A preparative high performance liquid chromatography method for separation of lecithin: comparison to thin-layer chromatography

S. Bahrami, H. Gasser, and H. Redl

Ludwig Boltzmann Institute for Experimental Traumatology, Donaueschingenstrasse 13, A-1200 Vienna, Austria

Summary We have developed a high performance liquid chromatography (HPLC) method to separate lecithin from other phospholipid classes and to obtain lecithin from biologic materials. The separation was performed on a preparative 10-μm Spherisorb column with an optimized solvent system consisting of the following components: acetonitrile, isopropanol, methanol, water, and trifluoroacetic acid. The advantages of this method are the use of an isocratic solvent system limited to about 30 min and the very good separation of the phosphatidylcholine fraction from the sphingomyelin fraction. Furthermore, the HPLC method has a better recovery rate than the thin-layer chromatography method, and it can be run under automatic control. — **Bahrami, S., H. Gasser, and H. Redl.** A preparative high performance liquid chromatography method for separation of lecithin: comparison to thin-layer chromatography. *J. Lipid Res.* 1987. **28:** 596–598.

Supplementary key words phosphatidylcholine • dipalmitoyl-phosphatidylcholine • sphingomyelin • rat lung phospholipids • rat lavage lecithin

Thin-layer chromatography (TLC) on silica gel is used widely for separation and identification of lipids extracted from biological samples (1–4). During the last decade, numerous high performance liquid chromatography (HPLC) methods for the separation of phospholipids have been published (5–13). However, the use of HPLC for the analysis of phospholipids present in biological samples is still limited. None of the currently used methods is able to completely separate all of the natural phospholipids usually present during one run.

Our special interest was focused on phosphatidylcholine as a critical component of lung surfactant phospholipids. We developed this HPLC method for the preparative separation of lecithin from all other phospholipid classes. The preparative separation of lecithins was desired for the sake of their further analysis and classification. In the present investigation we identified and collected the lecithin fraction. Quantitation of lecithin species was achieved by gas-liquid chromatography (GLC) using an internal standard (dimyristoyl-phosphatidylcholine). Recovery was assessed by determining the inorganic phosphorus content of HPLC fractions relative to the amount in injected standards.

METHODS

Materials

Lecithin (egg), sphingomyelin (bovine), phosphatidylserine (bovine), phosphatidylinositol (plant), phosphatidylethanolamine (egg), lysophosphatidylethanolamine (egg), lysolecithin (egg), phosphatidylglycerol (synthetic) were purchased from Supelco (Bellefonte, PA). Acetonitrile, isopropanol, and methanol were purchased from Mallinckrodt (Paris, KY). Trifluoroacetic acid was of spectroscopic grade from Merck (Darmstadt, FRG). Dimyristoylphosphatidylcholine (synthetic) was purchased from Serva (Heidelberg, FRG).

Sample preparation

Sprague-Dawley male rats weighing 250 g were killed by exsanguination under halothane anesthesia. The lungs, except for one piece used for tissue analysis, were lavaged 5 times with physiological saline solution (3 ml per lavage). The lavage fluid was centrifuged (500 *g*, 20 min) to remove cells and debris. The supernatant and the unlavaged minced lung were lyophilized and processed as described by Folch, Lees, and Sloane Stanley (14). The extracted lipids were dried under a stream of nitrogen and redissolved in chloroform before HPLC or TLC analysis.

Chromatographic conditions

HPLC. We used a liquid chromatographic system consisting of two model 510 pumps, a model 116 K Injector, and a model 680 automated gradient controller (Waters Associates, Milford, MA) coupled to a 2158 Uvicord SD detector (LKB, Bromma, Sweden), Model 4270 integrator (Spectra Physics, San Jose, CA) and a Frac 100 fraction collector (Pharmacia, Uppsala, Sweden). The chromatographic stainless-steel columns were 250 mm × 16 mm I.D. prepacked with 10 μm Spherisorb S10W (Knauer Berlin, FRG), and a precolumn guard pack RCSS silica (Waters Associates) was used. The acetonitrile–isopropanol–methanol–water–trifluoroacetic acid 135:20:10:6.7:0.85 (v/v) solvent was delivered to the column at a flow rate of 10 ml/min at a pressure of

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine.

approximately 1000 psi (70 bars) at 22°C. Effluent absorbance was monitored at 206 nm. The reference cell contained air. Lecithin fractions were identified by virtue of: *1*) retention times similar to those of lecithin standards, and *2*) co-elution with internal lecithin standards added to the tissue extracts. The collected lecithin fractions were dried under a stream of nitrogen and re-evaporated after addition of 2 ml of methanol. This procedure was repeated twice to remove trifluoroacetic acid contained in the solvent system. The residue was used for determination of inorganic phosphorus content or suspended in Tris-buffer (pH 7.4) for GLC analysis. An equal volume of HPLC solvent was treated identically and used as a reagent blank in the phosphorus assay.

TLC. TLC aluminum sheets silica gel 60 (Merck Darmstadt, FRG) were used after being washed with chloroform-methanol 1:1. All solvents were the highest analytical grade (Merck Darmstadt, FRG). The following solvent system was used for development: chloroform-methanol-water 70:40:3 (v/v). The extracts were applied to thin-layer plates using a Linomat III (Camag, Muttenz, Switzerland) under a stream of nitrogen.

The plates were developed to a height of 15 cm above the solvent level. The silica gel was scraped from the areas of the chromatogram corresponding to the phosphatidylcholine of a reference chromatogram. The phosphatidylcholine was extracted from the silica gel 4 times with chloroform-methanol (1:1) and once with 2 ml of methanol. The collected fractions were dried under a stream of nitrogen and used for determination of inorganic phosphorus or suspended in Tris-buffer (pH 7.4) for GLC analysis.

GLC analysis. The following procedure for GLC analysis was identical in all samples preseparated by HPLC or TLC. GLC was performed with a Carlo Erba Fractovap 4160 and a Crompack fused silica Cp Sil 5 column.

The lecithin fractions were incubated with phospholipase C after being suspended in Tris-buffer (pH 7.4). The diacyl glycerols thus formed were assayed by capillary GLC as the corresponding trimethylsilyl ether (H_2 as carrier gas, temperature program 260-320°C) according to Lohninger and Nikiforov (15).

Total phospholipid. Quantification of total phospholipid was done according to Bartlett (16).

RESULTS AND DISCUSSION

Several previously described methods (5-9) were tested for their ability to produce pure lecithin fractions from lung tissue and/or from lavage fluid samples. None allowed a complete separation of the lecithin fraction from other phospholipid classes, in particular from sphingomyelin.

Therefore, many different solvent systems, composed of acetonitrile-isopropanol-methanol-water-trifluoroacetic

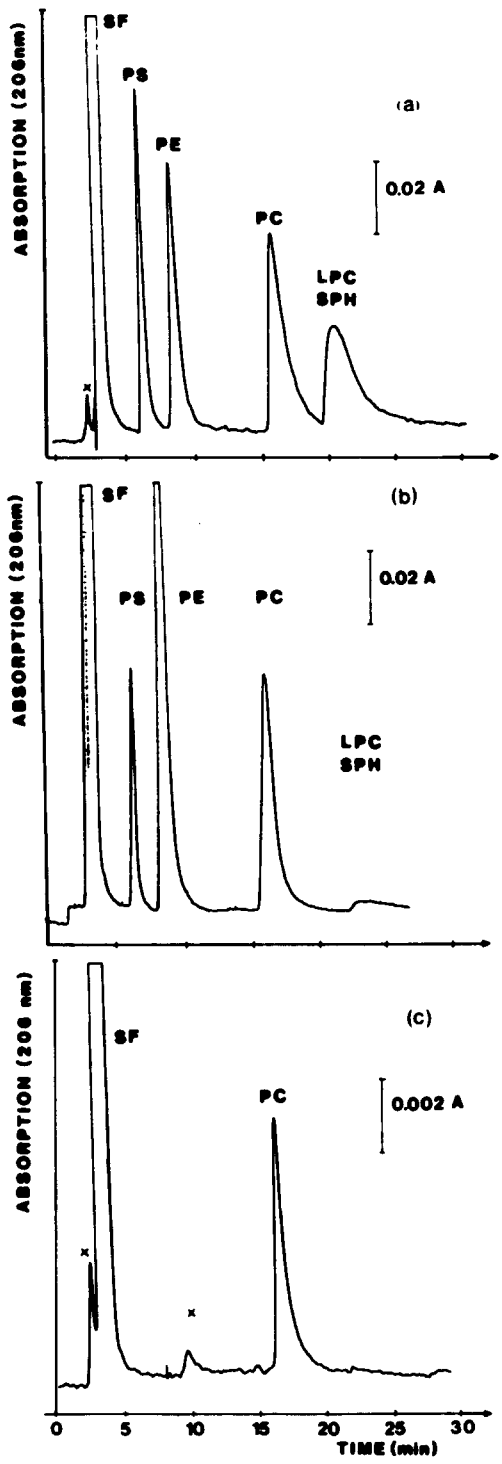

Fig. 1. HPLC separation of phospholipids on 10 μm Spherisorb column 25 cm × 16 mm I.D. The solvent was composed of acetonitrile-isopropanol-methanol-water-trifluoroacetic acid 135:20:10:6.7:0.85 (v/v) at a flow rate of 10 ml/min. Detection was at 206 nm. a): Forty μl of a standard mixture containing 60 μg each of PI and PS, 90 μg of PE, 130 μg of PC, and 900 μg each of LPC and SPH. b): Seventy μl of lung tissue extract in chloroform-methanol containing about 300 μg of phospholipids. c): Two hundred μl of lung lavage extract in chloroform-methanol containing about 100 μg of phospholipids. Peaks: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SPH, sphingomyelin; SF, solvent front containing phosphatidylinositol (PI) and/or natural lipids; X, unidentified peaks.

acid in various proportions, were tested for their ability to separate mixtures of phospholipid standards and phospholipids in biological samples. An isocratic mobile phase containing acetonitrile–isopropanol–methanol–water–trifluoroacetic acid 135:20:10:6.7:0.85 (v/v) was found to successfully separate both standards (**Fig. 1a**), the lecithin fraction from phospholipids present in lung tissue (Fig. 1b), and lavage fluid (Fig. 1c) extracts. The advantages of this method include isocratic run conditions, a run time less than 30 min, and excellent purification of the lecithin fraction with no TLC-detectable impurities from other phospholipids, particularly sphingomyelin and lysolecithin. Furthermore, it is remarkable that the recovery is approximately 100% (100.02 ± 5.52%) with HPLC compared to 75–80% with the TLC method (73.94 ± 1.50% after four elution steps or 81.96 ± 0.94% after fivefold elution). This high recovery rate is of critical importance in the analysis of biological samples of limited size. The ease of evaporation and the direct use for GLC analysis are further advantages of this analytical technique. However, one must be aware that a direct quantification of lipid mass from the peak area at 206 nm is not possible as was documented by Jungalwala, Evans, and McCluer (8). An incomplete separation of all phospholipids during one run is the major disadvantage of this method and other methods currently used. In our method the natural lipids, phosphatidylglycerol and phosphatidylinositol, coeluted with the solvent front as checked by TLC separations of the solvent front. Also, lysolecithin and sphingomyelin were not separated, resulting in one peak with a retention time of 22–24 min.

The quantification and characterization of separated lecithin fractions were done by GLC (15) using an internal standard. Comparison of samples preseparated either by HPLC or TLC (data not shown) revealed no significant differences (Student's *t*-test) between the two methods used, which leads to the conclusion that the two methods are equivalent and interchangeable when used with an internal standard. However, one achieves significantly ($P < 0.005$) higher recovery with the HPLC separation, even when the elution procedure for TLC is fivefold. The HPLC method described has additional advantages in easier handling, less time, and less possibility of oxidative reactions. Since the separation of phospholipid classes is a prerequisite for further analysis, rapid sample processing is necessary. In fact, with this rather fast technique automatization is possible, which is unlikely with TLC separations.

We thank Dr. R. Spragg, San Diego, CA, for reviewing the manuscript.

Manuscript received 10 February 1986 and in revised form 15 December 1986.

REFERENCES

1. Freeman, C. P., and D. West. 1966. Complete separation of lipid classes on a single thin-layer plate. *J. Lipid Res.* **7:** 324–327.
2. Blass, K. G., and C. S. Ho. 1981. Sensitive and highly reproducible quantitative fluorescent thin-layer chromatographic visualisation technique for lecithin and sphingomyelin. *J. Chromatogr.* **208:** 170–173.
3. Gilfillan, A. M., A. J. Chu, D. A. Smart, and S. A. Rooney. 1983. Single plate separation of lung phospholipids including disaturated phosphatidylcholine. *J. Lipid Res.* **24:** 1651–1656.
4. Korte, K., and M. L. Casey. 1982. Phospholipid and neutral lipid separation by one-dimensional thin-layer chromatography. *J. Chromatogr.* **232:** 47–53.
5. Hax, W. M. A., and W. S. M. G. Van Kessel. 1977. High performance liquid chromatographic separation and photometric detection of phospholipids. *J. Chromatogr.* **142:** 735–741.
6. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.
7. Chen, S. S. H., and A. Y. Kou. 1982. Improved procedure for the separation of phospholipids by high-performance liquid chromatography. *J. Chromatogr.* **227:** 25–31.
8. Jungalwala, F. B., J. E. Evans, and E. H. McCluer. 1976. High-performance liquid chromatography of phosphatidylcholine and sphingomyelin with direct detection at 200 nm. *Biochem. J.* **155:** 55–60.
9. Nissen, H. P., and H. W. Kreysel. 1983. Analysis of phospholipids in human semen by high-performance liquid chromatography. *J. Chromatogr.* **276:** 29–35.
10. Paton, R. D., A. I. McGillivray, T. F. Speir, M. J. Whittle, C. R. Whitfield, and R. W. Logan. 1983. HPLC of phospholipids in biological fluids — application to amniotic fluid for the prediction of fetal lung maturity. *Clin. Chim. Acta.* **133:** 97–110.
11. Kaitaranta, J. K., P. J. Geiger, and S. P. Bessman. 1981. High-performance liquid chromatographic separation of phospholipid precursors and their direct measurement by automatic phosphorus analysis. *J. Chromatogr.* **206:** 327–332.
12. Kaduce, T. L., K. C. Norton, and A. A. Spector. 1983. A rapid isocratic method of phospholipid separation by high-peformance liquid chromatography. *J. Lipid Res.* **24:** 1398–1403.
13. Yandrasitz, J. R., G. Berry, and S. Segal. 1983. High-performance liquid chromatography of phospholipids: quantitation by phosphate analysis. *Anal. Biochem.* **135:** 239–243.
14. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226:** 497–509.
15. Lohninger, A., and A. Nikiforov. 1980. Quantitative determination of natural dipalmitoyl lecithin with dimyristoyllecithin as an internal standard by capillary gas liquid chromatography. *J. Chromatogr.* **192:** 185–192.
16. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
17. Rivnay, B. 1984. Combined analysis of phospholipids by high-performance liquid chromatography and thin-layer chromatography analysis of phospholipid classes in commercial soyabean lecithin. *J. Chromatogr.* **294:** 303–315.