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Note

Separation of molecular species of phosphatidylcholine by high-performance liquid chromatography on a PLRP-S column

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There are two general approaches to the separation of molecular species of phospholipids (reviewed by Christie[1]). The most widely adopted consists in conversion to non-polar derivatives, *e.g.* by removal of the phosphoryl moiety by means of phospholipase C hydrolysis and acetylation or silylation of the resulting diacylglycerols for subsequent fractionation by silver nitrate thin-layer chromatography (TLC), high-temperature gas–liquid chromatography (GLC) or reversed-phase high-performance liquid chromatography (HPLC). The second approach consists in fractionation of the intact phospholipids by either silver nitrate TLC and/or reversed-phase TLC or reversed-phase HPLC, and is generally much more difficult technically. Methods of the latter type are particularly valuable in metabolic studies when the turnover of the phosphoryl group is measured with isotopically-labelled precursors. In all the published studies of the fractionation of molecular species of phosphatidylcholine by HPLC, bonded octadecylsilyl (C_{18} or ODS) reversed-phase columns have been used. It has then almost invariably been necessary to use eluents with an aqueous component containing salts, such as choline chloride or phosphate[2–7]. Removal of these salts, prior to analysis or quantification by other procedures, is time-consuming and can lead to losses. In this study, it has been shown that useful separations of molecular species of intact phosphatidylcholines can be achieved by reversed-phase HPLC on a polystyrene-based PLRP-S column. The fractions were identified and quantified by GLC of the methyl ester derivatives of the constituent fatty acids.

EXPERIMENTAL

Lipids were extracted, by means of chloroform–methanol (2:1, v/v), from the livers of female Wistar rats on a standard laboratory diet. A fraction consisting of the choline-containing phospholipids was prepared by column chromatography on DEAE-cellulose[8], and pure phosphatidylcholine was obtained from this by preparative HPLC on a silica column[9].

A Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics, St. Albans, U.K.) was used together with an ACS 750/14 mass detector (Applied Chromatography Systems, Luton, U.K.), with a stream splitter (approx. 10:1) between the end of the column and the detector. In preliminary experiments, a Knauer dif-

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ferential refractometer (Dr. H. Knauer, Oberursel/Taunus, F.R.G.) was used. The column used for the fractionation of the molecular species of phosphatidylcholines was of stainless steel (150 × 4.6 mm) and was packed with 5 μm PLRP-S, a polymeric styrene–divinylbenzene (Polymer Laboratories, Church Stretton, U.K.). Isocratic elution was possible and the optimum solvent combination was acetonitrile–methanol–water (70:15:15, v/v/v) at a flow-rate of 1 ml/min. The sample (0.5–2 mg) was dissolved in the eluting solvent (2–5 μl) for valve injection onto the column. All solvents were Analar or HPLC grades (Fisons, Loughborough, U.K.).

Fractions eluting via the stream splitter were collected manually and the solvent was evaporated in a stream of nitrogen prior to methylation of the fatty acid derivatives for GLC analysis[10]. Methyl eicosenoate (Sigma, Poole, U.K.) was added to each fraction as an internal standard for quantification purposes[1]. For GLC analysis, a fused-silica capillary column (25 m × 0.25 mm I.D.) containing Silar 5CP (Chrompak UK, London, U.K.) was used in a Carlo Erba Fractovap 4130 gas chromatograph (Erba Science UK, Swindon, U.K.) with hydrogen as carrier gas; peaks were quantified by electronic integration.

RESULTS AND DISCUSSION

Solvent systems based on acetonitrile, methanol and water have proved valuable both for adsorption and reversed-phase separations of phospholipids by means of HPLC (reviewed by Christie[11]. Such a combination was found to give a fractionation of phosphatidylcholine from rat liver into at least six species under isocratic elution conditions and at ambient temperature as illustrated in Fig. 1. Although the peaks were not entirely symmetrical, they were fairly distinct.

The mass detector (or "evaporative analyser" or "light-scattering detector")[12] was favoured for the work as the recorder baseline was not sensitive to changes in solvent composition or in ambient temperature. Satisfactory results were also ob-

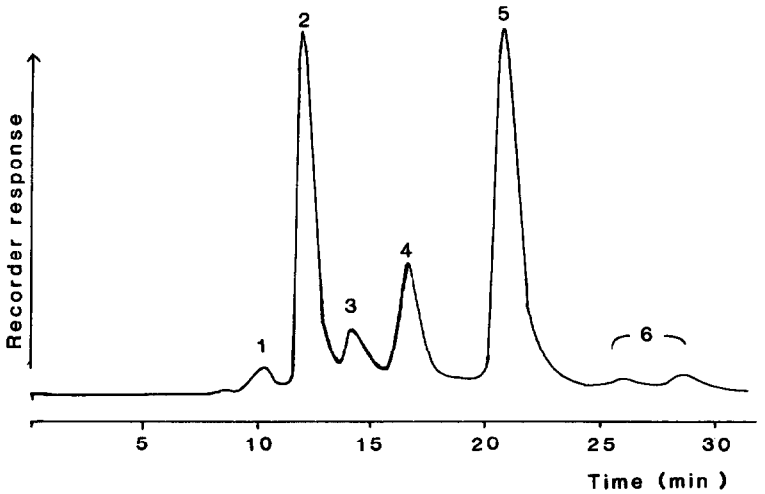

Fig. 1. Reversed-phase HPLC separation of molecular species of phosphatidylcholine from rat liver on a PLRP-S column with mass detection. For conditions, see the Experimental section. The numbers above the peaks correspond to the fractions in Table I.

tainable with care with a differential refractometer after the optimum elution conditions were determined. Great care is also necessary in calibrating the mass detector if it is to be used quantitatively, however, as the response is not linear but tends to fall off towards the lower mass range[12–14]. It is also influenced greatly by the concentration of the eluting components and therefore by the widths of the peaks, which in this instance were variable as each represented more than one component. For this reason, the fractions were collected via the stream splitter both for identification and for quantification purposes.

The results are listed in Table I, and represent the mean of duplicate analyses. None of the fractions, as might have been anticipated, contained a single molecular species, but considerable simplification had occurred. Most of the 16:0 fatty acid was in fractions 1–4, while most of the 18:0 fatty acid was in fractions 5 and 6. Thus fraction 2, for example, consisted mainly of species with 16:0 and di- or poly-unsaturated fatty acids, and fraction 5 of 18:0 and the same unsaturated components. Good replication of the results was possible and when the fatty acid composition of the intact lipid was computed as a check by reconstitution of the analyses of individual fractions, excellent agreement with the true values was obtained. When a second rat liver phosphatidylcholine sample was analysed, the fatty acid compositions of the fractions were very similar to those in Table I although the amounts of each fraction varied considerably (data not shown).

As with C_{18}-bonded phases, some separation had been achieved both according to the degree of unsaturation and the chain-lengths of the constituent fatty acids of the lipids. The chain-length of the saturated fatty acids appeared to exert the predominant effect. It is possible that the position of these fatty acids on the glycerol moiety is important as both tend to be located on the primary position in phosphatidylcholine (reviewed by Holub and Kuksis[15]; the fatty acid in position *sn*-2 could be less accessible to the stationary phase because of its proximity to the polar phos-

TABLE I

FATTY ACID COMPOSITIONS AND RELATIVE PROPORTIONS (mol-% OF THE TOTAL) OF MOLECULAR FRACTIONS OF RAT LIVER PHOSPHATIDYLCHOLINE SEPARATED BY HPLC

Fatty acid	*Intact lipid*	*Fraction*					
		1	*2*	*3*	*4*	*5*	*6*
14:0	0.4	4.0	0.4	–	–	–	1.0
16:0	21.6	23.4	44.4	34.0	37.7	5.1	3.2
16:1	2.2	17.6	2.2	–	1.6	–	–
18:0	25.4	4.4	1.0	–	7.7	47.2	49.5
18:1	12.8	6.5	4.3	19.7	33.5	5.6	17.1
18:2	12.3	17.2	14.8	16.0	4.9	16.2	7.5
20:3 (*n*−9)	2.1	0.7	0.3	3.6	2.4	–	10.2
20:4 (*n*−6)	15.9	12.9	20.7	18.7	5.8	19.7	8.5
20:5 (*n*−3)	1.2	8.1	0.5	–	4.2	–	
22:5 (*n*−3)	0.4	0.7	0.1	–	–	–	
22:6 (*n*−3)	5.8	4.5	11.3	8.0	2.2	6.2	3.0
Proportions		5.2	18.8	7.8	14.5	44.2	9.5

phorylcholine moiety. An analogous, if less marked effect, has been noted by others with a C_{18}-bonded phase[6].

The results reported above confirm that useful separations of molecular species of intact phosphatidylcholine can be obtained by means of HPLC on a PLRP-S column without having inorganic species in the solvents. The resolution obtained was perhaps not as good as that recorded by Patton *et al.*[6], but is at least as good as those recorded by others[2–5,7]. Elution conditions could probably be developed for the analysis of other phospholipid classes with this column, but it has not proved suitable for non-polar lipids such as triacylglycerols.

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