Preparative scale separation of neutral lipids and phospholipids by centrifugally accelerated thin-layer chromatography

Synnøve Bergheim,*† Karl Egil Malterud,[1,*] and Thorleif Anthonsen†

Institute of Pharmacy, Section Pharmacognosy, University of Oslo, P. O. Box 1068, Blindern, N-0316 Oslo 3, Norway, and Department of Chemistry,† University of Trondheim, N-7055 Dragvoll, Norway*

Summary Mixtures of lipids and phospholipids were separated by centrifugally accelerated thin-layer chromatography on a preparative scale (300–500 mg lipid mixture per run). The isolated lipids and phospholipids were identified by ^{1}H and ^{13}C NMR spectroscopy and their fatty acid composition was determined by GLC and GLC-MS of their methyl esters. —**Bergheim, S., K. E. Malterud and T. Anthonsen.** Preparative scale separation of neutral lipids and phospholipids by centrifugally accelerated thin-layer chromatography. *J. Lipid Res.* 1991. **32:** 877–879.

Supplementary key words phosphatidylcholine • phosphatidylethanolamine • phosphatidylinositosol • chromatotron

Phospholipids form an important part of cell membranes in animal and plant tissue. Due to their biological and commercial importance, they have for many years been the subject of intensive research in biology and biochemistry.

In the preparation of pure phospholipids, separation into classes such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositosol, etc. forms an important first step. Methods for lipid/phospholipid separation have been the subject of several books and reviews (e.g., refs. 1–3). On a preparative scale, this has traditionally been carried out by column chromatography, a method that is time-consuming and, therefore, may lead to deterioration of sensitive substances. This technique is solvent-consuming as well, and separation may be less than optimal (4). Other methods, such as preparative TLC and preparative HPLC, share some of these disadvantages, and may be difficult to carry out on a preparative scale. Gradient elution is not easily carried out with TLC methods, and manipulation of samples during extraction from the TLC medium may be deleterious to substances of low stability. Preparative HPLC may require complex and/or expensive instrumentation. Overpressured thin-layer chromatography (5, 6) may prove efficient, but apparatus for this technique is not yet common.

In this communication, we report the separation on a preparative scale of phospholipid classes from crude mixtures by centrifugally accelerated thin-layer chromatography on a Chromatotron^{R}. Briefly, the Chromatotron consists of a polycarbonate housing (connected to an N_2 source) in which is placed a circular glass plate coated with an adsorbent, placed at a 45° angle to the horizontal and connected to an electric motor. The plate rotates at ca. 700 rpm. The solution to be separated is applied near the center of the plate, followed by eluent. Movement of eluent and dissolved substances through the adsorbent layer is speeded up by centrifugal force, and when the liquid reaches the outer edge of the adsorbent layer, it is thrown off, runs down to an outlet at the bottom of the instrument and is collected into fractions.

To our knowledge, this method has not been described for preparation of phospholipids, although it is commonly used for other natural products, and its efficiency is well recognized (7).

MATERIALS AND METHODS

The lipid/phospholipid mixture used was a sample of crude lecithin of soybean origin obtained from a soap manufacturer (Denofa, Oslo, Norway). In addition, a phospholipid preparation from egg yolk (phosphatidylcholine type XV-E, ca. 60% pure, Sigma, St. Louis, MO) was separated. All solvents used were of reagent grade quality.

Liquid chromatography

The Chromatotron, model 7924T, was from Harrison Research, Palo Alto, CA. Layers of silica gel PF_{254} with $CaSO_4$, 2-mm thickness (Type 7749, Merck, Darmstadt, Germany) were used. In analytical TLC, 0.2-mm layers of silica gel F_{254} on aluminium foils (Type 5554, Merck) were used.

Eluents on the Chromatotron were chloroform–petroleum ether (b.p. 60–80°C) 1:1 (v/v) (solvent 1); the organic phase of chloroform–methanol–water 175:22:3 (solvent 2); and chloroform–methanol–water 75:22:3 (solvent 3). In analytical TLC, solvent 3 was used as mobile phase, and spots were visualized, after air-drying, by spraying with $Ce(SO_4)_2$ (1%, w/v) in 10% aqueous H_2SO_4 and heating at 110°C for 5 min.

Spectroscopy and gas-liquid chromatography

NMR spectra were recorded on a Varian Gemini 200 instrument (Varian, Palo Alto, CA) at 200 MHz for ^{1}H and 50 MHz for ^{13}C in $CDCl_3$ or CD_3SOCD_3 solutions. Gas chromatography was carried out on a Vega 2 series GC6000 instrument (Carlo Erba, Milan, Italy) with

Abbreviations: CA-TLC, centrifugally accelerated thin-layer chromatography; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed.

flame ionization detection, using a 30-m fused silica capillary column (Supelco, Bellefonte, PA), ID 0.25 mm, wall coated (0.2 μm thickness) with SP-2340. The apparatus was connected to a HP3396A integrator (Hewlett-Packard, Palo Alto, CA). Column temperature was 160°C initially, increasing by 2°C/min to 220°C. Injector temperature was 240°C and detector temperature 250°C. A gas flow of 1.0 ml/min was used (split 20:1), and FAME standards were run for comparison. In GLC-MS experiments, a Hewlett-Packard 5970B mass selective detector coupled to a Hewlett-Packard 5890A gas chromatograph was used with column and chromatographic conditions as described above.

Separation of lipid/phospholipid mixtures

Separation was carried out by dissolving 300 mg of the crude lecithin in ca. 1 ml of solvent 1, applying it to the Chromatotron, and eluting with 350 ml of solvent 1, 510 ml of solvent 2, and 330 ml of solvent 3. During this time, a flow of N_2 (0.5-1.0 ml/min) was supplied to the Chromatotron. Fractions of 15 ml were collected and combined as indicated by analytical TLC. The combined samples were taken to dryness on a rotary evaporator at reduced pressure (temperature 35-40°C) and kept at −20°C under N_2.

Hydrolysis and methylation

Hydrolysis of esters and methylation of acids were carried out by the one-step method described by Metcalfe and Wang (8), using methanolic tetramethyl ammonium hydroxide. Samples of 1-3 mg were used for hydrolysis/methylation.

RESULTS AND DISCUSSION

By separation of 300-mg batches of a crude lipid/phospholipid mixture, substances were obtained as shown in **Table 1**. The major phospholipids as well as most of the triglyceride and sterol ester fractions in the sample were obtained in a pure state (TLC). They were identified by ^1H and ^{13}C NMR and comparison of the spectra with those reported in the literature.

Some minor components were obtained as mixtures of two or three substances. The sum of the fractions corresponded to about 86% of the sample. While seemingly empty fractions and volatile compounds in the sample may contribute to the loss, we believe that a considerable part of it is due to moisture in the sample. For the phosphatidylcholine preparation from egg yolk (see Table 1 for results), a recovery of 92% was obtained.

Table 2 shows the fatty acid composition of the substances obtained as determined by GLC and GLC-MS. By using centrifugally accelerated TLC, amounts of several hundred milligrams of each class of phospholipids can be obtained in one working day. We found that with 500-mg samples, separation was nearly as good as with a sample size of 300 mg, while separation deteriorated markedly when samples of 800 mg phospholipid were applied to the Chromatotron.

In applications of equal amounts of the same sample, contents of fractions varied somewhat; usually less than 10%. This may be due to variations in the amount of substance in fractions immediately before and after the ones combined and weighed. The purity of the substances isolated seemed fairly constant from spectroscopical and chromatographical data.

TABLE 1. Fractionation of phospholipid mixtures by CA-TLC

Fraction Number[a]	Weight in mg (Per cent of total amount separated)	R_f Values (TLC)	Composition
1 (4-11)	64.8 (21.6)	1.00	Triglycerides
2 (12-14)	27.0 (9.0)	1.00, 0.94	Triglycerides, sterol esters
3 (21-22)	11.1 (3.7)	0.81	Sterol esters
4 (25)	3.5 (1.2)	0.86	Free fatty acids
5 (26-27)	10.5 (3.5)	0.86, 0.61	Free fatty acids, sterol esters
6 (28-29)	5.7 (1.9)	0.61	Sterol esters
7 (31-32)	11.3 (3.8)	0.55, 0.53	Unidentified
8 (33-37)	8.4 (2.8)	0.53	Unidentified
9 (40-49)	36.9 (12.3)	0.50	Phosphatidylethanolamine
10 (54-57)	53.2 (17.7)	0.27	Phosphatidylcholine
11 (59-67)	27.0 (9.0)	0.15	Phosphatidylinositol
1 (9-14)	16.2 (5.4)	0.96	Cholesterol
2 (24-25)	5.6 (1.9)	0.87	Unidentified
3 (34-43)	42.7 (14.2)	0.48	Phosphatidylethanolamine
4 (45-51)	200.4 (66.8)	0.28	Phosphatidylcholine
5 (54-58)	10.9 (3.6)	0.12	Phosphatidylinositol

Upper part of table: separation of crude lecithin (Denofa), average of five 300-mg runs. Lower part: separation of crude egg yolk phosphatidylcholine, average of three 300-mg runs.

[a]Fraction numbers in parentheses refer to fractions obtained from the Chromatotron. Fractions missing from the Table did not contain visible amounts of substances (TLC).

TABLE 2. Fatty acid composition (% by weight) of phospholipid mixtures and main fractions from crude soybean lecithin (A) and egg lecithin (B)

Fatty Acid	A	1	2	9	10	11	B	3	4
14:0	0	0	0	0	0	0	0	0	1
16:0	16	12	12	19	16	31	22	18	25
16:1	0	0	0	0	0	0	1	0	1
18:0	3	4	4	2	4	6	17	32	15
18:1	14	24	22	7	14	5	29	18	30
trans-18:1	1	0	0	1	1	1	0	0	0
18:2	59	52	52	61	58	49	17	14	17
18:3	7	7	10	6	6	5	0	0	0
20:4	0	0	0	0	0	0	9	18	5

Distribution of fatty acids in crude lecithin (Denofa) (A), in crude egg yolk phosphatidylcholine (Sigma) (B), and in the main fractions (>4 % of the total weight of sample) obtained by CA-TLC.

For separation of fairly simple mixtures, such as egg lecithin, each plate could be used at least five times. For more complex ones, such as the lipid/phospholipid mixture, plates should probably be changed and re-coated with adsorbent after 3–4 runs. It is inadvisable to use the same plate for different mixtures.

Somewhat unexpectedly, it turned out that the origin of the chloroform used was critical. The separation described was obtained with chloroform from Merck (puriss.; Registry No. 2431), while chloroform from other sources with equivalent purity criteria yielded poorer separation. The difference in separating ability is probably not due to different HCl content in the chloroform, since extractions of the different chloroforms used with equal volumes of 0.9% aqueous NaCl all gave pH values of 6.41–6.42. The water content in the chloroform is insignificant compared with the amount added to the eluent. Variations in the ethanol content of the chloroform (stated as 0.6–1.0% or 0.4–1.0%) may be responsible for the difference in separation ability.

The solvent consumption is ca. 1200 ml per run. Since the separation takes place in an inert atmosphere (N_2), autoxidation is inhibited. We noticed, however, that while samples were devoid of odor directly after separation and evaporation of solvent, there was a slightly rancid odor after samples were kept at −20°C for 1 week under N_2. This phenomenon was most pronounced for the triglyceride fraction.

We conclude that the separation of phospholipids from crude mixtures described is a quick (time per run ca. 2 h) and efficient method for obtaining phospholipids separated from each other and from extraneous material. In contrast to classical TLC methods, no scraping off of stationary phase and extraction of sample is required. For this reason, and since the separation takes place in an inert atmosphere, oxidative denaturation of sensitive substances is minimized.

Thanks are due to Torun Aslaksen and Finn Tønnesen (Institute of Pharmacy, University of Oslo, Norway) for skilled technical assistance, and to Denofa, Oslo, for a sample of crude lecithin. The Chromatotron employed was financed through a grant (to KEM) from the Norwegian Research Council for the Sciences and Humanities.

Manuscript received 15 October 1990 and in revised form 22 January 1991.

REFERENCES

1. Kates, M. 1986. Techniques of Lipidology—Isolation, Analysis and Identification of Lipids. 2. Ed. Elsevier, Amsterdam, New York, Oxford.
2. Smith, G., and J. Stein. 1982. Lipid preparation. *Tech. Lipid Membr. Biochem.* **B406:** 1-16.
3. Horrocks, L. A., L. L. Dugan, C. J. Flynn, G. Goracci, S. Porcellati, and Y. Yeo. 1987. Modern techniques for the fractionation and purification of phospholipids from biological material. *Adv. Behav. Biol.* **33:** 3–16.
4. 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.
5. Kovács, L., J. Pucsok, A. Zalka, R. Dobó, and I. Györe. 1988. New TLC and OPTLC methods for the separation of serum lipids. *Fat Sci. Technol.* **90:** 195–199.
6. Pucsok, J., L. Kovács, A. Zalka, and R. Dobó. 1988. Separation of lipids by new thin-layer chromatography and overpressured thin-layer chromatography methods. *Clin. Biochem.* **21:** 81–85.
7. Hostettmann, K., M. Hostettmann, and A. Marston. 1986. Preparative Chromatography Techniques: Application in Natural Product Isolation. Springer-Verlag, Berlin.
8. Metcalfe, L. D., and C. N. Wang. 1981. Rapid preparation of fatty acid methyl esters using organic base-catalyzed transesterification. *J. Chromatogr. Sci.* **19:** 530–535.