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New HPLC method for separation of blood plasma phospholipids

Zofia Suchocka [a,*], Dorota Gronostajska [a], Piotr Suchocki [b,c], Jan Pachecka [a]

[a] Department of Biochemistry and Clinical Chemistry, The Warsaw Medical University, 1 Banacha Str., 02-097 Warsaw, Poland
[b] Department of Drug Analysis, The Warsaw Medical University, 1 Banacha Str., 02-097 Warsaw, Poland
[c] Department of Pharmaceutical Chemistry, Drug Institute, 30/34 Chelmska Str., 00-725 Warsaw, Poland

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Abstract

The aim of the present work was to develop a new HPLC method for separation of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC) from small-volume samples of blood plasma. Human plasma glycerophospholipids were separated by liquid–liquid extraction method followed by solid phase extraction (SPE) on aminopropyl columns. Reversed-phase Sephasil C8 column (10 cm × 2.1 mm, I.D. 5 μm) and micropreparative chromatograph "SMART" were used for separation of PC, PE, LPC and PI from SPE phospholipids extract. Binary-step gradient of eluent A: acetonitrile–methanol (130:5, v/v) and B (0.01% trifluoroacetic acid) provided good, fast and reproducible resolution of investigated phospholipids classes in 12 min at 30 °C. Eluted phospholipids were detected at wavelengths $\lambda = 235$ and 254 nm. This method made it possible to determine quantitatively: 5 μg ml^{-1} PC, 1 μg ml^{-1} LPC, 4 μg ml^{-1} PE and 3 μg ml^{-1} PI in blood plasma samples.

Keywords: Phosphatidylcholine; Phosphatidylethanolamine; Phosphatidylinositol; Lysophosphatidylcholine; HPLC of phospholipids

1. Introduction

There is a need for a good biochemical marker indicating an increased risk of premature arteriosclerosis. To predict risk of cardiovascular diseases, various parameters (e.g. total plasma cholesterol and high-density lipoprotein (HDL) cholesterol concentrations and apolipoproteins concentrations can be used, yet there is a lack of a reliable indicator of reverse cholesterol transport efficiency. As suggested by Fournier, concentrations of individual classes of HDL phospholipids can be used for prediction of HDL capacity to accept cellular cholesterol [1] and endothelium relaxation [2]. However, the quantitative separation of individual species is a common problem concerning most analytical procedures. Several cases of successful separation of individual underivatized and derivatized phospholipids species by HPLC method were reported [3–5]. A detection system is a limiting factor in these techniques. A popular, conventional UV detection method is

* Corresponding author. Tel./fax: +48-2-2572-0735.
E-mail address: zos@farm.amwaw.edu.pl (Z. Suchocka).

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characterized by high sensitivity, but measurement of phospholipids absorption at $\lambda = 200–237$ nm is hardly possible when using commonly applied HPLC solvents that are good eluents of lipids, as chloroform or dichloromethane. They are not transparent in this region (cutoff $\lambda = 245$ and 235 nm, respectively) and deteriorate the sensitivity of assay. Because of small volume of blood plasma specimens taken from prepubertal children, the sensitivity of assay was crucial in the present work. Another reason to avoid the above-mentioned eluents was that the chromatographic micropreparative system "SMART" is not resistant to chlorinated hydrocarbons.

The aim of the present work was to develop a new HPLC method for separation of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC) from blood plasma phospholipids without the use of chlorinated hydrocarbons as eluents.

2. Materials and methods

2.1. Materials

Solid phase extraction (SPE) of total lipid mixture was performed on ChromabondNH_2 SPE columns (Macherey-Nagel, Dűren). HPLC eluents: methanol, acetonitrile (both gradient grade), dichloromethane, propanol-2, *n*-hexane and diethyl ether (HPLC grade) were obtained from J.T. Baker (Deventer, Holland), glacial acetic acid and chloroform (HPLC grade) were obtained from BDH. Ethylenediamine disodium salt dihydrate was obtained from Sigma-Aldrich Co. and trifluoroacetic acid (TFA) from Ubichem Ltd. (UK). Water was supplied by the NANOpure water purification system of Barnstead (Resistivity: 18.3 $M\Omega\ cm^{-1}$). Blood plasma from healthy premature children was used.

2.2. HPLC system

The Pharmacia-LKB "SMART" micropreparative liquid chromatography system was used for separations of phospholipids. Absorbance of phospholipids in standard mixtures and blood plasma extracts was measured at wavelengths $\lambda = 235$ and 254 nm. A mixture of PC, LPC, PE and PI was separated on a Sephasil C8 column (100 × 2.1 mm I.D., particle size 5 μm (Pharmacia-Biotech) maintained at 30 °C.

2.3. Assay conditions

The binary step gradient was used for the separation of phospholipids (Table 1). The mobile phase contains eluent A: acetonitrile–methanol (130:5, v/v) and eluent B: 0.01% trifluoroacetic acid. A volume of an injected sample was 5 μl.

2.4. Standard solutions

Soybean phospholipids mixture for HPLC containing L-α-PC (1.5 mg ml^{-1}), L-α-PE (1.2 mg ml^{-1}), L-α-PI, ammonium salt (0.9 mg ml^{-1}) and L-α-LPC (0.3 mg ml^{-1}) (from Sigma-Aldrich Co.) was used as a stock standard solution. Working solutions of phospholipids were prepared from the stock standard solution by dilution with eluent A: acetonitrile–methanol (130:5, v/v) and used within 1 day. The solutions were stored under argon at −80 °C.

2.5. Blood collection

After venipuncture blood was collected into glass test tubes containing ethylenediamine disodium salt dihydrate as anticoagulant (concentra-

Table 1
Running procedure

Time (h)	Method parameters
00:00	Flow 150 μl min^{-1}, concentration of eluent B = 0.00
02:00	Injection of sample
02:01	Autozero
03:00	Concentration of eluent B = 1%
06:00	Concentration of eluent B = 10%
06:00	Flow 250 μl min^{-1}
07:00	Concentration of eluent B = 10%
08:00	Concentration of eluent B = 0.00
08:01	Flow 350 μl min^{-1}
12:00	The end of the method

tion 1 μmol ml^{-1} of sample) and immediately centrifuged (1500 × *g*/5 min/4 °C). Plasma was filtered through a Millex-AA membrane (diameter 0.8 μm; Millipore) and then frozen in small portions at −80 °C until further processing.

2.6. Extraction of phospholipids from blood plasma

A total lipids fraction was obtained after liquid–liquid extraction of 50 μl specimen of blood plasma with chloroform–methanol mixture (2:1, v/v), according to the method of Folch [6]. Blood plasma was shaken with 2 ml of an organic phase: chloroform–methanol (2:1, v/v) for 20 min at room temperature. Then, 0.9% sodium chloride solution was added there to, the extract was shaken for 30 s and immediately centrifuged (2000 × *g*/5 min/4 °C). The lower layer of the lipid extract was separated and dried under N_2. The dried extract was then dissolved in 500 μl of chloroform and fractionated by SPE according to the Kalużny method [7] with a use of aminopropyl-bounded silica SPE columns. ChromabondNH_2 columns (500 mg) were placed in the BAKERBOUND spe System and washed twice under vacuum (~ 10 kPa) with 2 ml portions of *n*-hexane. Then, a collection rack with receiving tubes was placed in the BAKERBOUND system. The vacuum was released immediately after the second hexane wash to avoid drying the columns completely. Lipid mixtures in 500 μl of chloroform were applied to the column under vacuum and the chloroform was pulled through it. This resulted in leaving the entire lipid mixture on the column. Then, the column was eluted with 4 ml of chloroform–propanol (2:1, v/v) to elute neutral lipids, and then, eluted with 4 ml of 2% acetic acid in diethyl ether to elute fatty acids. New collecting tubes were placed in the rack and then, the column was eluted with 4 ml of methanol. The last eluat containing phospholipids was dried under nitrogen. The phospholipids fraction after SPE was stored under N_2 in a tapered glass vial with a teflon cap (Supelco) in the dark at −80 °C in methylene chloride–methanol mixture (1:1, v/v).

2.7. Preparation of a sample

Prior to analysis, a sample stored in methylene chloride–methanol mixture (1:1, v/v) was dried under nitrogen, dissolved in 100 μl of methylene chloride and diluted with 2.4 ml of eluent A.

2.8. Calibration curves

Blank plasma samples were spiked with 12 various working solutions to obtain appropriate final concentrations (5–300 μg ml^{-1} for PC, 4–240 μg ml^{-1} for PE, 3–180 μg ml^{-1} for PI and 1–60 μg ml^{-1} for LPC). Then, liquid–liquid extraction and solid-phase extraction were used to prepare samples containing blood plasma. Calibration curves were obtained by plotting the peak heights versus the nominal concentrations. The relevant equations were fitted by linear regression (the least-squares method).

2.9. Validation of the method

The precision and accuracy of the method were evaluated by repetitive analysis of extracts from blood plasma samples spiked at the lowest (LOQ), medium and the highest concentration levels of calibration samples, respectively. Intra-day precision and accuracy data were obtained by the analysis of these samples (n = 6) in 1 day by the same operator. Inter-day precision and accuracy data were obtained by assaying these three concentration levels on different days by two operators (n = 8). The recoveries were measured by comparing the response of extracted blood plasma samples spiked before extraction with the response of extracted blank samples spiked just before injection. Phospholipids were quantified by comparison of peak area and height with those of standards.

3. Results and discussion

3.1. Extraction of phospholipids

Phospholipids, neutral lipids and sterols are components of blood plasma lipoproteins. Nature

of their interactions with proteins is hydrophobic and of van der Waals type. To isolate them from the plasma we used chloroform–methanol system (2:1, v/v) as suggested by Folch [6]. The endogenous water in the plasma constituted the third component of the system. After shaking the extract followed by equilibrating it with a saline solution (its volume being one fifth of the extract volume), the lower layer was composed of the chloroform–methanol system and a small volume of water in proportions, which provided satisfactory partitioning of lipids into the organic phase. The addition of sodium chloride minimized a loss of lipids during extraction [8] and permitted to extract gangliosides and a more polar prostaglandin into the upper phase. The lower phase contained virtually all of the lipids, while the upper phase contained much of the non-lipid contaminants. Preliminary extraction of total lipids fraction according to Folch reduced non-lipid contamination, such as amino acids, and made it possible to concentrate blood plasma lipids fast. The resulting extract can be evaporated easily. A dry extract after liquid–liquid extraction was prepared by subsequent SPE on aminopropyl columns in order to separate lipid species according to their polarity. Due to complexity of lipid extracts from natural sources, it is rarely possible to separate them into classes with a single chromatographic procedure. Fractionation of total lipids fraction using SPE enabled us to obtain three fractions containing seven different lipid classes with recovery 70–95% [7]. Although similar in chemical nature, lipids show subtle and exploitable differences. Thus, varying the solvent environment (pH, polarity, etc.) in eluents for SPE, compounds can be selectively isolated with a high degree of purity and recovery. Even a minor variation (1–2%) of solution composition can drastically change the character of a column and the resulting separation [7]. In our patients plasma samples the recoveries of investigated phospholipids classes were good (i.e. 92–96% for PC and LPC, 92–97% for PE and 93–96% for PI (Table 4). The phospholipids fraction produced by this method included combined phospholipids (PC, PE, phosphatidylserine, PI, lysophospholipids sphingomyelin) and neutral glycolipids [9].

3.2. Chromatography

Due to lipophility of the phospholipids, we attempted to separate them on a reversed-phase column (Sephasil C8, 5 μm). The TFA gradient was utilized because of varying chemical characteristics of the phospholipids investigated as well as to ensure the elution of all the four analytes. Secondary interactions between basic and acidic compounds with acidic silanols and the stationary phase surface may result in a strong analyte retention and peak tailing in HPLC. Therefore, we added TFA to neutralize the charged phosphate group in phospholipids as well as to reduce secondary interactions between the polar functionality of the phospholipids and any acidic silanols on the stationary phase. Under the above-described conditions, the complete separation of the four investigated glycerophospholipids was achieved in 12 min. Fig. 1 shows a typical chromatogram obtained for a standard mixture of PC, PE, LPC and PI at two different wavelength ($\lambda = 235$ and 254 nm). Peak shapes were improved by both the addition of 0.01% TFA and increasing the temperature. When the temperature of assay was stable (30 °C), the peak appearance and retention times were highly reproducible.

3.3. Linearity

As a criterion of the linearity of the method a correlation coefficient of the corresponding curve determined by the least squares analysis was used (Table 2). At $\lambda = 235$ nm, the mean correlation coefficient was 0.9965 for PC, 0.9941 for PE, 0.9978 for LPC and 0.9938 for PI, respectively. For PC the method was found to be linear at the concentrations from 5 to 300 μg ml^{-1}, for PE from 5 to 250 μg ml^{-1}, for PI from 2 to 180 μg ml^{-1} and for LPC from 1 to 60 μg ml^{-1}. The assay exhibits the necessary sensitivity and linearity to cover the physiological concentrations of phospholipids in blood plasma.

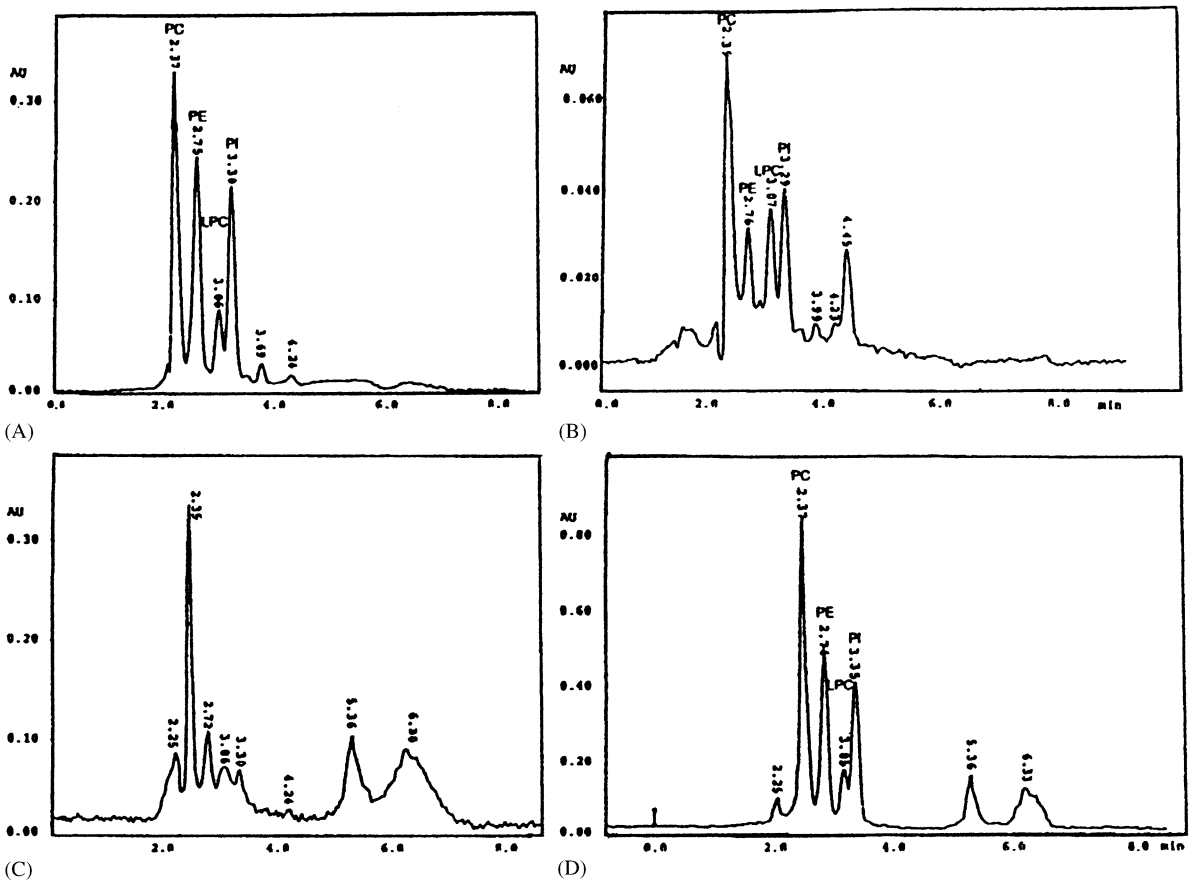

Fig. 1. HPLC separation of glycerophospholipids using Sephasil C8 column Chromatogram of a standard mixture of phospholipids: 150 μg ml^{-1} PC, 120 μg ml^{-1} PE, 30 μg ml^{-1} LPC and 90 μg ml^{-1} PI. (A) at λ = 235 nm, (B) at λ = 254 nm. (C) Chromatogram of a phospholipids extract from 50 μl of blood plasma, dissolved in a solvent (volume of the solvent being 50-fold the blood plasma sample volume). (D) Chromatogram of a phospholipids extract obtained from 50 μl of blood plasma spiked with phospholipid standards (50 μg ml^{-1} PC, 40 μg ml^{-1} PE, 30 μg ml^{-1} PI and 50 μg ml^{-1} LPC, respectively). Peaks refer to the following components: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophospatidylcholine; PI, phosphatidylinositol.

Table 2
Linearity parameters of calibration curve $y = (a \pm S_a) \cdot x + (b \pm S_b)$ at λ = 235 and 254 nm

Parameter	PC λ = 235 nm	PC λ = 254 nm	PE λ = 235 nm	PE λ = 254 nm	PI λ = 235 nm	PI λ = 254 nm	LPC λ = 235 nm	LPC λ = 254 nm
Slope (a)	2.1466	0.3336	1.7995	0.1920	2.0941	0.3836	2.9937	1.0217
Intercept (b)	25.4290	16.8618	46.2282	9.4451	43.3357	9.8952	5.9351	7.8856
S_a	0.0566	0.0112	0.0623	0.0078	0.0743	0.0129	0.0623	0.0331
S_b	8.0706	1.6040	7.1072	0.8902	6.3471	1.0715	1.7909	0.9531
$S_{(x,y)}$	65.1344	2.5730	50.5121	0.7925	40.2860	1.1480	3.2072	0.9084
R	0.9965	0.9944	0.9941	0.9919	0.9938	0.9947	0.9978	0.9948

S_a, mean square error of slope; S_b, mean square error of intercept; $S_{(x,y)}$, the root-mean square error; r, linear correlation coefficient. Values were obtained from 12 data points.

3.4. Limit of quantification

The limits of quantification for PC, PE, LPC and PI were found to be 5, 4, 1 and 3 μg ml^{-1}, respectively.

3.5. Precision and accuracy

The precision and accuracy data are shown in Table 3. The precision of the method was measured at wavelength $\lambda = 235$ nm. The intra-day coefficient of variation (R.S.D.) were found to be within 8.88 for PC, 7.11 for PE, 6.11 for LPC and 5.66 for PI at the lowest level, respectively. The inter-day coefficients of variation determined by repeating analysis on 4 separate days, were found to be within 17.69 for PC, 9.59 for PE, 8.59 for LPC and 5.31 for PI at the lowest level, respectively. Data presented in Table 3 show that the precision of the method is good at medium and higher concentrations, which covered concentrations found in blood plasma samples, and accep-

Table 4
Recovery

Compound	Nominal concentration (μg ml^{-1})	Recovery (mean ± S.D.) (%)
PC	5.00	95.53 ± 4.44
	100.00	94.64 ± 3.23
	300.00	92.41 ± 1.08
PE	4.00	96.74 ± 4.46
	100.00	93.04 ± 5.80
	240.00	92.50 ± 3.03
LPC	1.00	93.39 ± 2.74
	22.00	95.01 ± 4.13
	60.00	96.06 ± 4.20
PI	3.00	93.14 ± 4.69
	90.00	93.75 ± 3.75
	180.00	95.82 ± 3.13

Values were obtained from six measurements.

Table 3
Precision and accuracy of phospholipids assays

Compound	Nominal concentration (μg ml^{-1})	Concentration found (μg ml^{-1})	Precision R.S.D. (%)	Accuracy (%)
Intra-day variability ($n = 6$)				
PC	5.00	5.47	8.88	9.48
	100.00	93.98	8.87	3.98
	300.00	307.62	1.30	2.54
PE	4.00	3.61	7.11	9.75
	100.00	105.93	1.98	5.93
	240.00	233.39	3.45	2.75
LPC	1.00	1.09	6.11	19.20
	22.00	21.12	4.99	4.00
	60.00	60.49	1.55	0.82
PI	3.00	2.58	5.66	13.99
	90.00	96.39	1.75	5.75
	180.00	194.60	3.11	8.11
Inter-day variability ($n = 8$)				
PC	5.00	5.62	17.69	12.40
	100.00	95.25	10.12	4.75
	300.00	303.72	1.04.	8.88
PE	4.00	3.55	9.59	11.25
	100.00	105.96	2.22	5.96
	240.00	224.00	2.09	8.89
LPC	1.00	1.10	8.59	10.00
	22.00	22.11	6.96	4.05
	60.00	62.17	3.30	3.62
PI	3.00	2.59	5.31	13.67
	90.00	92.25	1.31	2.50
	180.00	166.76	3.12	7.36

table in the lowest concentrations of investigated phospholipids.

The accuracy was expressed as the percentage mean deviation between the mean concentration found and the theoretical concentration. The intra-day accuracy for PC ranged from 2.54 to 9.48 for PC, from 2.75 to 9.75 for PE, from 0.82 to 19.2 for LPC and from 8.11 to 13.99 for PI, respectively. The inter-day accuracy for PC ranged from 4.75 to 12.40 for PC, from 5.96 to 11.25 for PE, from 3.62 to 10.00 for LPC and from 2.5 to 13.67 for PI, respectively. The least accuracy was found in case of PI, however, all the values are acceptable for bioanalytical methods [10].

4. Conclusions

The method developed by the present authors is well suited for assays of PC, PE, LPC and PI in small volumes of blood plasma. The good sensitivity found in case of determination of these phospholipids in our samples, high precision and accuracy as well as short analysis time suggest that the method can be successfully applied for fast evaluation of pro- or antiatherogenic properties of blood plasma lipoproteins.

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