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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND MASS DETECTION OF INDIVIDUAL PHOSPHOLIPID CLASSES

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the separation of individual classes of phospholipids into subfractions. The separation was achieved with isocratic elution using a Nucleosil-5 C_{18} column and a mobile phase consisting of methanol, acetonitrile and water. Three commercial C_{18} packing materials were evaluated before Nucleosil was chosen as the stationary phase. The compounds were detected with a combination of an UV detector and a light-scattering mass detector, which provided quantitative chromatograms reported for the first time. The mass detector also allowed location of the most relevant peaks of the UV chromatogram. Phosphatidylcholine and phosphatidylethanolamine species from egg, and phosphatidylcholine species from rat liver, were resolved into more than 20 peaks. The method can be applied to the separation of phospholipids from different biological sources.

INTRODUCTION

Phospholipids are complex molecules containing a variety of fatty acyl components which are difficult to analyze[1,2]. One method is to determine the total fatty acid distribution, which can be accomplished by total hydrolysis of the acyl ester linkage, followed by methylation and gas chromatography (GLC) of the fatty acid methyl esters. To obtain more information, selective partial hydrolysis with phospholipases can be performed, and the fatty acid distribution at positions sn-1 and sn-2 of the phospholipid molecule can be determined. However, the identity of the two fatty acids in individual molecular species is not obtained.

Reversed-phase high-performance liquid chromatography (HPLC) can be utilized to separate a single phospholipid class into species according to differences in the fatty acid composition. Methanol–water[3] and methanol–water–chloroform[4] were used as solvent systems in some early investigations. Better resolution was achieved when buffers[5–7] and choline chloride[8–10] were incorporated into the solvent system. Intact phospholipids were analyzed in these studies; derivatives of hydrolysis products have also been analyzed[11–14].

The use of buffers and other solvent modifiers may complicate the identifica-

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tion of the separated species. In addition, quantitation is complex and time-consuming. In this study a simple and volatile solvent system was utilized which enabled the use of a light-scattering mass detector with improved quantitation capability. Species of egg phosphatidylcholine and phosphatidylethanolamine, as well as rat-liver phosphatidylcholine, were separated.

MATERIALS AND METHODS

Reagents

HPLC grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, U.K.). Water was doubly distilled and deionized before use. Purified egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were obtained from Lepharm Scandinavien (Copenhagen, Denmark). Nucleosil-5 C_{18} used as the stationary phase for HPLC was purchased from Macherey-Nagel (Düren, F.R.G.). Spherisorb-5 ODS-1 was purchased from Phase Sep (Queensferry, U.K.), Lichropshere-5 RP-18 from E. Merck (Darmstadt, F.R.G.).

Rat-liver PC preparation

Rat-liver phosphatidylcholine (PC) was prepared as described elsewhere[15]. Hexane-isopropanol (3:2, v/v) was used to extract the lipids from homogenized rat liver. The total lipid extract was fractionated by normal-phase HPLC, and the PC fraction was collected for subsequent analysis.

High-performance liquid chromatography

Chromatography was performed with a Model 5060 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) and a Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.). Phospholipids were detected using a SPD-2A variable wavelength spectrophotometric detector (Shimadzu, Kyoto, Japan) operated at 214 nm, and a mass detector (Applied Chromatography Systems, Luton, U.K.) with an evaporator temperature of 40°C. The detectors were connected in series. The chromatograms were recorded using a Shimadzu Chromatopac C-R2A integrator. The separation was achieved with a Nucleosil-5 C_{18} reversed phase column, 25 cm × 4.6 mm I.D., packed in our laboratory. The temperature of the column was kept constant at 30°C using a water circulating bath (Heto Lab Equipment, Birkeröd, Denmark). The mobile phase was methanol–water–acetonitrile (8:1:1, w/w/w) at a flow-rate of 1.5 ml/min (isocratical). The amount of sample injected was 200 μg.

RESULTS

Comparison of stationary phases

A comparison was made between three C_{18} stationary phases. Fig. 1 shows chromatograms of egg PC, separated under the same conditions, on Spherisorb, Nucleosil and Lichrosphere columns packed in our laboratory. The test parameters for these columns are shown in Table I. The columns had different degrees of octadecylsilane coverage; Lichrosphere had the most and Spherisorb the least. Thus, Lichrosphere was the most non-polar stationary phase, and Spherisorb the least, as demonstrated by the Rs values. Accordingly, the shortest retention times were ob-

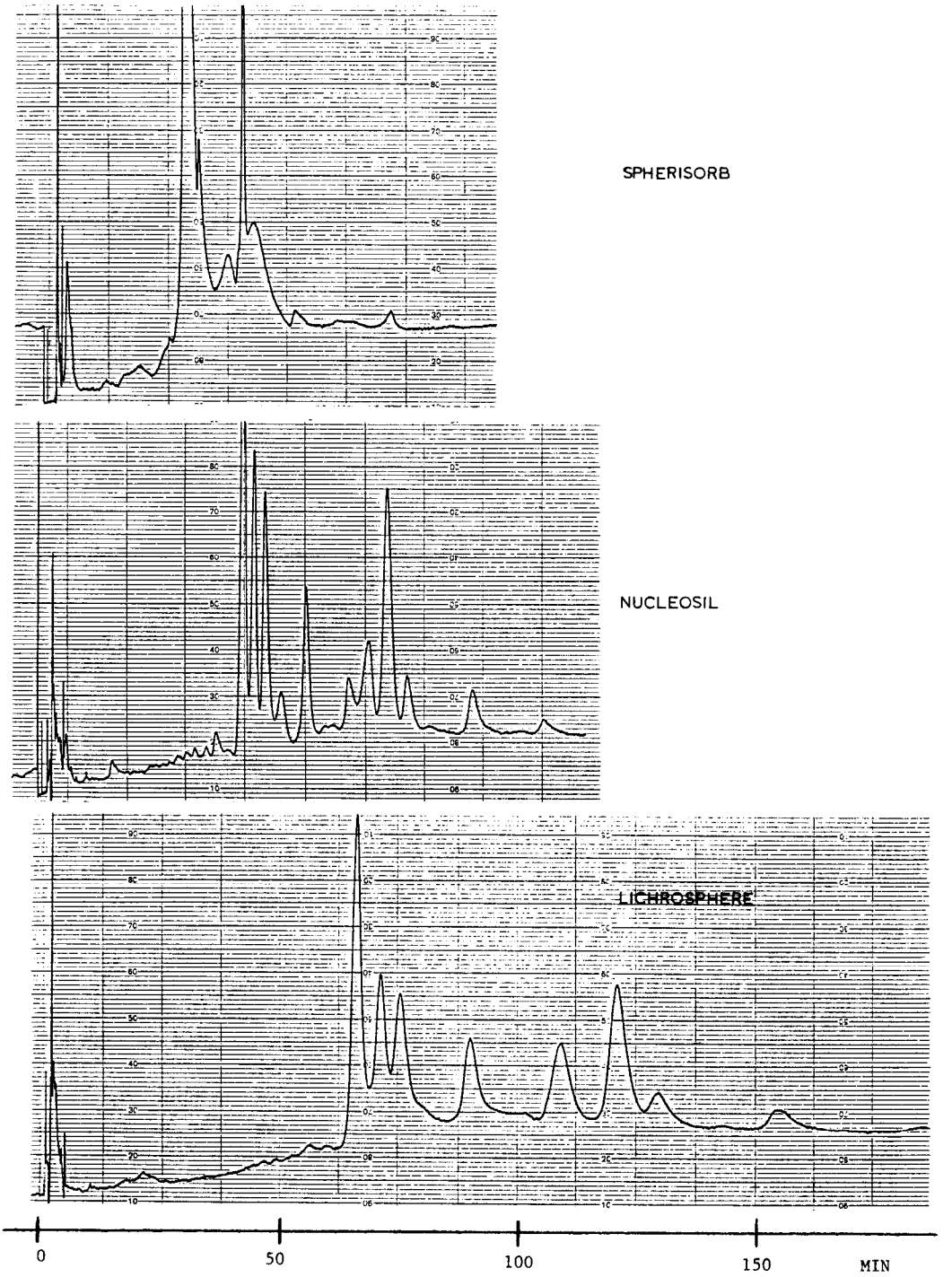

Fig. 1. HPLC separation of egg PC on three C_{18} reversed-phases. UV detection at 214 nm. Chromatographic conditions as described in Materials and Methods.

TABLE I

CALCULATED VALUES OF REDUCED PLATE HEIGHT AND RESOLUTION FOR THE THREE C_{18} PACKING MATERIALS

Eluent: methanol. Test substances: lauric and myristic acid methyl esters.

$$h = \frac{L}{5.54\left(\frac{t_1}{w^{1/2}\ t_1}\right)^2 \cdot d_p}$$

$$R_s = \frac{2\,(t_2 - t_1)}{wt_1 + wt_2}$$

where L = column length (mm); d_p = particle size (mm); t_1 = retention time of first solute; t_2 = retention time of second solute; w = peak width at base; $w^{1/2}$ = peak width at half height.

Packing material	*Reduced plate height, h*	*Resolution factor R_s*
Nucleosil C_{18}	3.11	5.92
Lichropshere RP-18	2.99	7.43
Spherisorb ODS-1	4.90	3.33

tained with Spherisorb, and the longest with Lichrosphere. The best selectivity, in terms of the largest number of peaks, was achieved with Nucleosil.

In order to make a comparison based on similar retention times, the conditions were changed for each column individually. Although the separation using Spherisorb and Lichrosphere improved, the best separation using these chromatographic conditions was obtained with Nucleosil.

Separation of egg PC and PE

A chromatogram of egg PC separated into subfractions is shown in Fig. 2. More than 20 peaks were detected. The upper chromatogram represents UV detection at 214 nm, and the lower, mass detection. More peaks were detected with the UV detector due to its high sensitivity. However, the mass detector provided a more quantitative representation since the peak size is largely a function of the concentration, whereas with the UV detector it is a function of the extinction coefficient of the constituents which make up a given absorbance peak.

The effect of concentration is clear when the two chromatograms in Fig. 2 are compared. One of the minor peaks in the UV chromatogram, retention time 70 min, actually contained the most mass. Conversely, the highest UV-absorbing compound was only a minor component. Egg PC had previously been detected only by UV spectroscopy[7,9], which provided a non-quantitative distribution.

Fig. 3 shows egg PE separated into subfractions using the same chromatographic conditions. Again, more than 20 peaks were detected. The mass detector indicated the peak with retention time of 50 min as having the highest concentration. The same component had a relatively small UV absorption.

Separation of rat-liver PC

Rat liver was also separated, and a chromatogram is shown in Fig. 4. Several peaks were resolved. The major constituents were indicated by the mass detector, and shown to be different from those of egg PC.

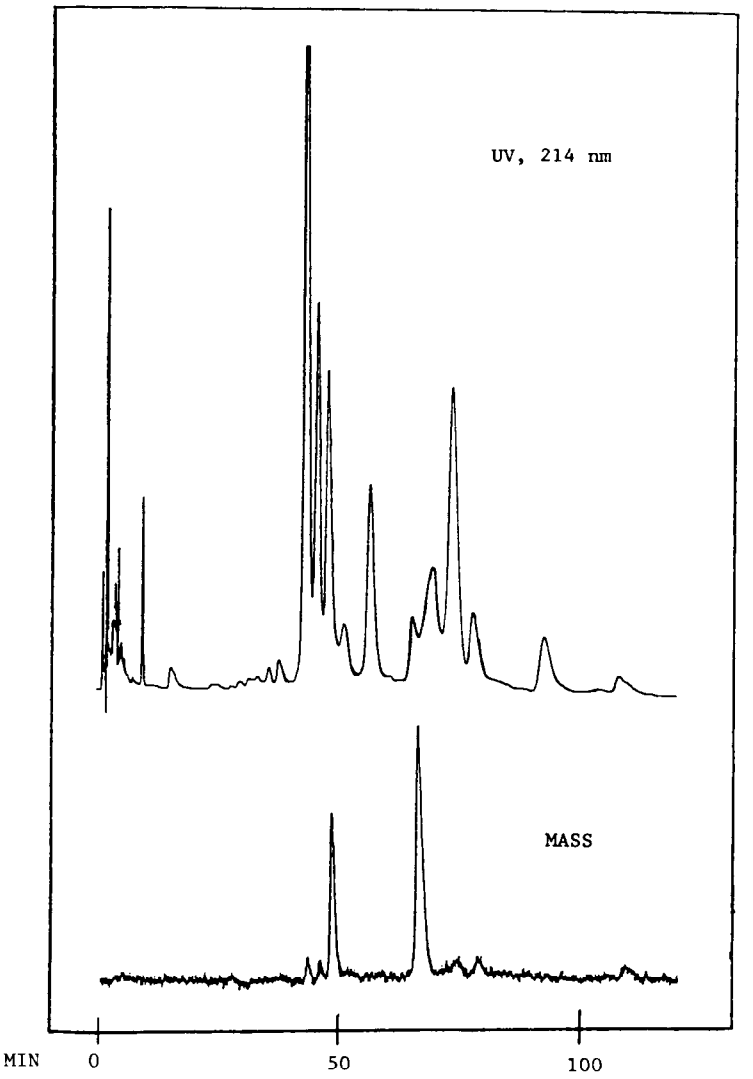

Fig. 2. HPLC separation of egg PC on Nucleosil C_{18}, with simultaneous UV and mass detection. Chromatographic conditions as described in Materials and Methods.

DISCUSSION

Comparison of stationary phases

Several variables should be considered when comparing columns. In this case, these include: column efficiency, coverage by bonded phase, amount of free silanol groups and column sensitivity to temperature, pH, solvents and modifiers. All these affect the separation.

The reduced plate height, *h*, was higher for Spherisorb than for the other two

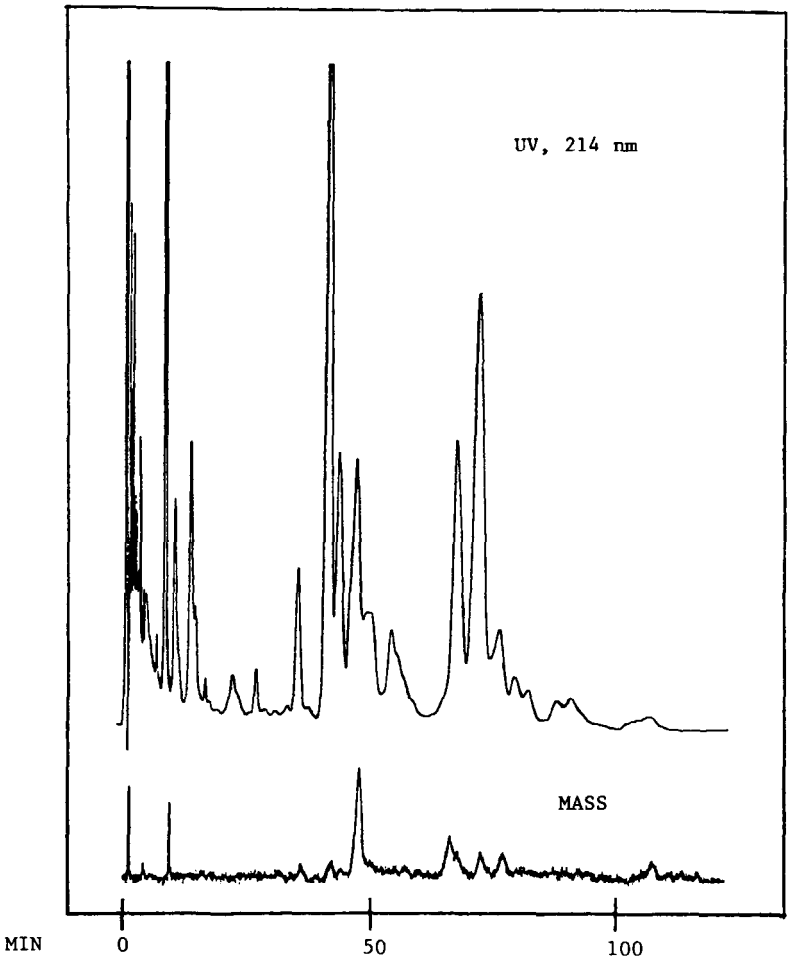

Fig. 3. HPLC separation of egg PE on Nucleosil C_{18}, with simultaneous UV and mass detection. Chromatographic conditions as described in Materials and Methods.

columns, indicating that the packing procedure was not optimized for Spherisorb. Better results could be expected with a lower h value.

An increase in octadecylsilane coverage did not increase resolution with Lichrosphere. Less coverage as in the case of Spherisorb was not desirable either. This confirms that separation does not occur only through interaction of the non-polar part of the molecule with the non-polar stationary phase; the polar end of the molecule is involved as well[8]. Free silanol groups in the stationary phase can interact with the polar end.

The utilization of solvent modifiers affects some stationary phases more than others. In this work solvent modifiers were avoided.

A comparison could be made only for a particular separation, based on a particular set of chromatographic conditions. In this case, Nucleosil was the best

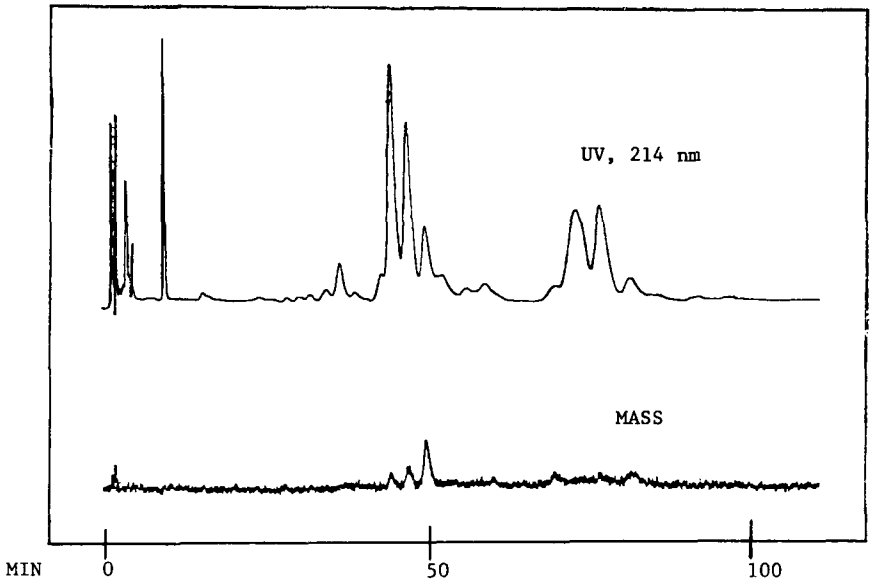

Fig. 4. HPLC separation of rat-liver PC on Nucleosil C_{18}, with a simultaneous UV and mass detection. Chromatographic conditions as described in Materials and Methods.

stationary phase, having adequate coverage and allowing interaction with the polar end of the molecule.

Separation of egg PC and PE

The analysis of complex, natural mixtures such as phospholipis is not an easy task. One problem is the identification of the separated species. Identification of the fatty acid pairs requires fractionation of the HPLC peaks followed by hydrolysis and GC analysis. Buffers or solvent modifiers presnet in the HPLC mobile phase can interfere. The described method has simplified this problem by achieving good separation without modifiers.

Another problem is quantification. Phospholipid quantitation by HPLC is limited due to the absence of a universal detector to measure molar quantities. Although UV detection is convenient, the area under a given absorbance peak does not reflect the molar amount of phospholipid eluted, but rather primarily the quantity of double bonds present in constituents within the fraction[16]. Phospholipid fractions have been quantitatively measured using a complex indirect method taking into account the percentage contribution of PC molecular species to each fraction isolated by preparative HPLC, and the total PC amount present in the fraction[7,9]. Colorimetric determination of phosphorus in the fractions collected by preparative reversed-phase HPLC was also used[8]. This is a tedious and time-consuming procedure. A more simple and indirect procedure would be advantageous.

The described method utilizes a light-scattering mass detector previously described[17]. The advantages of this detector are: (a) no baseline drift with gradients, (b) any volatile solvent can be utilized since solvents are eliminated before the eluate

reaches the light source and (c) most solutes are detected, and peak masses are measured directly from peak areas.

Although mass detection has several advantages, the usefulness of UV detection should not be overlooked. UV detection gives non-quantitative results in this case, but its high sensitivity enables the detection of minor components. We therefore suggest the use of both detectors.

The advantages of the method are the simple solvent system and the improved quantification ability. To our knowledge, this is the first time that mass detection has been utilized to analyze phospholipids. The method is useful for separation of different types of phospholipids as demonstrated with rat-liver PC and egg PE. In future work we will fractionate and identify these peaks as well as optimize the quantitative analysis based on mass detection.

REFERENCES

1 A. Kuksis, *Prog. Chem. Fats Other Lipids*, 12 (1972) 1.
2 B. J. Holub and A. Kuksis, *Adv. Lipid Res.*, 16 (1978) 1.
3 C. G. Crawford, R. D. Plattner, D. J. Sessa and J. J. Rackis, *Lipids*, 15 (1980) 91.
4 N. A. Porter, R. A. Wolf and J. R. Nixon, *Lipids*, 14 (1979) 20.
5 F. B. Jungalwala, V. Hayssen, J. M. Pasquini and R. H. McCluer, *J. Lipid Res.*, 20 (1979) 579.
6 F. B. Jungalwala, S. Sanyal and F. LeBaron, *Satell. Symp. Int. Soc. Neurochem.*, 1 (1982) 91.
7 M. Smith and F. B. Jungalwala, *J. Lipid Res.*, 22 (1981) 697.
8 G. M. Patton, J. M. Fasulo and S. J. Robins, *J. Lipid Res.*, 23 (1982) 190.
9 A. Cantafora, A. DiBiase, D. Alvaro, M. Angelico, M. Marin and A. F. Attili, *Clin. Chim. Acta*, 134 (1983) 281.
10 W. N. Marmer, T. A. Foglia and P. D. Vail, *Lipids*, 19 (1984) 353.
11 J. Y.-K. Hsieh, D. K. Welch and J. G. Turcotte, *J. Chromatogr.*, 208 (1981) 398.
12 J. Y.-K. Hsieh, D. K. Welch and J. G. Turcotte, *Lipids*, 16 (1981) 761.
13 Y. Nakagawa and L. A. Horrocks, *J. Lipid Res.*, 24 (1983) 1268.
14 M. L. Blank, M. Robinson, V. Fitzgerald and F. Snyder, *J. Chromatogr.*, 298 (1984) 473.
15 B. Herslöf, D. Ikonomou, L. Svensson and N. Sothirhos, in preparation.
16 F. B. Jungalwala, J. E. Evans and R. H. McCluer, *Biochem. J.*, 155 (1976) 55.
17 J. M. Charlesworth, *Anal. Chem.*, 50 (1978) 1414.