Journal of Chromatography, 142 (1977) 735–741

CHROM. 10,294

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND PHOTOMETRIC DETECTION OF PHOSPHOLIPIDS

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SUMMARY

A rapid and efficient method for the separation of (phospho)lipids by high-performance liquid chromatography using *n*-hexane–2-propanol–water mixtures as the solvent system is described. The lipid separation occurs on silica gel columns and the individual components are monitored directly by UV absorption at 206 nm. Of a total lipid extract from erythrocytes as well as suboesophageal ganglia of the snail *Helix pomatia*, a complete separation is achieved of cholesterol, phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine and lysophosphatidylethanolamine, whereas phosphatidylcholine and sphingomyelin are partly separated under these circumstances. In addition to separation of phospholipids in different classes, separation of molecular species can also be achieved in some instances, as is shown for phosphatidylcholines and sphingomyelins.

INTRODUCTION

The recently developed technique of high-performance liquid chromatography (HPLC) can be used for the separation of particularly non-volatile or thermally unstable material, and it has considerable potential for the separation of neutral lipids[1–16]. However, HPLC of molecules with a strong amphipathic character, such as phospholipids, is more complicated. Furthermore, continuous peak monitoring, which is a prerequisite for a rapid procedure, is difficult for phospholipids. Refractive index and flame-ionization detection methods are insensitive and are incompatible with virtually all gradient elution techniques. The photometric method is very sensitive and easily applicable because of its insensitivity to changes in flow-rate, temperature and mobile phase composition. Lipids lack specific absorption peaks but, owing to the presence of unsaturated centres and functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium, there is a strong absorption in the 203–214-nm region, commonly referred to as "end absorption".

The application of modern HPLC with ultraviolet detection failed for phospholipids, because chloroform–methanol mixtures, the eluents commonly used in adsorption chromatography on silica gel, have a strong absorption below 245 nm. We recently described a sensitive method for the separation and direct ultraviolet detection of phospholipids[17]. In this method, *n*-hexane–2-propanol–water mixtures were used as a good substitute for the chloroform–methanol mixtures. These eluents not only provide high resolution, but are also transparent in the 200-nm region, so that direct, non-destructive detection of lipids with a high degree of sensitivity is possible. However, owing to the complex absorption behaviour of phospholipids, there will be problems in the direct quantification of lipid classes which contain a great variety of fatty acid constituents[17]. The molar extinction coefficients have to be estimated for each source before the chromatograms can be interpreted quantitatively.

The aim of this paper is to show what has been attempted and what has been achieved with respect to the HPLC separation and direct ultraviolet detection of phospholipids.

EXPERIMENTAL

Apparatus

The equipment used comprised the following units, all obtained from Pye Unicam (Cambridge, Great Britain): Model LC 3 liquid chromatograph, having a maximum operating pressure of 160 bar and a Model LC 3 UV detector with a wavelength range of 190–380 nm and a flow cell volume of 8 μl. Absorption measurements were made at 206 nm.

Additional items of equipment included a multi-range pen recorder (Philips PM 8221) and a Model 7105 universal septumless injector (Rheodyne, Berkeley, Calif., U.S.A.). For gradient elution the LKB (Bromma, Sweden) 11300 Ultrograd gradient mixer was used. The eluents in chambers 1 and 2 were linearly mixed as indicated in the figure legends.

The chromatographic packings used were LiChrosorb SI-60, 10 μm, LiChrosorb SI-60, 5 μm (Merck, Darmstadt, G.F.R.) and Spherisorb S 10 W (Phase Separations, Queensferry, Great Britain). Standard stainless-steel chromatographic columns were 25 cm long with an I.D. of 0.42 cm and were pre-packed by Chrompack B.V. (Middelburg, The Netherlands). For preparative separation of sphingomyelins, special columns of length 25 cm and I.D. 0.9 or 1.6 cm were obtained from Chrompack and Knauer (Berlin, G.F.R.), respectively.

Solvents

For column elution, different mixtures of *n*-hexane, 2-propanol and water were used. *n*-Hexane and 2-propanol (both Uvasol) were purchased from Merck and showed transmittances at 206 nm of 58% and 18%, respectively (optical pathlength 1 cm).

Prior to use, the air from the eluting solvents was removed with a bath sonicator (Philips Type 2100/10). This procedure was repeated every hour.

Lipid preparation

The syntheses of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-

sn-glycero-3-phosphocholine were carried out according to established procedures[18].

Sphingomyelin was isolated from beef erythrocyte ghost by the method of Dodge *et al.*[19]. The extraction and purification procedure was described by Van Dijk *et al.*[20].

A modified Folch extraction[21] was applied to suboesophageal ganglia of the snail *Helix pomatia* in order to obtain a total lipid extract.

A total lipid mixture of human erythrocyte membranes was isolated from erythrocyte ghosts prepared by the method of Dodge *et al.*[19].

RESULTS AND DISCUSSION

For reasons outlined previously[17], *n*-hexane–2-propanol–water mixtures were chosen as eluting solvents, and absorption measurements were made at 206 nm. The polarity of the solvent system was varied by changing the amount of water, whereas the *n*-hexane–2-propanol ratio was kept at 3:4 (v/v). The required ratio (polarity) for optimal separation depends on the composition of the phospholipid mixture and is indicated in the figure legends.

Before sample analysis, the columns were first washed with a *n*-hexane–2-propanol–water (2:6:1.5, v/v/v). The adsorbent was then equilibrated with the solvent system until a stable baseline was obtained. Most of the experiments were performed at an average flow-rate of 1 ml/min. Unless otherwise indicated, the columns were not thermostated as the chromatographic process is insensitive to small temperature changes. In the set-up described above, the columns were used for more than 100 runs without loss of reproducibility.

Fig. 1 shows the separation by HPLC of a total lipid extract from human erythrocyte membranes using a LiChrosorb SI-60, 10 μm column with a gradient of *n*-hexane–2-propanol–water from 6:8:0.75 to 6:8:1.4 (v/v/v). The different fractions were collected and identified by thin-layer chromatography. Peaks a and b are cholesterol and phosphatidic acid, respectively, and c and d could not be identified. Peaks e and f are phosphatidylethanolamine and lysophosphatidylethanolamine. In order to separate the other lipids, the polarity of the solvent was not increased further but kept constant. The other peaks were identified as phosphatidylinositol (g), phosphatidylserine (h), a mixture of phosphatidylcholine and sphingomyelin (i), sphingomyelin (j) and lysophosphatidylcholine (k).

An efficient separation of a total lipid extract from suboesophageal ganglia of the snail *Helix pomatia* on a Spherisorb S 10 W column is shown in Fig. 2. Elution was carried out stepwise. First the column was eluted with *n*-hexane–2-propanol–water (6:8:0.75, v/v/v, Fig. 2a; or 6:8:0.9, v/v/v, Fig. 2b). Again the different fractions were collected and identified by thin-layer chromatography. In Fig. 2a, peaks a and b are cholesterol and phosphatidic acid, respectively, and peak c could not be identified. Phosphatidylinositol (d) was eluted prior to phosphatidylethanolamine (e, f). The eluent was then changed stepwise to *n*-hexane–2-propanol–water (6:8:1.75, v/v/v), which resulted in elution of phosphatidylserine (g), phosphatidylcholine (h) and lysophosphatidylcholine (j). Peak i, which does not contain phosphorus, is unidentified. The baseline change is due to the drastic increase in the water content of the eluent and is so pronounced because of the sensitive detection level being used. If *n*-hexane–2-propanol–water (6:8:0.9, v/v/v) is used in the first step, than the

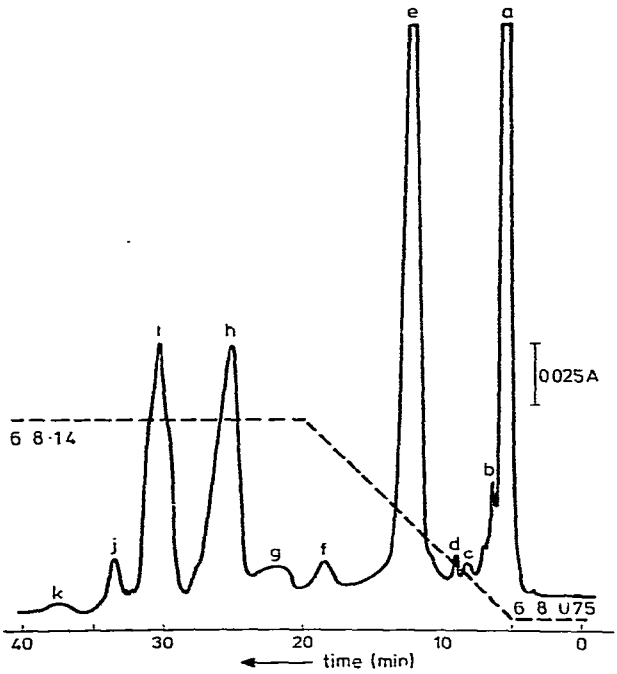

Fig. 1. Separation of a total lipid extract from human erythrocyte membranes. Sample load, 50 μg in 50 μl of solvent; temperature, 20°; flow-rate, 1.0 ml/min; detection at 206 nm; column, LiChrosorb SI-60, 10 μm. The *n*-hexane–2-propanol–water gradient used is indicated. Peaks are identified in the text. Reproduced from ref. 17.

separation is faster; the only disadvantage is a decreased resolution between peak b and c from Fig. 2a.

The problem of optimization of the elution parameters of phospholipids from adsorption columns is complicated. In comparing crucial parameters such as column packing and changes in the composition of the mobile phase, it is, in our experience, preferable to use a stepwise gradient in combination with the Spherisorb packing for the separation of phospholipid classes. In particular, the reproducibility is enhanced with the latter combination. Furthermore, we should emphasize that the elutions with *n*-hexane–2-propanol–water, as described above, give a better separation of the lipid classes than with acetonitrile–methanol–water as described by Jungalwala *et al.*[22].

In addition to the separation of phospholipids in different classes, in some instances it is even possible to achieve a separation of molecular species on LiChrosorb SI-60 columns. Fig. 3, for instance, shows a complete separation of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine with *n*-hexane–2-propanol–water (6:8:1.6, v/v/v) as the solvent system. Fig. 3 also shows the relevance of unsaturated centres for the value of the extinction coefficient (ε_{206}) of phospholipids[17,22]. It was also possible to separate 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine distinctly from 1,2-didocos-13′-*cis*-enoyl-*sn*-glycero-3-phosphocholine with the same solvent system[17]. Phospholipids with small differences in fatty acid chain length and unsaturation show only partial separation and in many instances

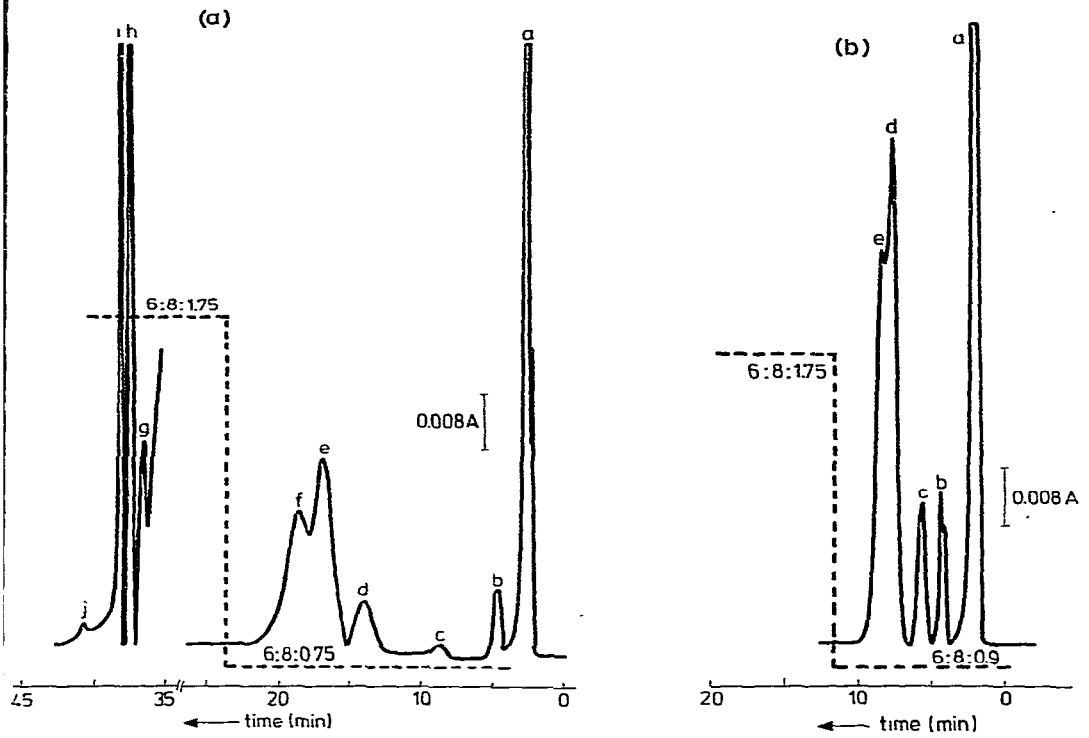

Fig. 2. Separation of total lipid extracts from suboesophageal ganglia of the snail *Helix pomatia*. (a) Sample load, 5 μg in 50 μl of solvent; room temperature; flow-rate, 1.1 ml/min; detection at 206 nm; column, Spherisorb S 10 W. The *n*-hexane–2-propanol–water gradient used is indicated. (b) Conditions as in (a), except for flow-rate, 1.25 ml/min. Peaks are identified in the text.

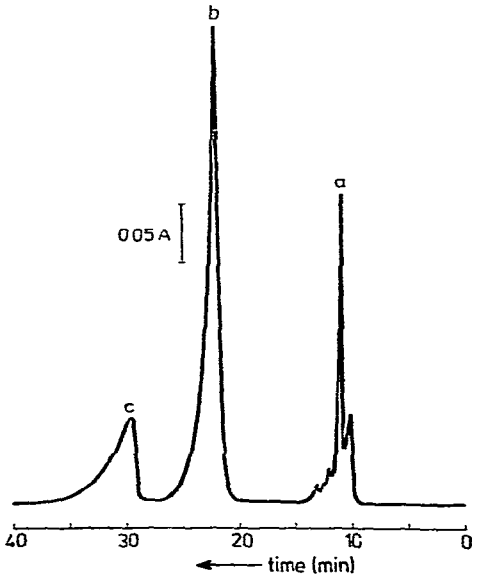

Fig. 3. Separation of a mixture of synthetic phosphatidylcholines. Solvent system, *n*-hexane–2-propanol–water (6:8:1.6, v/v/v); flow-rate, 2.5 ml/min; room temperature; detection at 206 nm; column, LiChrosorb SI-60, 5 μm (25 × 1.6 cm I.D.); 2.5 μmole of each component dissolved in 250 μl of solvent was applied to the column. Peaks: a, unidentified components; b, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; c, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

lead to shoulder formation. Indications of a different chromatographic behaviour of phospholipid species can be seen in Fig. 1 (peaks h and i). In Fig. 2, peak e and f, which are incompletely separated but which give both a positive phosphorus- and ninhydrin reaction, might be explained by the presence of phosphatidylethanolamine in suboesophageal ganglia of the snail *Helix pomatia*.

As mentioned above, it was found that in the separation of red cell lipids (Fig. 1) part of the sphingomyelin was present as a contaminant of the lecithin peak (i), whereas the main part of the sphingomyelin was eluted as a distinct fraction (peak j). Previously[17] we suggested that the large difference in the chain lengths of the fatty acids in the amide linkage, present in sphingomyelin from human erythrocyte membranes, might explain the separation into two fractions. This could be confirmed, when sphingomyelin obtained from beef erythrocytes, was applied to a LiChrosorb SI-60, 5 μm column (Fig. 4). Table I shows the fatty acid composition of the beef erythrocyte sphingomyelin species (peak d, f). It can be concluded that the main part of peak d contains sphingomyelin with the long-chain behenic and lignoceric acid. Sphingomyelins present in peak f mainly contain palmitic acid.

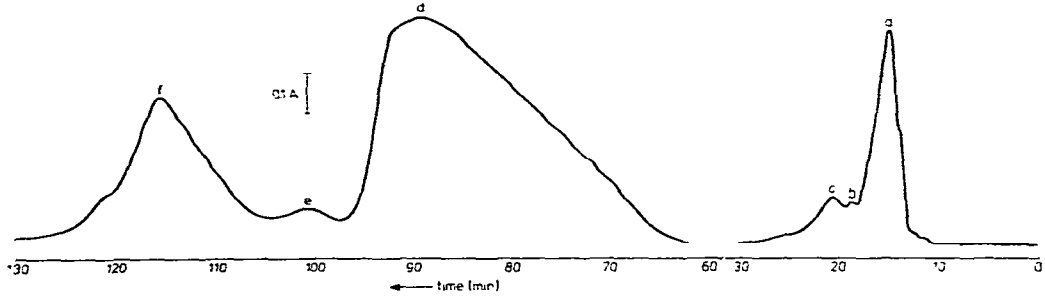

Fig. 4. Semi-preparative separation of sphingomyelin species of beef erythrocytes. Solvent system, *n*-hexane–2-propanol–water (6:8:1.5, v/v/v); flow-rate, 2 ml/min; room temperature; detection at 206 nm; column, LiChrosorb SI-60, 5 μm (25 × 0.9 cm I.D.); 10 mg of sphingomyelin dissolved in 250 μl of solvent was applied to the column. Peaks: a–c, unidentified components; d–f, sphingomyelin species.

TABLE I

FATTY ACID COMPOSITION OF DISTINCT SEPARATED SPECIES OF BEEF ERYTHROCYTES SPHINGOMYELIN

Chain length and unsaturation	*% in peak d (Fig. 4)*	*% in peak f (Fig. 4)*
14:0	1.9	1.4
16:0	4.5	85.8
18:0	0.8	1.4
18:1	4.8	5.7
18:2	4.8	5.7
22:0	16.3	
22:1	Trace	
22:2	3.6	
24:0 + 24:1	63.0	

CONCLUSION

It has been found that ultraviolet absorption at 206 nm provides a sensitive method of detection for phospholipids, offering the greatest opportunity for observing impurities in the sample. Consequently, HPLC and direct ultraviolet detection yield phospholipids of a higher purity than can be obtained with convential silicic acid column chromatography.

ACKNOWLEDGEMENT

Our thanks are due to Mr. F. Spijkerboer for his skilful technical assistance.

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