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Note

High-performance liquid chromatographic separation and quantitative analysis of synthetic phospholipids

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During recent years, high-performance liquid chromatography (HPLC) has been increasingly applied to analyses. The flame-ionization detector (FID), devised by Maggs[1], proved effective in the detection of lipids, the analysis of which had not been feasible with a UV or RI monitor. As the sensitivity of the FID is determined by the number of carbon atoms[2], synthetic lipids are essential for the quantitative HPLC separations. We have carried out quantitative separations of glycerides, fatty acids and sterols using HPLC[3] and applied the data to soybeans and soybean foods[4,5].

Phospholipids are specifically located in biomembranes and, together with proteins, form the main constituents. The rapid quantitative analysis of phospholipids is essential for biochemical research on biomembranes. Nevertheless, only a few separations by HPLC have previously been carried out. Erdahl *et al.*[6] analyzed qualitatively the lipid composition of soybean lecithins by HPLC using a silicic acid column, and Privett *et al.*[7] applied their data to the study of developing soybeans.

In a previous paper[3], we described the separation of soybean lecithin with a Micropak SH-10 silica gel packing (Varian, Palo Alto, Calif., U.S.A.), by which polar lipids were eluted in 18–23 min. For the further separation of individual polar lipids, the use of polar solvents was necessary. However, it is not feasible to use methanol and/or water for long periods with a silica gel packing. In this work, therefore, we have used as an alternative chloroform–methanol–water for the separation and quantification of synthetic phospholipids, employing an anion exchanger, μBondapak-NH_2, as an adsorption packing.

EXPERIMENTAL

Apparatus

A Varian Model LC 4200 chromatograph was employed, equipped with a Pye Unicam Model LCM2 flame-ionization detector (FID). The temperatures of the components of FID were controlled as follows: cleaner, 800°; oxidizer, 750°; evaporator, 210°; reactor, 405°; and FID, 160°. The speed of the moving wire was 10.0 cm/sec. The sensitivity of the detector was adjusted to $3.2 \cdot 10^{-11}$ A f.s.d.

The column was a porous anion exchanger, μBondapak-NH_2 (Waters Assoc., Milford, Mass., U.S.A.) with a 10-μm packing in a stainless-tube (300 mm × 1/4 in.

I.D.). The pre-column was inserted between the injector and the column. AX/Corasil, 37–75 μm diameter, was packed into the stainless-steel pre-column (100 mm × 1/8 in. I.D.) with a plastic rod by the incremental dry method.

Solvents

Chloroform (Guaranteed-reagent grade) and methanol (liquid chromatographic reagent grade) were purchased from Wako (Osaka, Japan).

Reagents

Synthetic dipalmitoyl phosphatidic acid (PA), dipalmitoyl phosphatidylglycerol (PG), dipalmitoyl phosphatidylserine (PS), dipalmitoylphosphatidylcholine (PC), dipalmitoylethanolamine (PE) and natural phosphatidylinositol (PI) were the products of Serdary Research Labs. (Ontario, Canada). As PA, PG and PS gave several spots in thin-layer chromatography, the main bands that corresponded to the spots detected with Dittmer's reagent, ninhydrin reagent and iodine vapour were scraped off and extracted with chloroform–methanol (2:1). Tristearin (TS) was a product of Nakarai Chemicals (Tokyo, Japan).

Analytical conditions

Two solvents were used as eluents: solvent A was chloroform and solvent B was methanol–water (25:1) (solvent I) or methanol–water (25:4) (solvent II). The composition of the eluent is expressed as % B_1 or % B_2, which indicates the percentage of solvent I or solvent II in the total solvent mixture. The flow-rate of the eluent was kept constant at 40 ml/h. The column was washed for 60 min prior to the subsequent analysis when the eluent was changed.

RESULTS AND DISCUSSION

In order to establish appropriate conditions for separations with short retention times (t_R), HPLC was carried out with various solvents. Fig. 1 shows the effect of the solvent on the t_R of the phospholipids PG, PA, PS, PC, PE and TS. Only TS was eluted when chloroform was used as the sole eluent (0% B_1). The t_R of TS was 4.0–4.2 min between 60 and 100% B_1 and 3.4 min with 30% B_2. The t_R of PS scarcely changed when solvent B_1 was employed, but it was 6.0 min with 30% B_2 and 4.7 min with 50% B_2.

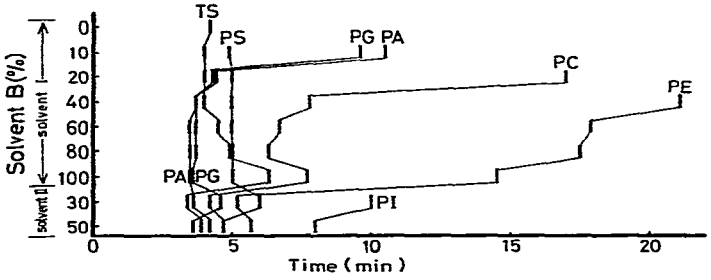

Fig. 1. Effect of solvent on retention time. Solvent B (%) indicates the concentration of solvent B in (solvent A + solvent B). Solvent A in both solvents I and II is chloroform; solvent B in solvents I and II is methanol–water (25:1) and methanol–water (25:4), respectively.

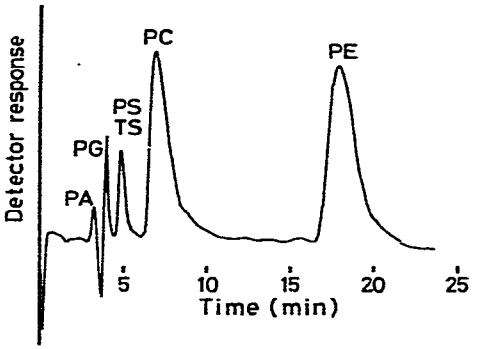

Fig. 2. Separation of phospholipids on μBondapak-NH_2.

PG and PA were eluted between 9.6 and 10.5 min with 10% B_1, and between 3.5 and 5.0 min with less than 20% B_1. PC was eluted at 17.0 min with 0% B_1 and at less than 8 min with more than 40% B_1. PI was eluted only if solvent II was employed and it was eluted at 10.0 min with 30% B_2.

Fig. 2 shows the high-performance liquid chromatogram of the five synthetic phospholipids and tristearin when they were eluted with 80% B_1. The phospholipids were eluted in the order PA, PG, PS, PC and PE; the peak of TS was inseparable from that of PS. The trough of the peak of PA was lower than the base-line.

Fig. 3 shows the recorder response with various amounts of TS, PS, PC and PE up to 250 ng, using 80% B_1. The plots of detector responses against the amounts of the samples were linear. The detector responses to equal amounts of TS and PS were identical; the response to PE was the highest of the four lipids. If the sensitivity to TS is given a value of 100, then those of PS, PC and PE were 100, 644 and 890, respectively.

Fig. 4 shows the relationship between retention time and flow-rate between 10 and 100 ml/h when lipids were eluted with 80% B_1. At each flow-rate the lipids were eluted in the order PA, PG, PS, PC and PE. TS and PS had similar retention times.

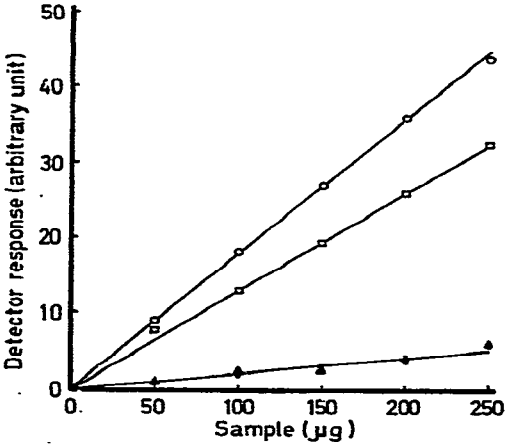

Fig. 3. Linearity of detector response for analysis of phopholipids. ● = TS; ▲ = PS; □ = PC; ○ = PE.

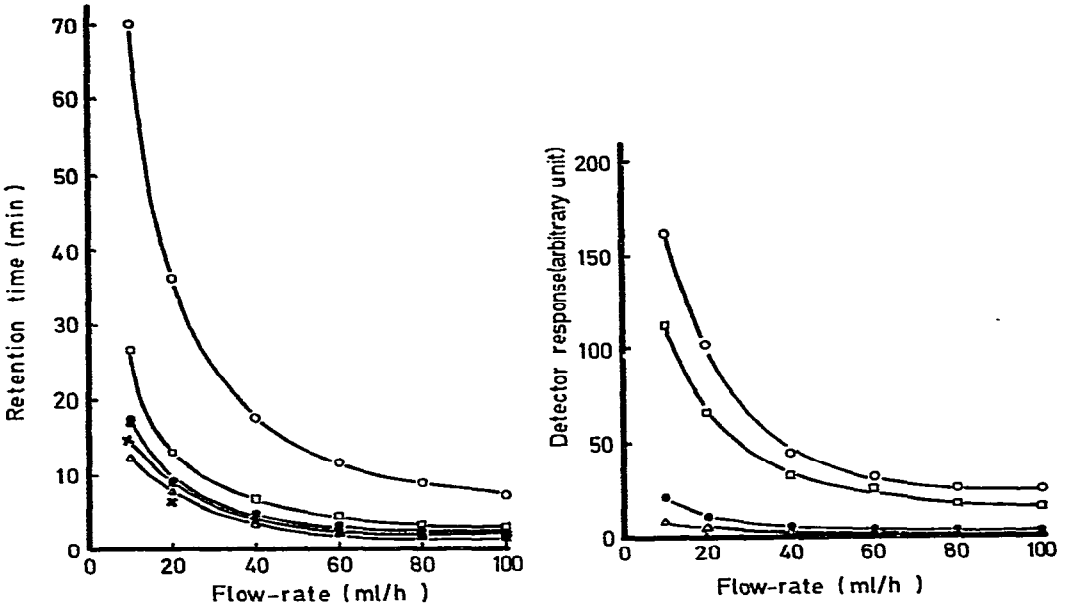

Fig. 4. Effect of flow-rate on retention time. ● = TS; × = PG; △ = PA; ▲ = PS; □ = PC; ○ = PE.

Fig. 5. Effect of flow-rate on detector response. ● = TS; △ = PS; □ = PC; ○ = PE.

The retention times became shorter as the flow-rate increased, and the relative retention times of the six lipids were almost the same at each flow-rate, being 0.69, 0.85, 0.96, 1.42 and 3.69 relative to TS = 1.0 at 40 ml/h.

Fig. 5 shows the relationship between the flow-rate and the recorder response. An increase in the flow-rate was accompanied by a decrease in the recorder response, although the relationship was not linear.

Theoretically, the detector response should be proportional to the number of carbon atoms in the material being analyzed[2]. Changes in the analytical conditions should not affect the peak area, *i.e.*, a certain number of carbon atoms should give a certain peak area. The reason why the type of lipids and the flow-rate can alter the recorder response remains to be investigated. However, as the conditions of the other components of the FID were held consistant, a possible reason is the mechanism of the coating assembly[8]. In spite of the linear relationship between flow rate and retention time, the relationship between flow-rate and recorder response was not linear, which demonstrates that strictly regulated conditions and a constant retention time are most important for the quantitative separation of unknown samples.

Fig. 6 shows the relationship between the flow-rate and plate height of the peaks of lipids eluted when solvent B was replaced with solvent I. The plate height of PA increased with increasing flow-rate. The lipids with the lowest plate heights were PG at a flow-rate less than 60 ml/h and TS at flow-rates between 80 and 100 ml/h. The plate heights of the five sythetic phospholipids were between 0.1 and 2.9.

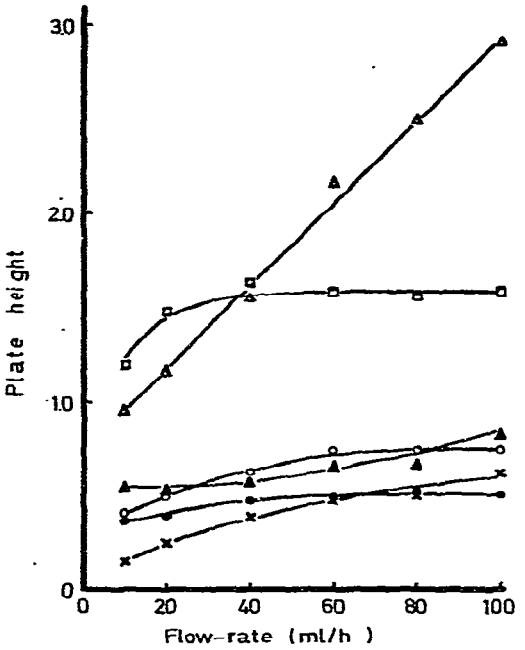

Fig. 6. Effect of flow-rate on plate height. ● = TS; × = PG; △ = PA; ▲ = PS; □ = PC; ○ = PE.

From the results presented above, the anion exchanger, μBondapak-NH_2 employed as the adsorption packing has been shown to be useful for the separation and quantification of phospholipids. By changing the composition of the chloroform–methanol–water solvent, retention times and relative retention times could be changed, so that the HPLC separation of phospholipids could be carried out easily.

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