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Note

A new rapid method for phospholipid separation by high-performance liquid chromatography with light-scattering detection

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The evaluation of membrane phospholipid molecular classes is especially useful for determining the physical and biological properties of cellular membranes.

Previous quantitations of phospholipids have been obtained with thin-layer chromatography (TLC) [1–4]. In recent years, numerous high-performance liquid chromatography (HPLC) methods for the separation of phospholipids have been reported [5–18]. These methods offer the same separation capability as TLC, in addition to several advantages, especially concerning the degree of reproducibility and speed of measurement. However, detection of molecules with strong amphipathic characteristics, such as phospholipids, is more complicated. Methods using refractive index and flame ionization detection are not sensitive enough and are incompatible with virtually all gradient elution techniques [5].

Conversely, UV detection (200–220 nm) is sensitive and non-destructive but other problems occur because the range of phospholipids absorbance limits the choice of eluting solvents. Thus, none of the currently published methods enables a complete separation of natural phospholipids and quantification is therefore extremely difficult [5–18]. For this reason, efforts have linked recent UV detection and mass spectrometry (MS) in an attempt to improve phospholipid detection and identification [19,20]. Unfortunately, extensive routine use of this method is limited by the complexity and cost of the material(s).

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To solve the problems associated with phospholipid detection, we developed a new HPLC technique with a light-scattering detector [21], which seems to be particularly convenient for phospholipids. An application of this detector with a ternary-solvent system for the separation of lipid classes has been previously described [22,23].

This paper describes a binary-solvent HPLC procedure that completely separates the main phospholipid classes.

EXPERIMENTAL

Materials

Bovine brain phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, egg yolk lecithin phosphatidylglycerol and bovine liver phosphatidylinositol were purschased from Sigma (St. Louis, MO, U.S.A.).

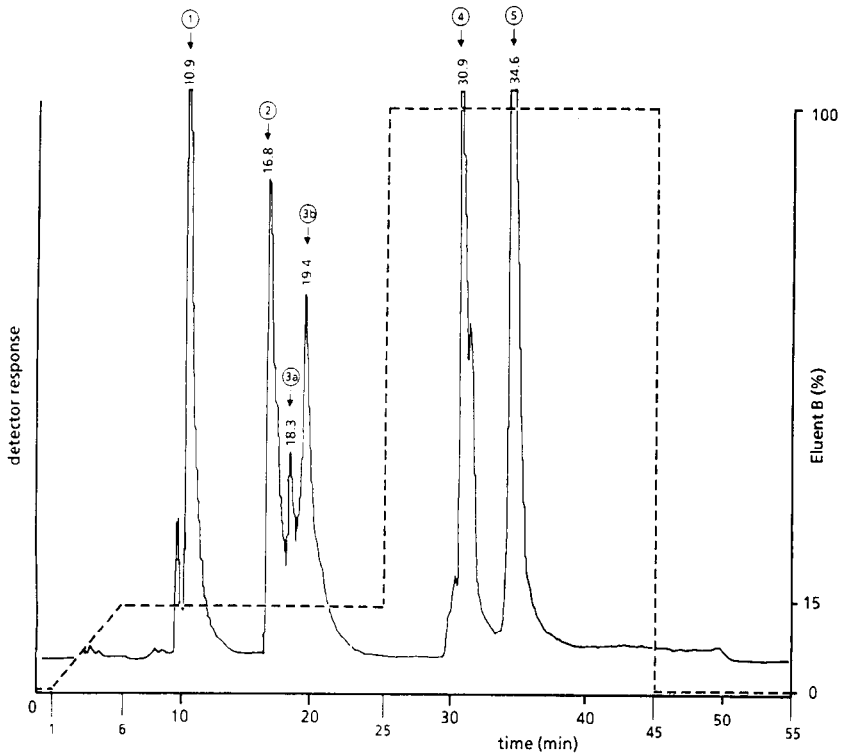

Fig. 1. Separation of natural standard phospholipids by HPLC with mass (light-scattering) detection. Peaks: 1 = phosphatidylglycerol; 2 = phosphatidylinositol; 3a and 3b = phosphatidylserine; 4 = phosphatidylethanolamine; 5 = phosphatidylcholine. Column and solvents A and B as described in Experimental. The dashed line indicates the gradient programme. Flow-rate, 1.0 ml/min.

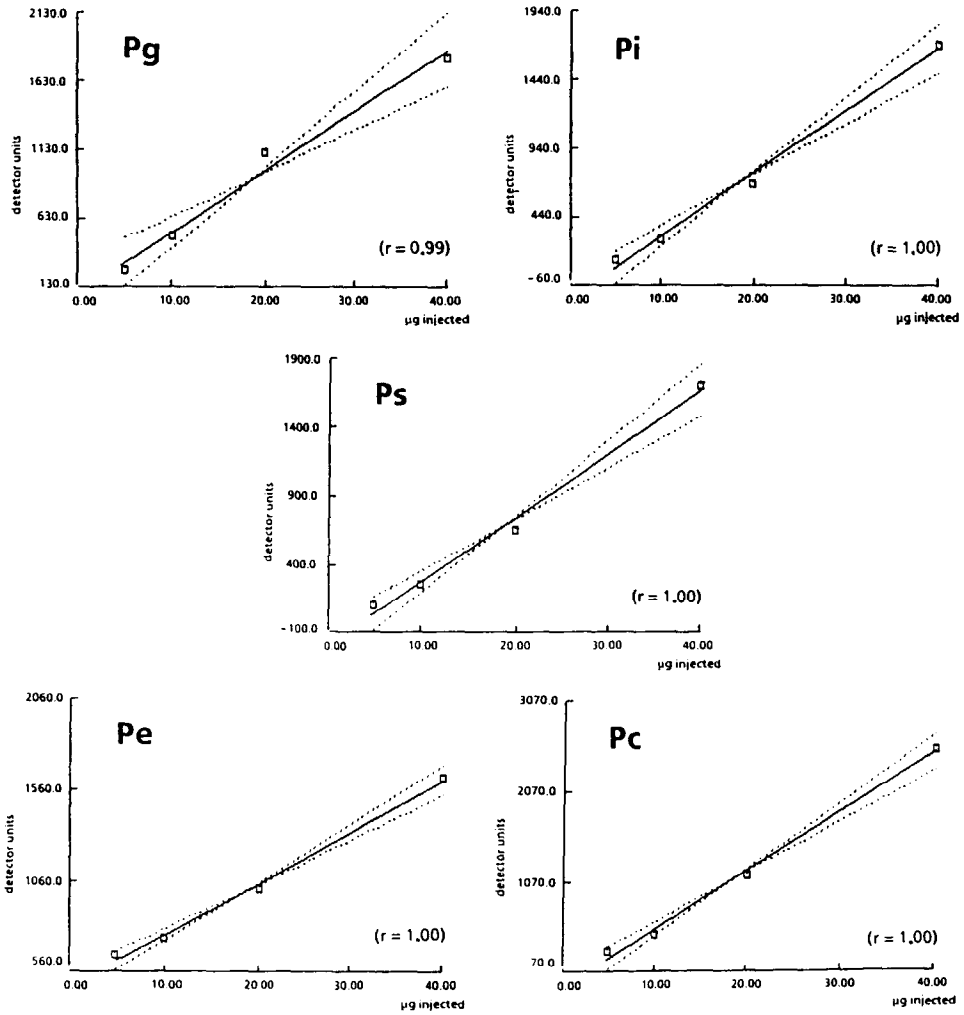

Fig. 2. Calibration graphs for individual phospholipids: phosphatidylglycerol (Pg), phosphatidylinositol (Pi), phosphatidylserine (Ps), phosphatidylethanolamine (Pe) and phosphatidylcholine (Pc). Each point represents the average of at least four determinations. Regression coefficients in brackets.

Methanol, chloroform, dichloromethane, trifluoroacetic acid (TFA) and *n*-heptane were obtained from Baker (Deventer, The Netherlands).

Chromatographic conditions

A Beckman liquid chromatographic Gold system consisting of a Model 126 pump, a Model 406 interface and an IBM microcomputer were used. The de-

tection was performed by a light-scattering detector (Model DDL 11, Cunow, Cergy-St. Christophe, France).

The column was a prepacked normal-phase Nucleosil 100 3-μm stainless-steel tube, 250 mm $\times$ 4 mm I.D. (Société Française Chromato Colonne, Neuilly-Plaisane, France).

Reagents

Two mobile phases were prepared: solvent A, chloroform–TFA (400:5), and solvent B, chloroform–methanol–*n*-heptane–water–TFA (100:400:50:15:5).

The water used was deionized and filtered through a Millipore system (Milli-Q). The chromatographic system was programmed for gradient elution using the two mobile phases. The flow-rate of the eluent was kept constant at 1 ml/min at room temperature (20–22°C).

In the light-scattering detector, the nebulization was performed at 40.5°C and a pressure of 2 bar. The solvent programme used is shown in Fig. 1. The first 45-min period of the elution scheme was designed to separate the phospholipid components, and the final 10 min regenerated the column prior to injection of the next sample.

RESULTS

The HPLC analysis of a standard mixture of five natural phospholipids in less than 1 h is shown in Fig. 1. A sample of 10 μg of each phospholipid in 10 μl of solvent was applied to the column. the phospholipids were well separated, with good peak shapes, in the following order: phosphatidylglycerol (retention time, $t_R = 10.9$ min), phosphatidylinositol ($t_R = 16.9$ min), phosphatidylserine ($t_R = 18.3$ and 19.4 min), phosphatidylethanolamine ($t_R = 30.9$ min) and phosphatidylcholine ($t_R = 34.6$ min). Phosphatidylserine produced two splits peaks, possibly caused by the separation of its two major subclasses.

Fig. 2 shows that a linear response, measured in terms of peak area, is obtained for amounts of 5–40 μg of the different phospholipids. The linear regression correlation coefficients were better than 0.99 for each of the five phospholipid concentration–response curves.

DISCUSSION

Numerous techniques have been published for phospholipid measurements. For instance, TLC has been used with various solvent mixtures and stationary phases, but most such methods are time-consuming and require tedious sample preparation(s). Moreover, there are the drawnbacks of poor reproducibility and the difficulty of routine automated use. To resolve these problems, many authors have turned to HPLC techniques for phospholipid analysis. Nevertheless, most of them have failed once again to solve separation and quantification

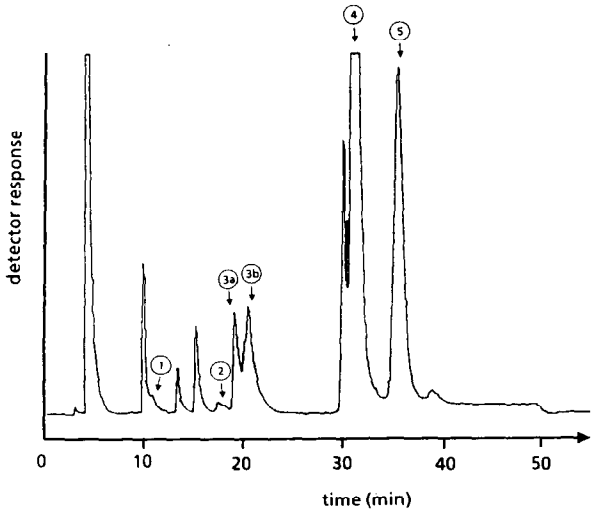

Fig. 3. Separation of a phospholipid extract from rat brain hippocampus. Peaks were identified by spiking the extract with standard phospholipids. Peaks: 1=phosphatidylglycerol; 2=phosphatidylinositol; 3a and 3b=phosphatidylserine; 4=phosphatidylethanolamine; 5=phosphatidylcholine.

problems. The techniques of Hax and Geurts van Kessel [10] and Geurts van Kessel et al. [7] fail to isolate phosphatidylcholine. The method of Jungalwala [6] allows the separation of phosphatidylcholine and sphingomyelin, but not other classes.

Moreover, each phospholipid may give rise to multiple peaks, reflecting different carbon chain lengths [24–26]. The method described herein has simplified these problems by achieving separation for all of the phospholipid classes.

In order to separate phospholipid classes with widely different polarities in a single step, we found it necessary to use gradient elution, starting with a solvent of low polarity and ending with a solvent mixture containing water. A polar solvent miscible both with chloroform and with water is required to mediate the transfer from one extreme to the other. Methanol was found to be best for this purpose.

The baseline was stable and needed little or no adjustment over a full day of use. The standardization of precisely identical reequilibration conditions between analyses prevents any shift in retention times of phospholipid compounds. This also maintains reproducible results in quantitative analysis with the light-scattering detector.

The good reproducibility for each eluted phospholipid and the simplicity of elution gradient are great improvements over a previous technique reported by Christie [22], who described how phosphatidylserine and phosphatidylinositol peaks were consistently migrating ca. 30 s later towards the end. The present method offers the advantage of being very reproducible (insofar as retention

time is concerned) whenever standardized equilibration times between subsequent runs were adhered to. This was confirmed largely by repeated injections of lipid mixtures at different concentrations during dose–response linearity studies. The other advantage of the present method is the relative simplicity of the elution solvent system over that of Christie [22].

High resolution and short analysis time are achieved by the case of 3-μm silica as column packing material, and much better resolution is obtained with longer columns.

This method has two major advantages. First is the absence of baseline drift with gradient. In this work, the adaptability of analytical silica columns was confirmed by comparison studies with different phase columns. Some of them, such as chemically bonded phases (amino groups), produced a non-linear baseline with drift caused by stationary phase dissolution. On the other hand, ca. 400 injections of lipid mixture were made on the silica column without any baseline drift or loss of reproducibility and sensitivity. The second advantage is the opportunity to use volatile solvents, which are eliminated before the eluate reaches the light source in the detector.

The HPLC system developed and characterized in this study appears to be well suited for rapid and efficient separation of most phospholipid constituents of biological membranes, as exemplified in Fig. 3. In the biological extracts the peaks were identified by spiking with each standard phospholipid. Nevertheless, other peaks remain to be identified. Further experiments are currently being carried out in our laboratory (particularly MS identification). Biological samples should be adapted to fall in the linear part of the concentration–response curve shown in Fig. 2 (i.e. between 5 and 40 μg of each lipid injected).

This HPLC method appears to be a promising approach for particular physiopathological conditions.

REFERENCES

1 C.P. Freeman and D. West, J. Lipid Res., 7 (1966) 324.
2 K.G. Blass and C.S. Ho, J. Chromatogr., 208 (1981) 170.
3 A.M. Gilfillan, A.J. Chu, D.A. Smart and S.A. Rooney, J. Lipid Res., 24 (1983) 1651.
4 K. Korte and M.L. Casey, J. Chromatogr., 232 (1982) 47.
5 K. Kiuchi, T. Ohta and H. Ebine, J. Chromatogr., 133 (19877) 266.
6 F.B. Jungalwala, J.E. Evans and R.H. McCluer, Biochem. J., 155 (1976) 55.
7 W.S.M. Geurts van Kessel, W.M.A. Hax, R.A. Demel and J. de Gier, Biochim. Biophys. Acta, 486 (1977) 524.
8 R.W. Gross and B.E. Sobel, J. Chromatogr., 197 (1980) 79.
9 V.L. Hanson, J.Y. Park, T.W. Osborn and R.M. Kiral, J. Chromatogr., 205 (1981) 393.
10 W.M.A. Hax and W.S.M. Geurts van Kessel, J. Chromatogr., 142 (1977) 735.
11 P. Juaneda and G. Rocquelin, Lipids, 21 (1986) 239.
12 G.M. Patton, J.M. Fasulo and S.J. Robins, J. Lipid Res., 23 (1982) 190.
13 S.S.H. Chen and A.Y. You, J. Chromatogr., 227 (1982) 25.
14 H.P. Nissen and K.W. Kreysel, J. Chromatogr., 276 (1983) 29.

15 J.R. Yandrasitz, G. Berry and S. Segal, Anal. Biochem., 135 (1983) 239.
16 T.L. Kaduce, K.C. Norton and A.A. Spector, J. Lipid Res., 24 (1983) 1398.
17 J.K. Kaitaranta, P.J. Geiger and S.P. Bessman, J. Chromatogr., 206 (1981) 327.
18 S. Bahrami, H. Gasser and H. Redl, J. Lipid Res., 28 (1987) 596.
19 N. Sotirhos, C. Thörngren and B. Herslöf, J. Chromatogr., 331 (1985) 313.
20 F.B. Jungalwala, J.E. Evans and R.H. McCluer, J. Lipid Res., 25 (1984) 738.
21 A. Stolyhwo, H. Colin and G. Guiochon, J. Chromatogr., 265 (1983) 1.
22 W.W. Christie, J. Lipid Res., 26 (1985) 507.
23 W.W. Christie, J. Chromatogr., 361 (1986) 396.
24 A.D. Postle, J. Chromatogr., 415 (1987) 241.
25 M. Schlame, H. Rabe, B. Rustow and D. Kunze, Biochim. Biophys. Acta., 958 (1988) 493.
26 S.J. Robins and G.M. Patton, J. Lipid Res., 27 (1986) 131.