METHODS

Evaluation of Phospholipid Composition of Erythrocytes from Healthy Volunteers by the Method of High-Performance Liquid Chromatography with a Light Scattering Detector

D. A. Korzhenevskiy and A. A. Selischeva

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 147, No. 4, pp. 473-476, April, 2009
Original article submitted September 24, 2008

Phospholipid composition of lipid extract from erythrocytes of healthy volunteers was evaluated by means of high-performance liquid chromatography with light scattering detection. The lower and upper limits of the major (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin) and minor phospholipids (phosphatidylserine) were estimated in 61 extracts. No significant differences were found between men and women.

Key Words: *phospholipids; high-performance liquid chromatography; light scattering detector; erythrocytes*

Phospholipid composition of cell membranes in organs and tissues can significantly vary under pathological conditions. These variations were revealed in the composition of high-density lipoproteins (Niemann—Pick disease [9] and hypertension [1]), blood plasma (type 2 diabetes mellitus [14]), and surfactant (inflammation of the upper respiratory tract [2]).

These changes concern not only the content of individual phospholipids (PL), but also their fatty acid composition. The development of various diseases is accompanied by oxidative stress, which results in accumulation of lipid peroxidation products [10, 15]. Apart from studies of the lipid composition of plasma lipoproteins, a perspective trend is evaluation of the effect of diabetes mellitus on the PL composition and rheological properties of blood cells [8]. However, the results were difficult to interpret due to weaknesses of experimental methods.

Institute of Biomedical Problems, Moscow, Russia. ***Address for correspondence:*** babischew@rambler.ru. D. A. Korzhenevskiy

Studying of the PL composition of blood components is an urgent problem of medical biochemistry. The content of various classes of PL in human blood should be precisely evaluated under normal conditions to use this parameter as an additional information about the development of that disease.

The composition and content of PL are estimated by various methods. Previous studies explored thin-layer chromatography followed by colorimetric analysis of PL in individual spots on plates or high-performance liquid chromatography (HPLC) with UV detection [3,12], which does not allow us to study PL with saturated fatty acid chains. This disadvantage can be avoided by using light scattering method [7,11]. However, this approach requires the development of a specific methodology. The signal/noise ratio varies and depends on technical parameters of the detector. Moreover, the peak area for any compound depends nonlinearly on its concentration [4,5].

Assessment of various classes of PL by means of HPLC with light scatter detection requires the use of

0007-4888/09/1474-0489

calibration curves for each class of PL in concentration range typical of a specific object of study.

The upper and lower limits of various classes of PL in erythrocytes from healthy volunteers were evaluated by means of HPLC with light scatter detection.

MATERIALS AND METHODS

The following synthetic compounds (Avanti Polar Lipids, 99% purity) were used as the standards for PL identification: phosphatidylcholines (PC; 16:0/16:0, 18:0/18:1, 18:0/18:2), phosphatidylethanolamines (PE; 16:0/16:0, 16:0/18:2), and phosphatidylserine (PS; 16:0/16:0). The study was performed with natural PL, which had a specific content of fatty acid residues (sphingomyelin (SM) from porcine brain; and phosphatidylinositols (PI) from porcine liver). The following organic solvents were used: hexane, isopropanol, methanol, chloroform (Merck KGaA), acetic acid, and aqueous ammonia (Khimmed). Deionized water (resistance >18 MΩ) was obtained on an UVOI-MF-7 device (Mediana-Fil'tr).

The venous blood was sampled from healthy volunteers (men and women, 22-65 years). They were selected by the results of biochemical blood tests. The concentrations of glucose, triacylglycerides, cholesterol, calcium ions, and glycosylated hemoglobin did not differ from normal. The blood (1 ml) was collected in tubes with EDTA (1.6 mg/ml) and immediately centrifuged at 300*g* and 4°C for 10 min.

To obtain crude lipid extract, 3 ml methanol and 0.2 ml deionized water were added to 0.5 ml erythrocyte mass. Extraction was performed under thorough agitation for 15 min. Chloroform (3 ml) was added. Agitation was performed for another 15 min. The mixture was centrifuged at 500*g* and room temperature for 15 min. The supernatant was collected. A chloroform—methanol mixture (2:1; 3 ml) was added to the pellet. Agitation was performed for 20 min. The mixture was centrifuged under similar conditions. The second supernatant was collected and mixed with the first supernatant. NaCl (1%, 0.5 volume) was added to the combined extract. The mixture was agitated for 10 min and centrifuged. The lower layer of chloroform was taken and evaporated to dryness on a rotor evaporator. A dry lipid film was dissolved in 0.6 ml chloroform-methanol 3:1 mixture. PL content in the sample was calculated relative to inorganic phosphate concentration [13].

PL were classified by HPLC on a ProStar liquid chromatograph (Varian) equipped with a Mono-Chrom DIOL 3 column (100×2 mm) at 35°C. The gradient of solvents A and B served as the eluate. Solvent A contained 49.8% hexane, 49.7% isopropanol, 0.3% CH_3COOH, and 0.2% aqueous ammonia (v/v). Solvent B contained 9.5% water, 45% hexane, 45% isopropanol, 0.3% CH_3COOH, and 0.2% aqueous ammonia (v/v). The gradient appeared as follows: 0-4 min, 85% A; 6 min, 65% A; 16-24 min, 45% A; and 26 min, 85% A; 0.2 ml/min elution rate; 10 μl sample volume was.

The substances were detected on a PL-ELS 2100 light scattering detector (Varian) under the following conditions: nebulizer temperature, 40°C; drying gas (nitrogen) temperature, 80°C; and gas flow rate, 1.2 liter/min.

Weighted samples of the standards were dissolved in a chloroform—methanol 3:1 mixture to construct the calibration curves. The concentration varied from 0.06 to 1 mg/ml. Standard solutions were stored at -12°C for not more than 1 month. Similarly to the lipid extract, they were subjected to a chromatographic analysis (individually or in the mixture). The detector response was recorded at 4 concentrations of the standard. These concentrations were measured at least 3 times. The response was approximated with a second-order calibration curve as follows:

$$S=a+b_1n+b_2n^2,$$

where S is the chromatographic peak area and n is the concentration of the standard.

Various classes of erythrocyte PL were studied quantitatively by the external standard method with calibration curves. The results were analyzed by means of Statistica 6.0 software.

RESULTS

Various classes of PL in erythrocytes from healthy volunteers were analyzed quantitatively with calibration curves. The curves were constructed for each standard. Figure 1 shows the results for 4 classes of PL. The detector response was nonlinear for all PL. Our findings are consistent with published data [4,5].

The total PL content in the lipid extract of erythrocytes from 1 ml blood was estimated from the amount of inorganic phosphorus (1-4 mmol/liter) [13].

Various classes of PL were determined by means of HPLC with light scattering detection. Figure 2 illustrates the chromatogram of erythrocytes from healthy volunteers. The order of elution (estimated by standards, Fig. 3) appeared as follows: PE, PS, PC, and SM (double peak). Some samples were characterized by a small-area peak of lysophospha-

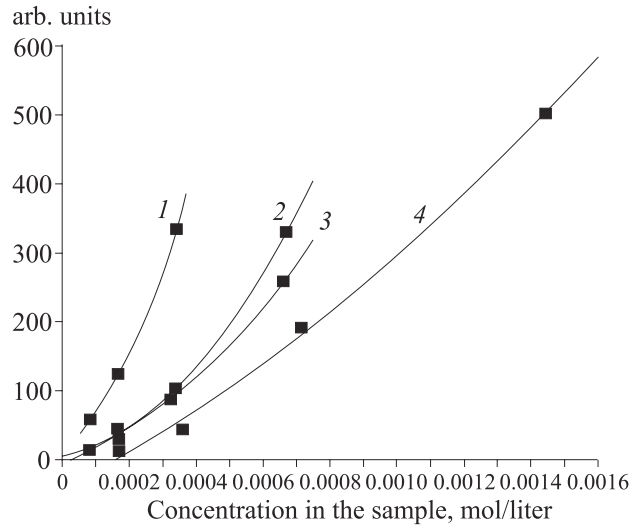

Fig. 1. Dependence of chromatographic peak areas for various classes of PL on the concentration in test sample. PC (*1*), SM (*2*), PS (*3*), and PE (*4*).

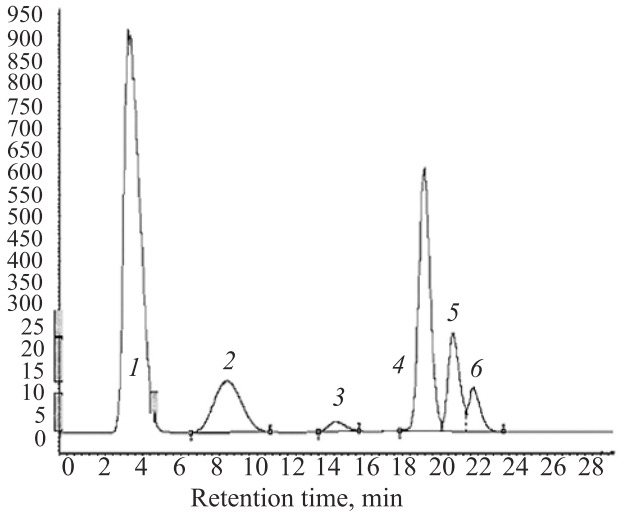

Fig. 2. Chromatogram of the lipid extract from human erythrocytes. Neutral lipids, cholesterol, and cholesterol esters (*1*); PE (*2*); PS (*3*); PC (*4*); and SM (*5* and *6*).

tidylcholines. This peak was not taken into account in further analysis.

Studying the lipid extracts of erythrocytes from healthy volunteers allowed us to evaluate the range of concentrations of PL classes. Tables 1 and 2 show the results of descriptive statistics. The phospholipid composition of erythrocytes did not differ in men and women. No differences were found in the PE/PS ratio. It should be emphasized that this ratio is extensively used to evaluate changes in PL composition [8].

The major classes include PC, PE, and SM. PS constitutes the minor class (Tables 1 and 2). These data are qualitatively consistent with the results of previous studies by thin-layer chromatography or HPLC with light scattering detection [6,8]. However, some differences were found in the content of individual PL (*e.g.*, PS). This discrepancy probably results from the fact that previous calculations were made only for the peak area [6]. Differences in the response to various classes of PL were not taken into account.

Our results show the limits of various classes of PL in erythrocytes from healthy volunteers. These data should be taken into account in the study of

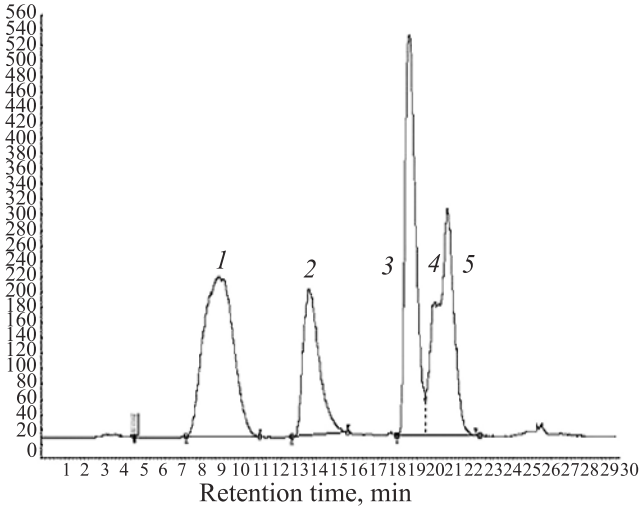

Fig. 3. Chromatogram of the mixture of standards. PE (*1*), PS (*2*), PC (*3*), and SM (*4* and *5*).

TABLE 1. Descriptive Statistics for the Distribution of Major Classes of PL in Erythrocytes from Healthy Women (*n*=35)

PL	M (SD)	CI (95%)	Me (q25; q75)	CV, %
PE, %	30.95 (2.54)	30.08; 31.82	30.99 (29.35; 32.16)	8.19
PS, %	9.10 (3.06)	8.05; 10.15	8.98 (6.78; 10.77)	33.61
PC, %	26.25 (3.49)	25.06; 27.45	25.33 (23.20; 28.90)	13.28
SM, %	33.70 (2.51)	32.84; 34.56	33.15 (31.91; 35.77)	7.45
PE/PS, arb. units	3.90 (1.79)	3.29; 4.52	3.40 (2.82; 4.67)	45.78

Note. Here and in Table 2: *n*, number of measurements with each sample; M, mean; SD, standard deviation; CI (95%), confidence interval; Me, median, q25, first quartile; q75, third quartile; CV, coefficient of variation.

TABLE 2. Descriptive Statistics for the Distribution of Main Classes of PL in Erythrocytes from Healthy Men (n=24)

PL	M (SD)	CI (95%)	Me (q25; q75)	CV, %
PE, %	31.95 (3.75)	30.37; 33.53	31.81 (30.15; 33.37)	11.73
PS, %	9.45 (3.67)	7.90; 11.00	8.61 (7.47; 10.88)	38.81
PC, %	26.03 (2.63)	24.92; 27.14	26.02 (24.15; 28.23)	10.09
SM, %	32.57 (2.64)	31.45; 33.69	32.14 (30.42; 34.62)	8.11
PE/PS, arb. units	3.97 (1.92)	3.16; 4.78	3.71 (2.86; 4.51)	48.33

lipid metabolic disturbances in the human organism.

REFERENCES

1. I. N. Ozerova, N. V. Perova, N. V. Shchel'tsyna, and M. N. Mamedov, *Byull. Eksp. Biol. Med*., **143**, No. 3, 289-292 (2007).
2. B. Barroso and R. Bischoff, *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **814**, No. 1, 21-28 (2005).
3. W. Bernhard, M. Linck, H. Creutzburg, *et al*., *Anal. Biochem*., **220**, No. 1, 172-180 (1994).
4. J. F. Brouwers, B. M. Gadella, L. M. van Golde, and A. G. Tielens, *J. Lipid Res.*, **39**, No. 2, 344-353 (1998).
5. A. M. Descalzo, E. M. Insani, and N. A. Pensel, *Lipids*, **38**, No. 9, 999-1003 (2003).
6. S. Mawatari, Y. Okuma, and T. Fujino, *Anal. Biochem*., **370**, No. 1, 54-59 (2007).
7. R. A. Moreau, *Lipids*, **41**, No. 7, 727-731 (2006).
8. S. Labrouche, G. Freyburger, H. Gin, *et al*., *Metabolism*, **45**, No. 1, 57-71 (1996).
9. C. Y. Lee, A. Lesimple, A. Larsen, *et al*., *J. Lipid. Res*., **46**, No. 6, 1213-1228 (2005).
10. T. S. Nagashima, S. Oikawa, Y. Hirayama, *et al*., *Diabetes Res. Clin. Pract*., **56**, No. 1, 19-25 (2002).
11. T. Seppanen-Laakso, I. Laakso, H. Vanhanen, *et al*., *J. Chromatogr. B. Biomed. Sci. Appl*., **754**, No. 2, 437-445 (2001).
12. Z. Suchocka, D. Gronostajska, P. Suchocki, and J. Pachecka, *J. Pharm. Biomed. Anal.*, **32**, Nos. 4-5, 859-865 (2003).
13. V. E. Vaskovsky and V. I. Svetashev, *J. Chromatogr*., **65**, No. 2, 451-453 (1972).
14. C. Wang, H. Kong, Y. Guan, *et al*., *Anal. Chem*., **77**, No. 13, 4108-4116 (2005).
15. J. L. Witztum and D. Steinberg, *J. Clin. Invest*., **88**, No. 6, 1785-1792 (1991).