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Note

Improved separation of phospholipids by high-performance liquid chromatography

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A number of methods for the separation of phospholipids by high-performance liquid chromatography (HPLC) have been reported [1—4]. Only a few can separate all the surface-active phospholipids (surfactant) usually found in amniotic fluid, lung lavage or lung tissue. Recently two groups have presented fast and efficient methods that use a combination of a DIOL column and a silica precolumn and an acetonitrile—water gradient system at elevated temperatures [1, 3]. We have used the same system, and have found that substitution of water by a 0.005 *M* phosphate buffer (pH 5.0) gave not only baseline separation of seven phospholipids but also much sharper and more symmetrical peaks than those reported in other communications.

EXPERIMENTAL

Two Knauer pumps, a Knauer programmer 50 B and a dynamic mixing chamber (Knauer, Berlin, F.R.G.) were used to form the solvent gradient. The columns used were a 250 × 4.6 mm I.D. LiChrosorb DIOL, 5 μm (Merck, Darmstadt, F.R.G.) fitted with a 30 × 4.6 mm I.D. LiChrosorb Si 60, 5 μm (Merck) precolumn. Both columns were slurry-packed under a pressure of 600 bar. The columns were maintained at 50°C (Knauer column oven), while the solvents were kept at room temperature. All solvents were HPLC grade (Merck) and prior to use degassed under water jet vacuum and mechanical stirring for 10 min. Water was distilled twice in an all-glass still. Sodium

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dihydrogen phosphate was analytical-reagent grade and supplied by Merck. Samples were injected via a Rheodyne sample loop at volumes between 2 and 20 μl. Eluted compounds were monitored at 195 nm with a Gynkotek SP-4 variable-wavelength UV recorder (Gynkotek, Munich, F.R.G.).

Phospholipid standards, phosphatidylglycerol (PG, egg yolk), phosphatidylinositol (PI, soy bean), phosphatidylserine (PS, bovine brain), phospatidylethanolamine (PE, egg yolk), phosphatidylcholine (PC, bovine brain), sphingomyeline (S, bovine brain) and lysophosphatidylcholine (LL, egg yolk) were obtained from Sigma (Munich, F.R.G.). Synthetic dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine were also obtained from Sigma.

HPLC procedure

Two solvents were used as eluents: solvent A was acetonitrile and solvent B was acetonitrile—0.005 *M* sodium dihydrogen phosphate buffer (pH 5.0) (80:20). Solvent B was freshly prepared every day, because on standing for extended time buffer salts may partly precipitate. The system for gradient elution was set up as shown in Table I. The flow-rate was held at 2.0 ml/min and the column back-pressure ranged between 14 and 19 MPa.

The separation achieved by this procedure is illustrated by Fig. 1. The pH influences the retention times of PB, PI and PS, whereas the other phospholipids were not affected by pH changes. An increasing pH decreased the retention times of PG, PI and PE; although an increase from pH 3.0 to pH 6.0 gave an improved separation of PE and PS, PG eluted very early with poor peak shape. The optimum value was found to be pH 5.0.

Extraction of phospholipids from lung tissue and lavage fluid

Lungs from foetal mice (day 17 of gestation) were homogenized for 2 min with 2 ml of chloroform—methanol (2:1) in an all-glass homogenizer at 0°C. The homogenate was transferred into an Eppendorf vial (1.5 ml) and centrifuged at 12 000 *g* (Eppendorf lab centrifuge) for 1 min. The supernatant was transferred into a glass vial and the solvent was evaporated under a stream of nitrogen at room temperature. The lipid residue (containing surfactant and cell membrane phospholipids) was dissolved in 100 μl of chloro-

TABLE I

SYSTEM FOR GRADIENT ELUTION

Time (min)	Percentage solvent B	Percentage solvent A
0	2	98
2	2	98
5	30	70
12	90	10
16	90	10
20	2	98

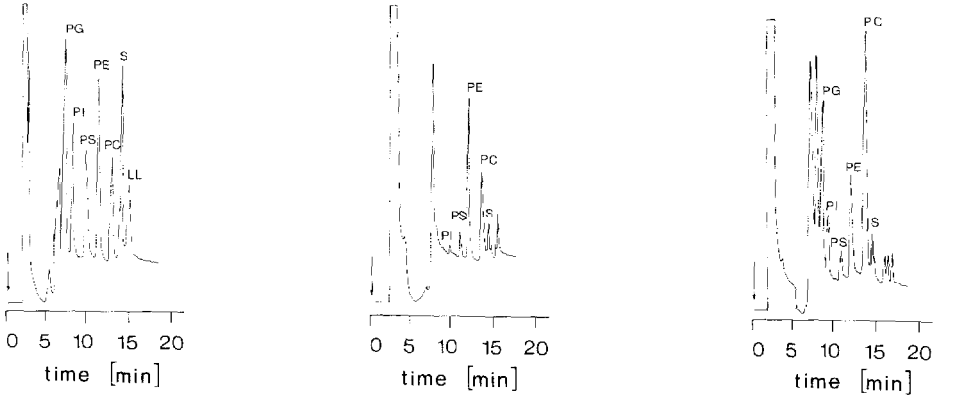

Fig. 1. Chromatogram of phospholipid standards. Detector, 195 nm 0.32 a.u.f.s. Approximately 10 μg of each compound were injected. For abbreviations, see Experimental.

Fig. 2. Chromatogram of phospholipid mixture extracted from foetal mouse lung. Detector, 195 nm 0.16 a.u.f.s. For abbreviations, see Experimental.

Fig. 3. Chromatogram of phospholipid mixture extracted from lung lavage fluid (1 ml), obtained from a 15-year-old patient. An L/S (PC = L) ratio of 12 was calculated from peak heights. Detector, 195 nm 0.16 a.u.f.s. For abbreviations, see Experimental.

form—methanol (2:1), and an aliquot (20 μl) of this solution was submitted to HPLC analysis (Fig. 2).

Lung lavage fluid (1 ml) was vortex-mixed with 3 ml of chloroform—methanol (2:1) for 1 min. After centrifugation for 10 min at 1500 *g*, the chloroform layer was aspirated and evaporated to dryness under a stream of nitrogen. The residue was dissolved and injected as described above (Fig. 3).

The sensitivity of our procedure was established by analysis of several concentrations of the pure phospholipid standards. The minimal detectable amount of each compound was 0.5 μg, and between 1.0 and 20.0 μg there was a linear correlation between the amount of substance injected and the peak height measured. The coefficient of variation for ten repetitive injections of the same sample ranged from 6 to 8% for the pure standards. This applies only to phospholipid standards obtained from biological sources with an unknown moiety of unsaturated fatty acids attached to the glycerol backbone. Sensitivity decreased considerably (approximately factor 10) when synthetic dipalmitoylphosphatidylglycerol or dipalmitoylphosphatidyllecithin were measured.

DISCUSSION

Recently two authors [1, 3] have published HPLC methods for separating phospholipids from biological sources, under similar chromatographic conditions. Briand et al. [1] obtained a separation of PG, PI, PE, PC, S and LL but were not able to separate PE satisfactorily from PS. Furthermore, PG, PI and particularly PS were eluted as broad asymmetric peaks. Chen and Kou [2] reported a separation on a silica column, but PG and PC coeluted partly and S eluted as a broad tailing peak. Better results were presented by Andrews [3], whose procedure is very similar to that published by Briand et al. [1] and our

own study. Although overall analysis time was 16 min (without reequilibration to initial conditions) and all compounds were resolved well, some gave still broad tailing peaks. In the present communication we used 0.005 *M* phosphate buffer instead of water and maintained a pH of 5.0. This resulted in a considerable improvement in both separation and peak shape. An improved resolution facilitates the collection of separated compounds for further analysis and symmetric peaks can be more easily quantitated by peak-height determination. Interestingly, only the acidic phospholipids (PG, PI, PS) are influenced by changes in pH whereas the neutral compounds PC, S and LL are not affected in their retention behaviour. When a gradient with increasing amounts of sodium dihydrogen phosphate was introduced, "ghost-peaks" appeared, mainly before PG was eluted. These peaks, however, also appeared when a gradient was started without sample injection and was therefore not due to degradation products of the phospholipids. The ghost-peaks were invariant with column temperature or pH. Furthermore, one complete analysis requires only 20 min, which includes equilibration of the column to initial gradient conditions. The achieved sensitivity in detection of phospholipids is comparable with procedures reported previously, using UV absorption at wavelengths of ca. 200 nm. This seems to be the limit of UV detection and is due to the lack of strong chromophores in the phospholipid molecules.

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