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Quantitation of individual molecular species of phosphatidylcholines by reversed-phase high-performance liquid chromatography with fluorometric detection

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Abstract

The multiplicity of phosphatidylcholines is caused by the presence of different pairs of fatty acids in their individual molecular species and at least 27 miscellaneous fatty acids were identified in phosphatidylcholines in the serum of healthy individuals by combined gas–liquid chromatography and mass spectrometry in our present experiments. A method is described for the separation and quantitation of molecular species of phosphatidylcholine in human serum. Total phosphatidylcholine is isolated from lipids extracted from the serum with chloroform–methanol (2:1) by reversed-phase liquid–liquid extraction and subjected to reversed-phase high-performance liquid chromatography with a discontinuous descending gradient of water. Separation is monitored by fluorometry (340/460 nm) and absorption at 205 nm, if required. Up to 25 different molecular species of phosphatidylcholine may be quantified with a satisfactory reproducibility (±5–8%). Data on the distribution of individual molecular species in phosphatidylcholine of 53 normal serums are presented. The method may be used for quantitation of these phospholipids also in other biological materials (cell lines, leukemic cells from patients), and on a micropreparative scale to isolate individual compounds. The speed of separation as well as a satisfactory reproducibility are its principal advantages.

Keywords: Phosphatidylcholines

1. Introduction

Phosphatidylcholines (PCs) play an important role in signal transduction and carcinogenesis [1] and are also involved in the synthesis of cholesteryl esters being substrates for lecithin-cholesterol acyltransferase (LCAT) (EC 2.3.1.43) [2]. Despite of their important functions, relatively little attention was paid up to now to individual molecular species of these phospolipids.

The reason for this is undoubtedly the fact that the separation and detection of these compounds are relatively complicated and time-consuming. Reversed-phase high-performance liquid chromatography (HPLC) seems to be the method of choice for such experiments. Satisfactory separations were reported using mixtures of methanol–acetonitrile–water [3,4]. An interesting approach was chosen by Blank et al. [5] who described a pre-column de-

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rivatisation of PCs by splitting them with phospholipase C into diglycerides and converting these to benzoate derivatives that were subsequently separated by reversed-phase HPLC with methanol–2-propanol mixtures. The elution was significantly more rapid than that in earlier methods but the preliminary derivatization seems to be rather complicated. The determination of lipid P was the only method available for the detection of PCs in earlier experiments [6] but it is complicated and time-consuming. Absorption at 205 nm may be used for monitoring column eluates [3] but it is not very sensitive and cannot be applied for quantitation since individual PC species give a widely differing response depending on the number of double bonds present in their molecule [7]. On the other hand light-scattering detection [8] and fluorimetery [7] which monitor the fluorescence (luminescence) of phosphatidylcholines at 340/460 nm are suitable for quantitation. The latter detection method yields a uniform response for PC species of any composition [7].

We have recently reported purification to apparent homogeneity of cholesterol-esterifying enzymes from rat liver cytosol that utilize PCs as substrate [9]. Addition of serum from patients with malignant tumors but not of healthy individuals significantly stimulated the activity of these enzymes in vitro [10]. These results indicated an alteration of PC patterns in cancer serum and we indeed recently demonstrated that total PC levels in serum of cancer patients is very significantly elevated when compared with healthy individuals [11]. For our further studies the development of a novel procedure seemed desirable because methods described up to now are time-consuming (they require a preliminary separation of PCs from other phospholipids by HPLC [3] before the quantitation of PC species by additional HPLC), and are scarcely suitable for routine analyses.

This paper describes a procedure for quantitation of individual molecular species of PCs in serum by reversed-phase HPLC in a descending gradient of water which follows after a preliminary separation of PCs from other phospholipids by a simple liquid–liquid extraction [13]. This method shows a satisfactory sensitivity, is relatively rapid and suitable for serial analyses. It may be easily adapted for other types of biological materials (cell lines, tumor and leukemic cells etc.). It is also useful for micropreparative purification of PC molecular species.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich, Prague, Czech Republic. Solvents were of HPLC grade. Stainless steel columns, 250×4 mm I.D., packed with Separon SGX C_{18}, 5 μm, with an efficiency of >65 000 theoretical plates/m were obtained from Tessek, Prague, Czech Republic.

2.2. Apparatus

The HPLC system consisted of Beckman Model 114M solvent delivery modules. The column was placed in a column oven (Ecom, Prague, Czech Republic) at 50°C and the effluent was monitored at 205 nm in a type 2082.1 variable-wavelength detector (Ecom). After this, the eluate passed through a polyether ether ketone (PEEK) mixing chamber where it was continuously mixed with the scintillator solution. The resulting mixture was passed through a 3 m×0.25 mm I.D. PTFE capillary kept at 50°C into the type 2092 fluorometric detector (Ecom). The excitation wavelength was 340 nm, emission was monitored at 460 nm.

2.3. Isolation of phosphatidylcholine

1.0 ml of serum from blood donors was extracted with chloroform–methanol [14], PCs were separated from the extract by liquid–liquid extraction and this solution was evaporated to dryness at 50°C under a stream of nitrogen in a Thermovap evaporator (Ecom). The residue was dissolved in 60 μl of ethanol and 50 μl of it was injected into the liquid chromatograph. The remainder was after a suitable dilution with ethanol used for triplicate assay for total PC by its complexation with rhodamin B [15].

2.4. High-performance liquid chromatography

PCs were separated in a discontinuous descending gradient of water at a flow-rate 1.0 ml/min. The

shape of the gradient is shown in Fig. 3. Solution A contained 40 m*M* choline chloride in methanol–acetonitrile (75:25, v/v), solution B contained an addition of 3.0 ml water in 100 ml of solution A. The scintillator mixture was 3.5 ml of 3 m*M* diphenylhexatriene in tetrahydrofuran per 1000 ml water containing 0.25 ml of Brij 35 [7]. The scintillator mixture was added at 2.0 ml/min. The gradient was controlled and results were evaluated using the CSW version 1.6. (DataApex, Prague, Czech Republic). For the micropreparative purposes, individual peaks were isolated using UV absorbance detection at 205 nm, evaporated to dryness, and after solution in methanol their purity was checked (see an example in Fig. 4). The fraction were further analysed by gas chromatography–mass spectrometry (GC–MS).

2.5. Transesterification of phosphatidylcholines

Fractions separated by HPLC were evaporated to dryness, dissolved in 1 ml methanol containing 20% benzene in an ultrasonic bath and 200 μl of the sample was taken for re-esterification. The re-esterification was performed by boiling the sample with 1.5 *M* HCl in methanol for 1 h in a 25-ml conical flask equipped with a reflux condenser and a drying tube. The solution of thus formed methyl esters in methanol was transferred to a 100-ml separatory funnel, and after adding 25 ml water the methyl esters were extracted with 2×10 ml *n*-hexane. The extract was washed with distilled water and evaporated to dryness. The methylesters were dissolved in 1 ml *n*-heptane and 1 μl of the sample was injected onto the gas chromatograph.

2.6. Gas–liquid chromatography–mass spectrometry

GC–MS analyses were performed using a Varian 3400 gas chromatograph (Varian, Zug, Switzerland) equipped with an ITD 800 ion trap detector (Finnigan MAT, San Jose, CA, USA) on a 30 m×0.25 mm I.D. capillary column, DB-5 stationary phase of a film thickness 0.25 μm (J&W Scientific, Folsom, CA, USA), at a temperature programme: 80°C, 0.8 min, 50°C/min, 210°C, 30 min, 3°C/min, 280°C, in a splitless mode with helium as a carrier gas at a flow 28 ml/s (at 80°C), with injection temperature 290°C and detector temperature 220°C. MS measurements were carried out in a full-scan mode in the mass range *m/z* 50–400. Available standards (mostly Sigma and Supelco) were used for methyl ester identification.

2.7. Identification and quantitation of PC molecular species

For the identification of molecular species, total PCs were separated by HPLC into fractions corresponding to individual peaks in which fatty acids were identified by GC–MS as described above. Fractions pooled from 5–7 isolations from the same material were used. Quantitation was performed by integration of individual peak areas.

3. Results

3.1. Separation of phosphatidylcholine molecular species by HPLC

Isocratic elution of total PCs by methanol–acetonitrile (75:25) monitored by fluorometry revealed the presence of 25 significant peaks that could be evaluated by integration. The analysis was rather rapid but fractions eluted earlier exhibited poor separation which could not be substantially improved by changing the methanol–acetonitrile proportion (Fig. 1). If chromatograms A and B are compared, significant differences in responses to individual fractions are found. This is due to the fact that absorption at 205 nm is significantly dependent from the number of double bonds in a particular component. This is not so if the elution is recorded by fluorometry where the response is same for all PC species [7].

However, addition of water to the elution mixture did improve the separation significantly although this resulted in a prolonged duration of the analysis (Fig. 2). Even fractions representing apparently minor impurities present in standard compounds which could not be separated in the absence of water (or at lower concentrations of water) at all could be easily evaluated after the addition of water. Less pure standard compounds were used deliberately in these

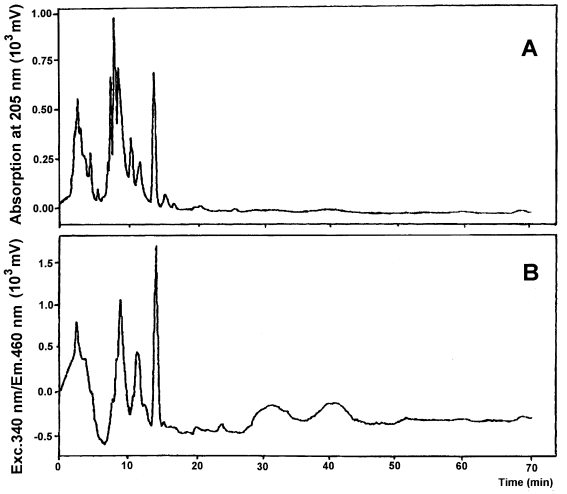

Fig. 1. Separation of total serum phosphatidylcholine by isocratic elution with 40 m*M* choline HCl in methanol–acetonitrile (55:25, v/v). The eluate was monitored at 205 nm (A) or by fluorometry (B).

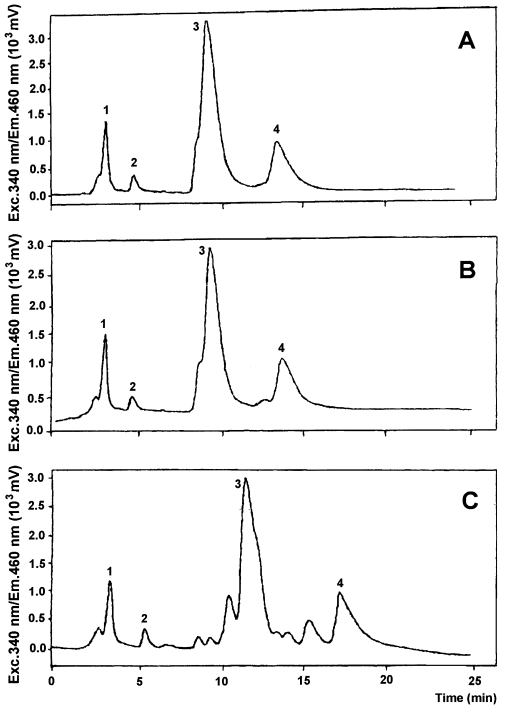

Fig. 2. Effect of water present in the elution mixture on the separation of model phosphatidylcholines [8–35 μg each of dipalmitoleoyl- (1), dilinoleoyl- (2), dipalmitoyl- (3) and dioleoyl-phosphatidylcholine (4)] by isocratic elution with 40 m*M* choline HCl in methanol–acetonitrile (75:25) containing (A) no water or (B) 0.2% or (C′) 3.0% of water. The separation was monitored by fluorometry.

experiments to demonstrate the effect of water present in the solution mixture on the separation of such minor impurities (Fig. 2).

On the basis of these results, the final system was developed using a discontinuous descending gradient of water (Fig. 3) that separated up to 48 integratable molecular species of PCs. In a general chromatogram (A and B) predominantly fractions with elution times 10–20 min are seen (which are most abundant) whereas minor components remain hidden. However, if sections of chromatogram B are enlarged, also these components become well visible. They represent well separated peaks that can be mostly easily integrated (Fig. 3C).

The reproducibility of results was tested using seven samples of the same serum. These were subjected separately to the complete procedure including extraction, liquid–liquid extraction and HPLC. Presentation of all results for each fraction would require too much space. However, the standard deviation (S.D.) values for more abundant fractions were approximately ±5% and for minor ones did not exceed ±8.5% of the mean value for individual components. Minor fractions representing less than 0.1% of total PCs were in most cases integratable but S.D. values were in most cases higher than ±9% and therefore these components were not quantified.

This method was found useful also for micropreparative isolations of PC species. Thus for the elucidation of individual molecular species of PCs, up to 5 mg of total serum PCs in 50 μl of chloroform–methanol (2:1) were injected into the chromatograph. Separation was monitored at 205 nm and fractions of column eluate corresponding to individual peaks were collected. Usually fractions from 3–5 isolations were pooled. They were subjected to HPLC and results showed a purity satisfactory for subsequent GC–MS analyses (Fig. 4). It should be emphasized that in this case methanol–acetonitrile only (without addition of water) was used for the elution to improve separation of these particular components and therefore the elution times do not

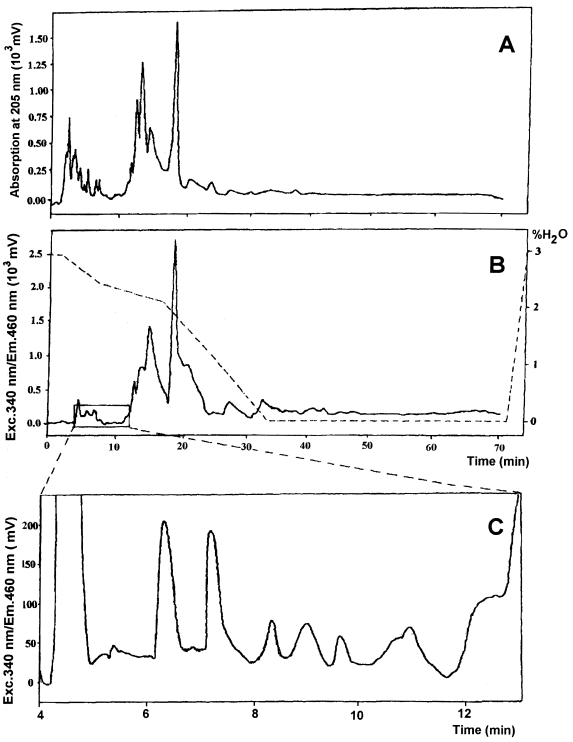

Fig. 3. Separation of total serum phosphatidylcholine in a descending gradient of water. The shape of the gradient is indicated by the dashed line and details are described in Section 2.4. The separation was monitored at 205 nm (A) and fluorometry (B). An enlarged section of chromatogram B is given as C (see Section 3.1 for details).

agree with those indicated in Table 1. Only some minor fractions showing more small peaks were not identified unequivocally (see Table 1).

3.2. Molecular species of PCs in normal human serum

Analysis of these compounds was performed in 53 serums of apparently healthy blood donors. The most abundant PC fractions contained 16:0-18:2, 16:0-18:1 and 18:0-18:2 fatty acids. Also moderate quantities of 16:0-20:4, 16:0-16:1, 18:1-18:2, 18:0-22:6 and 18:0-18:1 acids were found. Other fatty acids were present only in small or even trace concentrations. The composition of some fractions could not be identified as yet. They contain several minor fatty acids and a satisfactory resolution of individual PC molecular species could not be achieved (Table 1). Only fractions present in concentrations of more than 1.5 μg are included. The other minor (although integratable components) were omitted. As confirmed by the analysis of the individual fractions by HPLC, each of them contained at least 85% of the components indicated (Fig. 4). However, admixtures of minor species yielding no discrete peaks could not be avoided. Thus C12:1 acid accompanied peak Nos. 1 and 2 (see Table 1), C13:1 (peaks 5 and 6), 17:1 (peak 10), i-17:0 (peaks 14 and 16), ai-17:0 (peaks 15 and 16), 17:0 (peak 14), 18:3 (peak 8), 19:2

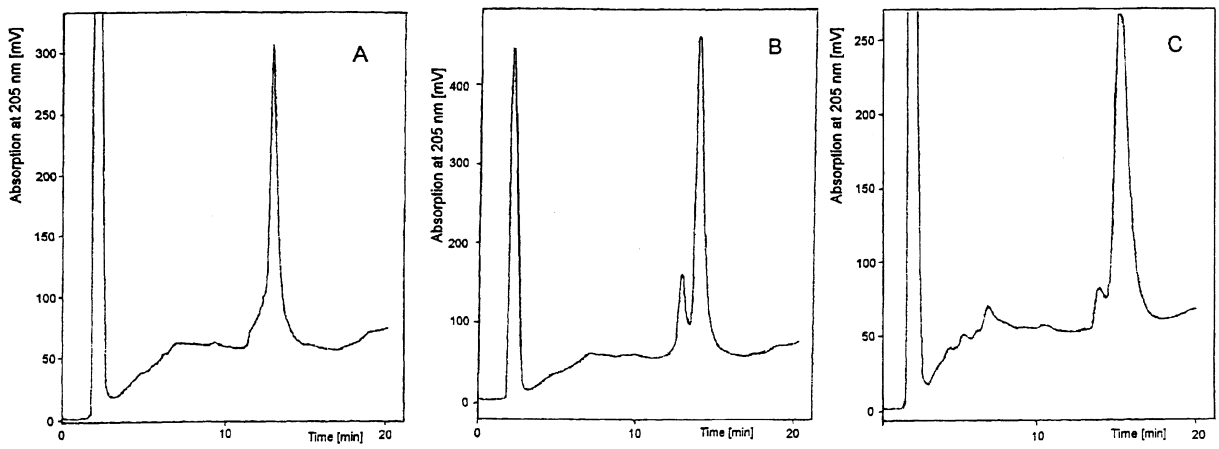

Fig. 4. Composition of fractions 10 (A), 11 (B) and 12 (C) (see Table 1) isolated from total serum phosphatidylcholines by micropreparative HPLC (see Section 2.4). The separation was performed in methanol–acetonitrile (75:25) without water. The absorbance was monitored at 205 nm. The first large peak belongs in each case to the solvent (chloroform–methanol).

Table 1
Molecular species of phosphatidylcholine in serum

No.	Retention time (min)	Fatty acids	Concentration			
			%	S.D.	μg/ml	S.D.
1	3.50	14:0-20:5	0.196	0.078	3.70	1.37
2	4.00	14.0-22.6	0.240	0.092	4.76	1.76
3	4.15	16:1-20:4	0.086	0.031	1.77	0.58
4	4.39	15:0-20:5	0.327	0.099	6.14	2.25
5	6.26	14:0-16:1	0.346	0.137	6.18	0.97
6	7.09	15:0-18:2	0.313	0.126	6.66	3.43
7	8.37	16:1-18:2	0.647	0.206	12.75	4.89
8	9.02	Unidentified	0.422	0.208	9.78	3.38
9	9.72	Unidentified	0.548	0.137	9.86	3.54
10	10.86	18:2-18:2	0.625	0.208	9.32	3.68
11	12.17	16:1-18:1	0.415	0.136	6.92	2.14
12	12.81	16:0-22:6	0.432	0.128	5.57	2.07
13	13.53	16:0-20:5	0.461	0.126	6.32	2.13
14	14.51	16:0-20:4+16:1-20:3	3.153	1.721	114.09	12.77
15	15.74	16:0-16:1	5.260	1.822	114.70	23.37
16	17.40	16:0-18:2	18.724	7.790	324.72	71.03
17	18.10	16:0-18:1+16:0-20:3	10.932	5.235	236.80	34.76
18	19.10	16:1-18:0+18:1-18:2	12.802	6.751	268.10	58.2
19	20.61	18:0-22:6	2.904	2.024	22.68	5.28
20	21.83	18:0-20:5	4.614	1.785	92.49	15.00
21	22.5	18:0-20:4	0.927	0.137	9.35	5.53
22	23.32	Unidentified	1.991	0.584	39.23	10.99
23	25.0	18:0-20:3	2.272	0.475	95.00	34.96
24	27.0	18:1-18:1	4.739	2.288	78.83	11.72
25	30.0	18:0-18:2	9.256	2.374	47.83	14.66
26	32.0	Unidentified	0.860	0.445	1.80	0.44
27	34.0	18:0-18:1	4.551	1.410	113.17	24.63
28	36.5	Unidentified	1.210	0.404	8.07	1.24
29	40.0	Unidentified	3.789	0.518	39.20	13.10
30	45.0	18:1-24:1	0.869	0.183	9.43	3.69
31	52.0	18:1-unsaturated acid>24	6.028	1.517	145.18	35.55

(peak 11), while occurrence of higher acids (>C20) was observed in fractions with retention times exceeding 35 min.

4. Discussion

The pattern of fatty acids present in serum PCs is apparently more complicated than that described in earlier papers [3,5,6]. Thus the presence of branched-chain saturated acids was not reported as yet. The presence of 14-methylhexadecanoic acid was to be expected since serum PC serves as the substrate for synthesis of cholesteryl 14-methylhexadecanoate in liver [9,12]. On the other hand, the appearance of 15-methylhexadecanoic acid is surprising since the iso-branching is believed to occur only in even-numbered but not in odd-numbered acids [16]. Furthermore, 17:1, 19:2 and saturated acids with more than 20 carbon atoms have not been described as yet to occur in biological materials.

The separation of molecular species of PCs by HPLC is rather difficult. Liquid–solid chromatography yields a good separation of individual classes of phospholipids [18,19] but it is not capable to separate individual PC species. Reversed-phase HPLC shows a good efficiency in this respect provided that the analyzed mixture contains only PCs without any admixture of other phospholipids. In particular, individual molecular species of phospha-

tidylethanolamine have very similar retention times as those of PCs [3], and would thus seriously interfere with the quantitation of PCs. In earlier papers this difficulty was overcome by a preliminary separation of PCs from all other phospholipids by solid–liquid HPLC [3]. This approach is tedious and time-consuming and quantitative recoveries can hardly be secured. Therefore, we prefer a preliminary separation of PCs by reversed-phase liquid–liquid extraction recently described [13].

Significant differences did exist in concentrations of individual molecular types of PC in the serum of 14 vertebrate species analyzed by Liu et al. [17]. Their results agree in general with those found in our experiments with the exception of PCs containing arachidonic acid that was relatively abundant in their analyses of human serum (7.27%) but found only in traces (0.37%) in our experiments. On the other hand they found only low concentrations (0.30%) of eicosapentenoic acid, which was relatively abundant (4.30%) in our samples.

The concentrations of molecular species vary in relatively broad ranges between individual persons similarly as described by Liu et al. [17]. However, some fractions (Nos. 5, 14 and 17; see Table 1) showed a rather small S.D. values. Apparently large differences when compared with normal individuals do exist in the quantitative distribution of individual species in serum of diseased persons.

Our preliminary results indicate such differences in patients with mammary carcinoma and leukemia (Hradec, unpublished data). These include also fractions not identified as yet. For this reason these molecular species are also given in Table 1.

The method described in this paper was successfully utilized for serial analyses of PC molecular species in serum of cancer patients. It was found that in patients with mammary cancer the pattern of PC molecular species is significantly different from that in healthy individuals. Similar differences were also demonstrated in patients with leukemia. Moreover, PC species in leukemic cells were entirely different from those in normal leucocytes and it was also possible to discriminate between acute and chronic forms of the disease. These changes were apparently related to the growth-rate and malignancy of these cells (Hradec, unpublished data). It seems that the method described here may be useful for monitoring the course of the disease and effects of therapy.

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