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METHOD FOR THE SENSITIVE ANALYSIS OF INDIVIDUAL MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN FLUORESCENCE DETECTION

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SUMMARY

Conditions are described for the efficient resolution by reversed-phase high-performance liquid chromatography on octadecylsilica stationary phases of individual molecular species of phosphatidylcholine on the basis of their fatty acyl composition. The effects are described of varying stationary phase carbon loading, mobile phase polarity and column temperature. Eluted peaks were detected by a sensitive post-column fluorescence system using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. This detection system permitted direct quantitation of phosphatidylcholine species irrespective of the degree of acyl unsaturation and eliminated the need for elaborate pre-column derivatization. The techniques were applied to the analysis of phosphatidylcholine isolated from rat liver and lung.

INTRODUCTION

Reports of the analysis of phospholipids in terms of molecular species composition have been sparse because of the lack of suitable, convenient analytical methods. Many studies have relied on indirect calculation from fatty acid analysis of fractions separated by argention thin-layer chromatography on the basis of acyl unsaturation [1]. A number of reversed-phase high-performance liquid chromatographic (HPLC) methods have been reported which provide more direct compositional information from the analysis of intact molecular species [2–4]. These methods have not gained wide acceptance because of problems associated with the quantitative detection of phospholipid molecules with differing degrees of acyl unsaturation. Phospholipid end absorption at 200–205 nm is a function of acyl unsaturation and so cannot be used for the direct calculation of concentra-

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tion. Moreover, disaturated species of phospholipid are virtually undetectable by ultraviolet absorbance.

Proposed solutions to this detection problem have included phospholipid phosphorous analysis of collected fractions [5] and mass detection by light scattering [4]. Both methods were too insensitive for most analytical purposes. The most widely described alternative detection techniques are all based on conversion of phospholipids to their respective diacylglycerol moieties by treatment with phospholipase C, coupled with the pre-column synthesis of a variety of derivatives. These include the benzoyl [6], *p*-nitrobenzoyl [7] and dinitrobenzoyl [8] chromophores, the dansylethanolamine phosphate [9] and napthylurethane [10] fluorescent compounds and the [^{3}H]acetate radioactive derivative [11]. Alternatively derivatives of diacyglycerol molecular species have been analysed by HPLC with mass spectrometry [12] and by gas chromatography (GC) on polar capillary columns [13]. Many of these techniques provide excellent resolutions of individual molecular species with detection limits as low as 20 pmol. Their common limitation is the extensive sample preparation they all require, with chemical modification, sequential extraction and chromatography steps. Such multi-stage procedures are potentially subject to many cumulative errors. The inconvenience of these methods is shown by the low number of reports analyzing the dynamic regulation of phospholipid metabolism in terms of molecular species. An additional significant drawback is that phospholipase C treatment precludes the analysis of metabolic studies using, for instance, the incorporation of [^{14}C]choline or ^{32}P.

An alternative detection technique is presented in this paper based on a post-column fluorescence derivatization using 1,6-diphenyl-1,3,5-hexatriene (DPH) which exhibits increased fluorescence in a lipid environment [12]. This technique has been proposed recently for the estimation of the lecithin/sphingomyelin ratio of amniotic fluid by normal-phase HPLC [14]. The results presented here extend this application to the analysis of individual molecular species of phosphatidylcholine (PC) separated by reversed-phase HPLC. The application of this technique to the analysis of the PC composition of adult rat liver and lung is also described.

EXPERIMENTAL

Chemicals

Both HPLC-grade and extraction solvents were obtained from Rathburn (Walkerburn, U.K.). Other chemicals and PC standards* were obtained from Sigma (Poole, U.K.).

Sample preparation

Samples of rat liver and lung were extracted with chloroform and methanol [14], dried under a stream of nitrogen and stored in a chloroform solution at

*PC nomenclature (acyl group in Sn1 position/acyl group in Sn2 position): 14:0, myristate; 16:0, palmitate; 16:1, palmitoleate; 18:0, stearate; 18:1, oleate; 18:2, linoleate; 18:3, α-linoleate; 20:4, arachidonate; 22:6, docosahexaenoate.

−20 °C under a nitrogen atmosphere until analysis. An internal standard of PC 14:0/14:0 (100 μl of a 1 μmol ml^{-1} chloroform solution) was added to the extraction mixture. Total PC was isolated from other phospholipid classes by normal-phase HPLC on μPorasil, 10 μm particle size, 30 cm×4.9 mm (Waters Assoc., Milford, MA, U.S.A.). The chromatography system employed a Waters 6000A pump, U6K injector and Model 450 spectrophotometer, operated at 200 nm. Using a mobile phase of hexane–propan-2-ol–water (6:8:0.9) at a flow-rate of 1 ml min^{-1}, PC eluted between 26 and 32 min. Eluted PC was collected, dried under nitrogen and dissolved in a minimum volume of trifluoroethanol for analysis by reversed-phase HPLC.

Molecular species analysis of phosphatidylcholine

Stationary phases evaluated were μBondapak C_{18}, 10 μm (Waters Assoc.), Spherisorb ODS 1 and ODS 2 (5 μm) and Apex ODS 1 and ODS 2, 5 μm (Jones Chromatography, Llanbradach, U.K.). All columns were 25 cm×4.6 mm I.D. stainless steel, with temperature regulation using a Model 7920 column block heater (Jones Chromatography). The mobile phase comprised 40 mmol l^{-1} choline chloride [2] in methanol containing varying proportions of water and acetonitrile at a flow-rate of 1 ml min^{-1}. The HPLC column eluate was passed initially through the Waters Model 450 spectrophotometer at 200 nm and then subsequently into the post-column detection system. This consisted of a second Model 6000A HPLC pump connected via the internal pulse dampner to a 15μl mixing chamber, a 3 m×0.5 mm I.D. PTFE string mixer at 50 °C and then a Model 1000 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.) fitted with a 100-μl flow-cell (Hellma, Westcliff-on-Sea, U.K.). The detection reagent was distilled water containing, per litre, 150 μl of 3 mmol l^{-1} DPH solution in tetrahydrofuran–0.001% (v/v) Tween 20, at a flow-rate of 3 ml min^{-1}. The detergent was necessary to prevent build up of background fluorescence due to adsorption of PC vesicles in the flow cell. The excitation and emission wavelengths were 340 and 460 nm, respectively, and the fluorescent and absorbance signals were both displayed on a dual-channel pen recorder (Tarkan W + W, Kontron Instruments, St. Albans, U.K.). Areas under eluted peaks were integrated gravimetrically.

Other analytical methods

The phospholipid concentration of lipid extracts was measured as lipid phosphorus using PC 16:0/16:0 as the standard [15]. Phospholipid acyl composition was measured by GC of their methyl esters. Methyl fatty acids were formed by transesterification using sodium methoxide. They were re-extracted with hexane and resolved by capillary GC using a stationary phase of CP-Sil 88 and hydrogen as carrier gas (Chrompack U.K., London, U.K.).

RESULTS

Post-column detection of phosphatidylcholine by DPH fluorescence

The optimal conditions for the post-column detection of PC species were determined empirically. The concentration of DPH used (0.45 μmol l^{-1}) gave the

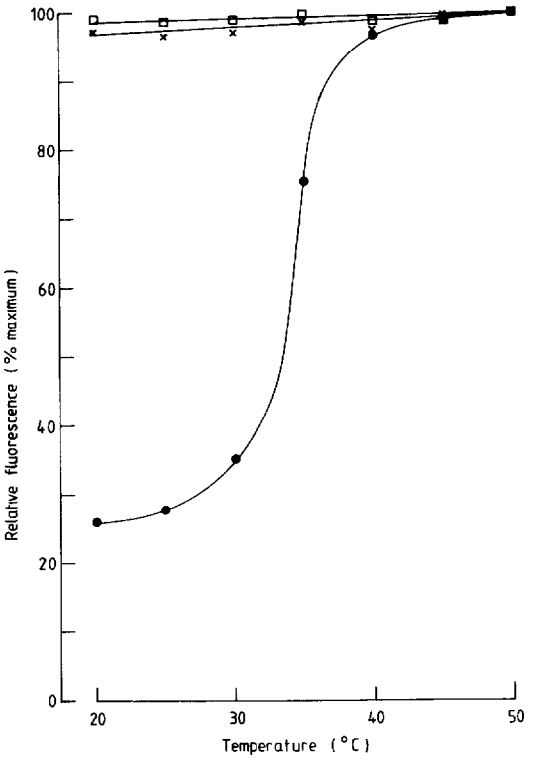

Fig. 1. The effect of temperature on the post-column DPH detection of PC standards. Eluted PC species were incubated at the indicated temperatures with the DPH detection reagent and their relative fluorescence intensities measured. (□) PC 14:0/14:0; (×) PC 16:0/18:2; (●) PC 16:0/16:0.

most favourable signal-to-noise ratio in response to an eluted HPLC peak containing 10 nmol of internal standard PC 14:0/14:0. Measurement of the fluorescence responses to a variety of standard PC species demonstrated that elevated temperatures were required for adequate post-column detection (Fig. 1). While the DPH fluorescence in response to 10 nmol of either PC 14:0/14:0 or PC 16:0/18:2 was not significantly affected by increasing the mixing temperature from 20 to 50°C, the response to 10 nmol PC 16:0/16:0 was increased four-fold. The post-column DPH fluorescence response at 50°C was linear from 0.5 to 100 nmol for all PC species analyzed. Slight variations in the fluorescence responses were observed between different molecular species of PC which were apparently due in part to acyl chain length. When expressed relative to PC 16:0/16:0 as 100% the integrated fluorescence response between 1 and 10 nmol for PC 12:0/12:0, PC 14:0/14:0 and PC 16:0/18.1 were 80, 94 and 101%, respectively. This variation in response had only a limited effect on the analysis of the major PC species present in rat liver and lung. The relative fluorescence response of eluted PC species correlated well with the concentration of phospholipid in collected fractions, measured as lipid phosphorus ($r=0.970$, $n=14$) (see Fig. 2).

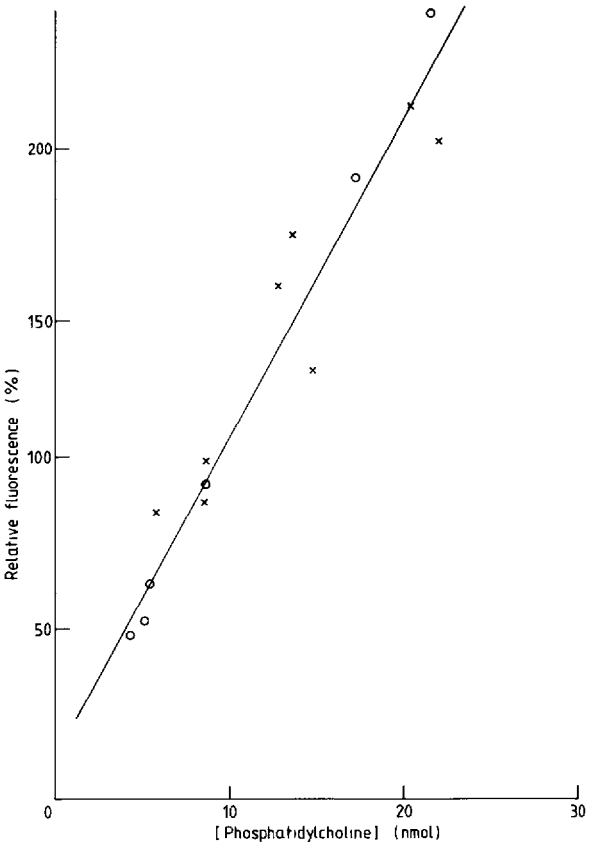

Fig. 2. The DPH fluorescence response to different concentrations of eluted PC species. Fluorescence was expressed relative to 10 nmol of internal standard PC 14:0/14:0 as 100%. Source of PC species: (×) liver; (○) lung.

Reversed-phase HPLC analysis of phosphatidylcholine

The application of post-column DPH fluorescence to the detection of molecular species of PC eluted by reversed-phase HPLC was investigated for rat liver and lung. These tissues were chosen to represent the extremes of PC composition, containing the highest proportions of unsaturated and saturated PC species, respectively. Typical chromatograms for the analysis of rat liver and lung PC are shown in Figs. 3 and 4, using an Apex ODS 2 stationary phase at 50°C and a mobile phase of 40 m*M* choline chloride in methanol–water (92.5:7.5). The upper trace in each case represents the UV absorbance at 200 nm, while the lower trace is the fluorescence response. Comparison of these chromatograms highlights the usefulness of the post-column fluorescence detection system. For liver PC most major species gave both UV absorbance and fluorescence responses (Fig. 3) and, despite the variation in the absorbance response, calculation of absolute concentrations would be feasible by the application of suitable conversion factors. By contrast, the major species of lung PC, PC 16:0/16:0 and PC 16:0/18:1, were virtually undetected by UV asorbance (Fig. 4) but were quantitatively detected by

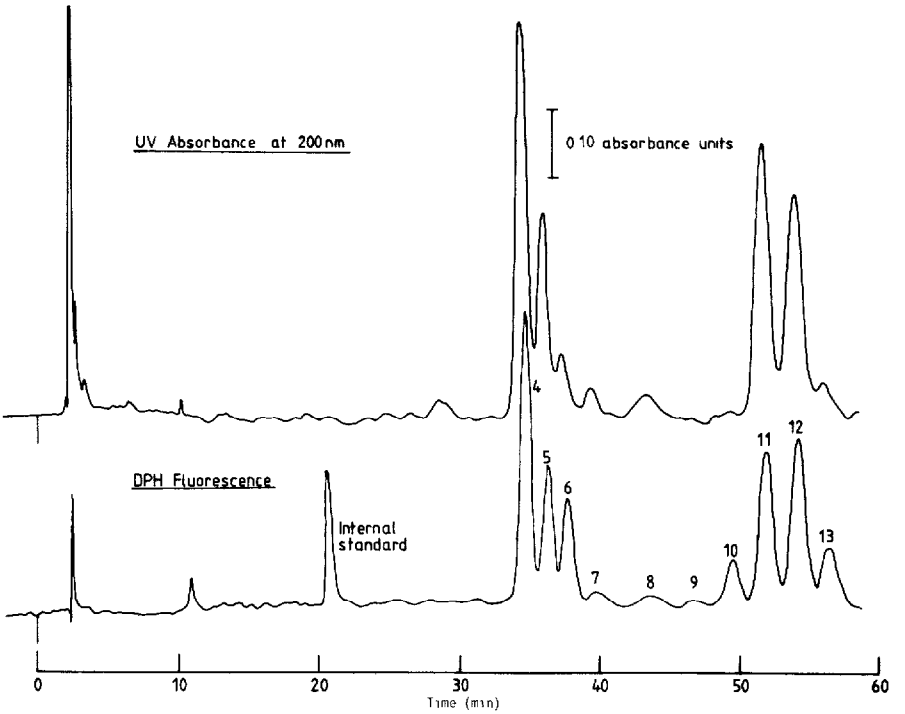

Fig. 3. The analysis of rat liver PC by reversed-phase HPLC. Column: 25 cm×4.6 mm Apex ODS 2, 5 μm at 50°C. Mobile phase: 40 mmol l^{-1} choline chloride in methanol–water (92.5:7.5) at 1 ml min^{-1}. Internal standard: PC 14:0/14:0. The numbered peaks refer to Table II.

DPH fluorescence. This difference is significant because the majority of tissues, and cells in culture, maintain a high proportion of relatively saturated PC species.

The ability to detect all PC molecular species allowed optimization of chromatography conditions for saturated as well as unsaturated species. Initially a series of reversed-phase C_{18} stationary phases were evaluated, using a mobile phase of 40 m*M* choline chloride in methanol–acetonitrile–water (90:7.5:2.5) at a flow-rate of 1 ml min^{-1} and a temperature of 20°C. Choline chloride was necessary to suppress ionic interactions between the choline group of PC and the stationary phase, which led to severe peak broadening and tailing [2]. Alternative agents investigated, 40 mmol l^{-1} triethylammonium sulphate and 50 mmol l^{-1} potassium phosphate pH 7.0, were both less effective. Stationary phase composition influenced the chromatography of PC considerably; resolution and peak symmetry were both better for fully end-capped phases with higher carbon loadings. Most efficient separations were achieved on Apex ODS 2, followed by Spherisorb ODS 2, μBondapak C_{18} and then Spherisorb ODS 1 (Table I). Under the chromatography conditions used, none of the stationary phases evaluated separated PC 16:0/16:0 and PC 16:0/18:1, but Apex ODS 2 and Spherisorb ODS 2 gave a reasonable resolution of PC 16:0/22:6 and PC 16:0/20:4. Both Spherisorb ODS 1 and μBondapak C_{18} gave inadequate resolution of all PC species. Apex ODS 2 was the stationary phase used for further methods development.

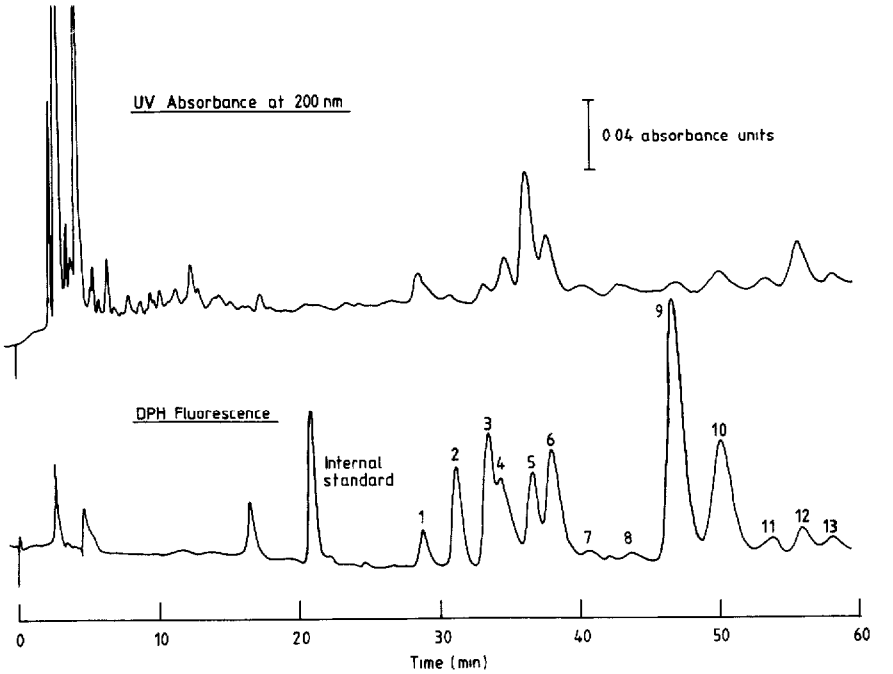

Fig. 4. The analysis of rat lung PC by reversed-phase HPLC. Conditions as in Fig. 3.

The column temperature was a very important parameter in achieving acceptable resolution, especially of more saturated PC species (Fig. 5a). Below 15°C PC 16:0/16:0 was effectively resolved from PC 16:0/18:1, but the latter species co-eluted with PC 18:0/20:4. No resolution of any of these three species was pos-

TABLE I

COMPARISON OF RESOLUTIONS OF STATIONARY PHASES

All columns were stainless steel, 25 cm × 4.6 mm I.D. Mobile phase A was 40 m*M* choline chloride in methanol–acetonitrile–water (90:7.5:2.5) at 1 ml min^{-1} and 20°C. Mobile phase B was 40 m*M* choline chloride in methanol–water (92.5:7.5) at 1 ml min^{-1} and 50°C. Resolution (R) was calculated from the equation: $R = \frac{1}{4}\frac{(\alpha-1)}{\alpha}(\sqrt{N})\frac{(k')}{k'+1}$ where α is the relative retention, N is the number of theoretical plates and k' is the capacity factor of an eluted compound.

Stationary phase	Mobile phase	Resolution	
		16:0/22:6 vs. 16:0/20:4	16:0/16:0 vs.16:0/18:1
Spherisorb ODS 1, 5 μm	A	0.20	0
Spherisorb ODS 2, 5 μm	A	1.00	0
μBondapak C_{18}, 10 μm	A	0.79	0
Apex ODS 2, 5 μm	A	1.20	0
Two Apex ODS 2 columns in series	A	2.13	0
Apex ODS 2, 5 μm	B	1.11	1.35

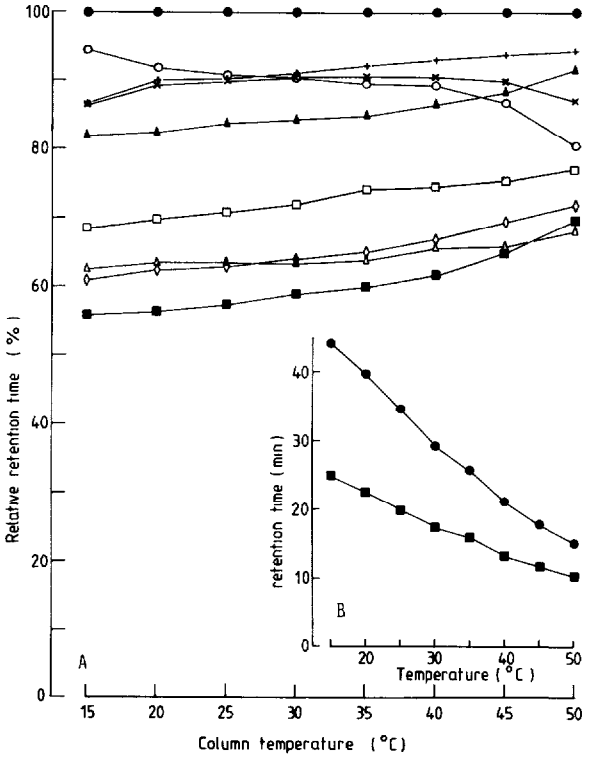

Fig. 5. (A) Effect of column temperature on the resolution of molecular species of PC by reversed-phase HPLC. Retention times were calculated relative to PC 18:0/18:2 as 100%. Column: 25 cm × 4.6 mm Apex ODS 2, 5 μm. Mobile phase: 40 mmol l^{-1} choline chloride in methanol-acetonitrile-water (90:7.5:2.5) at 1 ml min^{-1}. (△) PC 16:0/16:16:1; (■) PC 16:0/22:6; (◇) PC 16:0/20:4; (□) PC 16:0/18:2; (○) PC 16:0/16:0; (×) PC 16:0/18:1; (▲) PC 18:0/22:6; (+) PC 18:0/20:4; (●) PC 18:0/18:2. (B) Effect of column temperature on the absolute retention times of PC 16:0/22:6 (■) and PC 18:0/18:2 (●).

sible between 20 and 40 °C, which posed considerable problems during the initial methods development at ambient room temperatures. Good resolutions were obtained above 45 °C when their elution order was reversed. By contrast the elution order of the polyunsaturated species were unchanged between 15 and 50 °C. The other major effect of increased column temperature was to decrease the absolute retention times for all PC species (Fig. 5B) with an accompanying impaired resolution. Resolution at 50 °C was improved by increasing mobile phase polarity which prolonged retention times. Increasing the water content of the mobile phase from 2.5 to 7.5% did not change the elution order of any PC species from rat liver or lung but increased their retention times by a mean of 360%. The chromatograms of rat liver and lung PC shown in Figs. 3 and 4 were obtained under these conditions. The identities of the molecular species of PC separated by these conditions are given in Table II.

The reproducibility of the temperature-regulated chromatography was excellent, both in terms of retention times and peak areas. The coefficient of variation for retention time of the thirteen molecular species described in Table II in ten

TABLE II

MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE IDENTIFIED IN RAT LIVER AND LUNG

Molecular species of PC from rat liver and lung (Figs. 3 and 4) were numbered in elution order. Collected fractions were analyzed for acyl composition by GC. Identified species comprised at least 90% of the PC present in that fraction. N.D. = not determined.

Peak No.	Retention time (min)	Identity
1	31.0	N.D.
2	33.7	N.D.
3	34.4	16:0/16:1
4	35.5	16:0/22:6
5	36.8	16:0/20:4
6	38.2	16:0/18:2
7	40.4	N.D.
8	44.0	N.D.
9	46.7	16:0/16:0
10	49.9	16:0/18:1
11	52.6	18:0/22:6
12	54.9	18:0/20:4
13	57.1	18:0/18:2

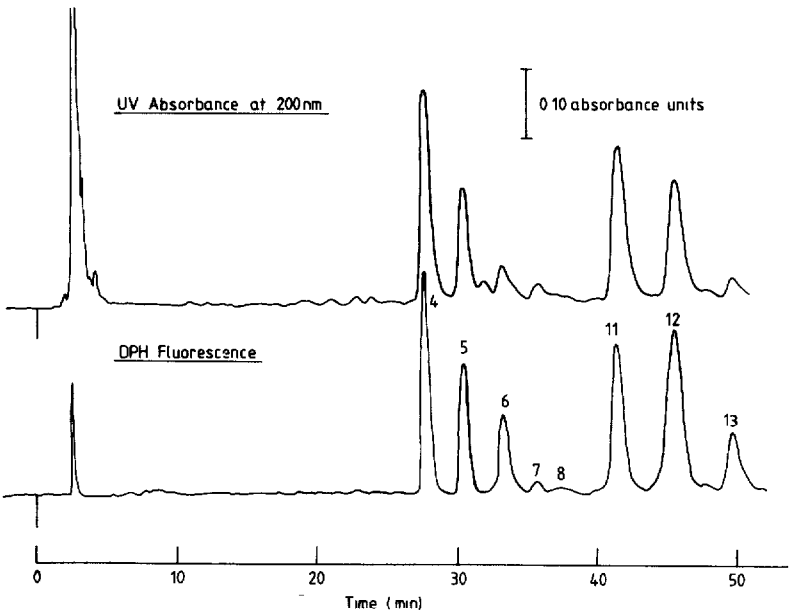

Fig. 6. Analysis of rat liver PC by reversed-phase HPLC. Columns: two 25 cm×4.9 mm Apex ODS 2, 5 μm, at 20°C. Mobile phase: 40 mmol l^{-1} choline chloride in methanol–acetonitrile–water (90:7.5:2.5) at 1 ml min^{-1}. The numbered peaks refer toTable II.

sequential chromatograms was 0.48±0.13% (mean±S.D.), range 0.32–0.68%. Comparable values for quantitation of six major PC species in rat liver, corrected for internal standard recovery, were 3.25±0.87% (mean±S.D.), range 2.15–4.78%. The concentrations of molecular species are not given. It was found, especially for liver PC, that there was a wide variability of composition under different nutritional and physiological conditions. For instance, the percentage contribution of PC 16:0/22:6 to total PC in maternal liver increased from 4.6 to 17.84% between days 14 and 21 of gestation. The mechanisms regulating this variability are currently under investigation.

While the chromatographic separations presented in Figs. 3 and 4 were optimized for the resolutions of PC 16:0/16:0 and PC 16:0/18:1 it was also possible to modify those conditions for the analysis of a different spectrum of PC species. This is shown for the analysis of liver PC by comparison of Figs. 3 and 6. Conditions used for the second analysis (Fig. 6) were two Apex ODS 2 columns in series at 20°C with a mobile phase of 40 m*M* choline chloride in methanol–acetonitrile–water (90:7.5:2.5) at a flow-rate of 1 ml min^{-1}. The separations of PC species containing polyunsaturated acyl groups were markedly improved, shown by the improved resolution between, for instance, PC 16:0/22:6 and PC 16:0/20:4 (Table I). Such improved resolution of relatively unstable compounds could have wide applications, especially in metabolic and preparative studies if the poorer resolution of more saturated PC species was less important.

DISCUSSION

The analysis of intact molecular species of PC by a technique that is sensitive, quantitative and reliable possesses considerable attractiveness. Sample manipulation was reduced to a minimum and errors resulting from elaborate pre-column derivatization procedures were eliminated. Extraction losses and solvent evaporation errors were corrected by recovery of PC 14:0/14:0 added as an internal standard, allowing direct calculation of concentration from the integrated fluorescence signal. The detection of saturated PC species enabled chromatographic conditions to be devised for their efficient and reproducible resolution.

The basis for the fluorescence detection of PC species relies on the formation of PC vesicles in the aqueous post-column system. The theoretical limiting factor is the critical micelle concentration of PC, the value of which is uncertain but is probably between 10^{-9} and 10^{-11} *M* [12]. However, the concentration of PC in the post-column system at the minimum detectable concentration (0.5–2 nmol per injection) was of the order 10^{-6}–10^{-7} *M*. This implies that there is inherently a potential improvement in sensitivity of DPH fluorescence detection of PC of about two orders of magnitude at least. Achievement of this greater sensitivity is dependent on noise reduction by improving the design of the post-column mixing equipment. Preliminary experiments using the present apparatus have indicated, however, that it is already sufficiently sensitive for the analysis of PC distribution in tissue culture cells.

The improved resolution of intact PC species facilitated by fluorescence detection has immediate advantages in metabolic studies involving the incorporations

of radioactively labelled precursors. It allows measurements to be made of the rates of incorporation of all radioactively labelled precursors of PC. This is particularly significant for the incorporation of [^{14}C]choline, which can give an estimate both of the relative rates of synthesis and turnover of individual molecular species of PC and of the degree and nature of acyl re-modelling. It avoids the problem of devising, for instance, efficient conditions for the dinitrobenzoyl–diacylglycerol derivatives of PC but relying on less efficient systems for the HPLC analysis of [^{14}C]choline incorporation into intact PC species [13].

This technique should find wide applications in the analysis of lung surfactant and cell culture metabolism, where there is a large proportion of more saturated PC species. It is also in principle equally applicable to the analysis of other classes of phospholipid.

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