6:8:1.55 (by vol) (solvent B), which was run isocratically for 25 min. Re-equilibration of the column was achieved by running solvent A for 20 min.

### General procedures

Prior to placing the phospholipids on the column, they were dissolved in HPLC-grade  $\text{CHCl}_3$  and filtered through a 0.45- $\mu$  fluoropolymer ACRO-L13 disposable filter assembly (Gelman Sciences, Inc., Ann Arbor, MI). All solvents were HPLC grade from Fisher Scientific and were filtered through a 0.45- $\mu$  FH type Millipore hydrophobic filter (Millipore Corp., Bedford, MA) and sparged with helium prior to use.

### Column maintenance

The preparative column was protected with an LC-Si Cartridge guard column (Supelco, Inc., Bellefonte, PA) and a 250 × 4.5 mm precolumn packed with 40- $\mu$  Vydac 101 SC Silica (The Separation Group, Hesperia, CA).

Regeneration of the preparative column was accomplished by running the following solvents through the column at 5 ml/min: hexane for 40 min, Supelco reactivation agent (Supelco, Inc., Bellefonte, PA) for 40 min, dichloroethane for 40 min, and finally hexane for 60 min. Columns not in use were stored in hexane. When a stored column was to be reused, it was subjected to a start-up program prior to any separation by flushing the following solvent mixtures through the column at 5 ml/min: solvent A for 10 min, then a 5-min linear gradient to achieve a solvent composition of solvent B, solvent B for 30 min, a 5-min linear gradient to return to the initial solvent A mixture, and finally solvent A for 1 hr. New columns were washed with hexane-2-propanol-water 2:6:1.5 until a stable baseline was attained as recommended by Geurts van Kessel et al. (2).

### Instrumentation

A Rainin-Gilson Autoprep preparative HPLC system with Rainin Rabbit HPX pump modules and 25-ml/min

Abbreviations: HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; CL, cardiolipin; PSI, pounds per square inch.

pump heads were used. Lipids eluted from the column were detected at a wavelength of 206 nm.

### Extraction and analysis of phospholipids

Rat liver microsomes (3) or inner mitochondria membranes (mitoplasts) were prepared (4), lipids were extracted (5), and the phospholipid fraction was separated from the neutral lipid fraction (6). Phospholipid phosphorus was determined by the Bartlett method (7). Phospholipids were chromatographed on Merck 5 × 5 cm high performance thin-layer silica gel 60 plates with a preconcentration zone (EM Science) in a solvent system of methyl acetate-*n*-propanol-chloroform-methanol-0.25% aqueous KCl 25:25:25:10:9 (by vol). Areas containing phospholipids were detected with I<sub>2</sub> vapor, ninhydrin reagent (8), Dragendorff's reagent (8), and a phosphate-detecting spray (9). Two-dimensional quantitative thin-layer chromatographic analysis of the phospholipids was accomplished as previously described (6). Standard phospholipids were purchased from Avanti Polar Lipids, Inc., Birmingham, AL.

## RESULTS AND DISCUSSION

With the preparative procedure, a minimum of 200 mg of standard phospholipids mixed in the same molar ratios as found in rat liver microsomes was separated in 1 hr (40 min for separation and 20 min for equilibration). The elution profiles of the standard phospholipids and the microsomal phospholipids were the same, and a complete separation of PS and PI was obtained (Fig. 1). Mitoplast phospholipids were also separated by the same procedure (Fig. 2). In both procedures, PE was separated from the other phospholipids, and PI was completely separated from PS (Figs. 1 and 2). Cardiolipin eluted with the solvent front (Fig. 2). When the phospholipid fraction applied to the column contains neutral lipids, the isolated CL will not be pure. When necessary, the CL fraction can be rechromatographed using hexane-isopropanol-water 6:8:0.65 to separate it from the solvent front. Sphingomyelin was not completely separated from PC. An isocratic procedure which separates PC from SPH has been described (10) and used on a semipreparative scale (11), so it could be used on a preparative scale.

The column can be equilibrated after the final run of the day and left overnight in the initial solvent without affecting the separation properties. As a result, the column can be used immediately in the morning allowing the maximum number of separations to be accomplished during the day.

The components in the eluted fractions were identified by comparing their retention times to those of standards and by comparing their *R<sub>f</sub>* values and reactions with spray reagents to those of standards on high performance thin-layer chromatograms. No hydrolysis products were observed on the chromatograms, indicating that lipid hydroly-

ysis did not occur during column chromatography or the evaporation process. The recovery from the columns was over 97%, and the mole percent composition of the eluted fractions was the same as determined by quantitative two-dimensional thin-layer chromatography for the microsomal or mitoplast lipid extracts.

The isolated phospholipids were suitable for making model membranes. When the HPLC-separated phospholipids were recombined in the same molar ratio as found in the intact membranes, they retained the same physical properties that they possessed in the intact membrane, in terms of their resistance to disordering by the *in vitro* addition of ethanol (12).

### Technical considerations

It is essential to prepare solvents A and B and form the gradient by changing from 100% solvent A to 100% solvent B. The following procedures do not work: 1) attempting to form the gradient in the instrument's mixing chamber by pumping hexane, 2-propanol, and water from separate reservoirs; and 2) attempting to form the gradient by pumping increasing amounts of water into the hexane-2-propanol mixture. The three solvents do not form a homogeneous mixture fast enough to use those procedures, and inconsistent elution profiles will occur.

When large quantities (100 mg or more) are to be separated, the lipids should be dissolved in 3–5 ml of HPLC-grade chloroform to prevent them from precipitating in the tubing and guard column during the column-loading process. Because of the high flow rates and high pressures in the preparative procedure, lipid samples are pumped onto the column rather than injected from a syringe.

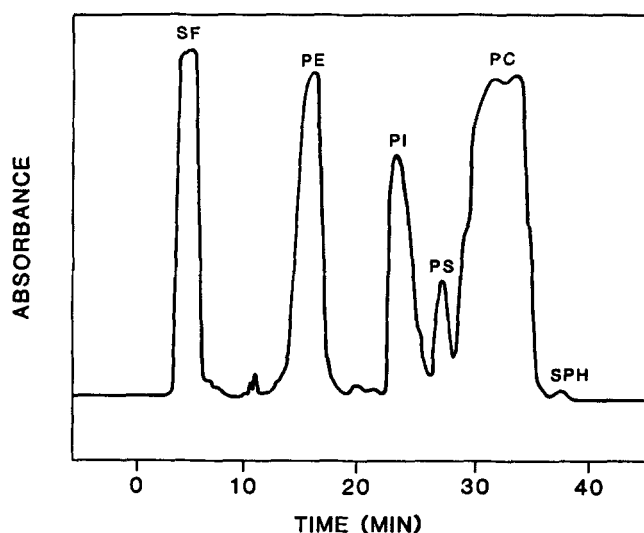


Fig. 1. Elution of extracted microsomal phospholipids by preparative HPLC. Seventy mg of phospholipids dissolved in 3 ml of CHCl<sub>3</sub> was pumped onto the column at 18 ml/min and separated by the preparative HPLC procedure. SF, solvent front.

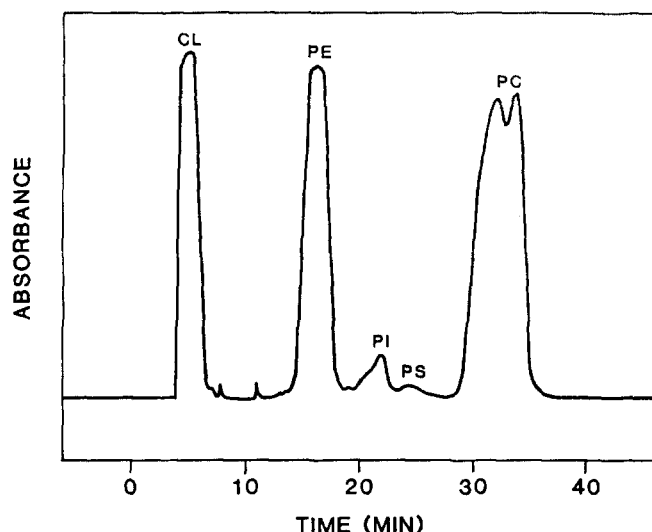


Fig. 2. Elution of extracted mitoplast phospholipids by preparative HPLC. Sixty mg of phospholipids dissolved in 3 ml of  $\text{CHCl}_3$  was pumped onto the column at 18 ml/min and separated by the preparative HPLC procedure.

These preparative procedures work well so long as the column is maintained properly. Because the mobile phases contain up to 10% water, a relatively high amount to use with silica gel, the resolution between PI and PS and/or PS and PC gradually deteriorates. We maintained the separations of the lipids by reactivating the column with the regeneration scheme when any of the separations could no longer be achieved. The ability to regenerate the expensive preparative column is essential for this procedure to be practical. It is very important to use the guard column and the precolumn. The packing in the precolumn and the cartridge in the guard column should be changed when excessively high pressures occur. At a flow rate of 18 ml/min, the initial pressure in our system is about 2200 PSI, and the final pressure is 2800–3100 PSI.

Using these procedures, we have now separated over 160 samples on one column and 130 on another, and both columns are still capable of separating the phospholipids. Therefore, this procedure should be useful to investigators

needing to rapidly isolate gram quantities of membrane phospholipids extracted and separated in the absence of exogenously added ions. ■■

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