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Rapid isocratic method for the separation and quantification of major phospholipid classes by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method for the quantification of major phospholipid classes is described. The separation was performed on Ultrasphere SI silica gel columns with a mobile phase of acetonitrile–methanol–85% phosphoric acid (100:10:1.8, v/v) using isocratic elution and UV detection at 203 nm. Complete separation of phosphatidylserine, phosphatidylethanolamine, plasmalogen, phosphatidylcholine and sphingomyelin was achieved within 8 min. The plasmalogen was resolved from phosphatidylethanolamine in hydrochloric acid-derivatized samples, or without derivatization using a mobile phase composition of 100:40:0.4. The phospholipids were quantified by peak-area integration by means of the calibration. The detection limit is 5 ng. Human erythrocyte ghost membranes, lymphocytes and thrombocytes were analysed for these phospholipids. This method is suitable for routine clinical studies of membrane disorders in health, toxicity and disease, as well as in research.

INTRODUCTION

In recent years, high-performance liquid chromatography (HPLC) has become a very useful tool for the measurement of phospholipids in health and disease studies as well as for research purposes. The HPLC detection of phospholipids is accomplished commonly by UV, refractive index or flame ionization detection. The last two detection methods lack sensitivity. UV detection has greater sensitivity, but absorption by phospholipids in the range 200–210 nm makes it difficult to use common chromatographic solvents, which are not transparent in this region. The choice of solvent in HPLC–UV studies has thus been confined to the two systems acetonitrile–methanol–water [1] and *n*-hexane–2-propanol–water [2]. These mobile phases have since been modified to improve the resolution of

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phospholipids by changing the proportions of the components, the pH, or both.

Because the resolution is improved by the acidic or basic environment of the solvent mixture used in silica gel TLC, Chen and Kou [3] added phosphoric acid to acetonitrile–methanol solvent system of their isocratic HPLC procedure. They were able to demonstrate efficient separation of major phospholipid classes on a silica gel-based column within 30 min. Kaduce *et al.* [4] reported the separation of phospholipids by isocratic HPLC in *ca.* 50 min. Owing to poor sensitivity these methods failed to resolve sphingomyelin in smaller sample sizes. Other available gradient elution methods are complex, laborious and time-consuming [5–7].

This paper describes a simple, rapid and sensitive isocratic method for a complete separation and quantification of major phospholipid classes, namely phosphatidylcholine, phosphatidylethanolamine (consisting of both phosphatidylethanolamine and plasmalogen), phosphatidylserine and sphingomyelin from the extracts of human erythrocyte ghost membranes, lymphocytes and thrombocytes.

EXPERIMENTAL

Chemicals

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) from bovine sources (Sigma, Munich, Germany) were used as phospholipid standards. All chemicals and reagents were of HPLC or highly purified grade.

Preparation of human erythrocyte ghost membranes and lipid extraction

Human blood was obtained from healthy donors. Heparin was used as an anticoagulant. Whole blood was centrifuged (2300 *g* for 10 min) in a swinging bucket rotor. Red blood cells were washed three times in five volumes of phosphate-buffered saline (150 m*M* NaCl, 50 m*M* sodium phosphate, pH 8.0) and centrifuged (2300 *g* for 10 min). The packed cells (1 ml) were hemolysed in forty volumes of 5 m*M* sodium phosphate (pH 8.0). The ghost membranes were obtained after three to four identical washes (at 22 000 *g* for 10 min) [8]. All procedures were performed at 0–4°C.

Phospholipids were extracted from erythrocyte ghost membranes by mechanical stirring in seven volumes of methanol–chloroform (2:1, v/v) for 45 min, followed by centrifugation (2000 *g* for 10 min). The supernatant was transferred to another tube. The pellet was mechanically stirred in ten volumes of chloroform–methanol–water (2:1:0.8, v/v) and centrifuged at 2000 *g* for 10 min. The supernatant was aspirated to the tube containing the first aliquot. After the addition of 2.5 ml each of chloroform and water, the tube was shaken and centrifuged at 2000 *g* for 10 min. The chloroform phase was aspirated to a sample tube and dried under a stream of nitrogen gas to prevent the oxidation of phospholipids. The residue was redissolved in a small volume of chloroform–methanol (2:1, v/v).

Phospholipid analysis by HPLC

A Waters (Milford, MA, U.S.A.) HPLC system, consisting of a Model 510 solvent-delivery system, a Model WISP 710B autosampler, a system interface module, and a Lamb-Max LC Model 481 spectrophotometer, was used. A Model 350 control station professional with Model LA50 printer plotter from Digital Equipment (Marlboro, MA, U.S.A.) was used for integration, calibration and report information, as well as to provide optimum control and data. The separation was achieved on a stainless-steel silica-based Beckman Ultrasphere SI 250 mm × 4.6 mm I.D. analytical column [with 5-μm spherical particles (80 Å pore)]. The mobile phase was acetonitrile–methanol–85% phosphoric acid (100:10:1.8, v/v or 100:40:0.4, v/v for method II), degassed in an ultrasonic bath. It was delivered to the column at a flow-rate of 1.5 ml/min and a pressure of *ca.* 75 bar at room temperature (25°C). The detector wavelength was set at 203 nm.

When standards or the aliquots of extracts in chloroform–methanol (2:1, v/v) were directly injected into the HPLC equipment, a tailed solvent front (SF) peak was produced that interfered with later-eluting peaks of PS and PE. This interaction was avoided by injecting the sample in *n*-hexane–2-propanol (3:1, v/v) to achieve a sharp SF peak and a zero baseline projection of the phospholipid peaks. For the separation of PL from PE, the sample tube containing the dried extract was kept inverted at the mouth of fuming hydrochloric acid bottle for 10 min. The sample was dissolved in a small volume of benzene and dried under nitrogen gas. Finally, the sample was redissolved in *n*-hexane–2-propanol (3:1, v/v) before injection. An alternative method II was used for the separation of PC, PE, PL, PS and SM without derivatization of sample.

RESULTS

The injection of the commercial phospholipids or aliquots of the original extract in chloroform–methanol produced a tailing SF peak, which interfered with the baseline processing of the later-eluting peaks of PS and PE (Fig. 1a). A sharp SF peak as well as complete distinct base-line resolution of all the phospholipids was achieved by injecting the sample in *n*-hexane–2-propanol (3:1, v/v) (Fig. 1b). The separation of the PL, containing vinyl ether bonds, is possible from diacyl-alkylacyl-phospholipids following the cleavage of 1-alk-1-enyl 2-acyl-phosphoglycerides by exposure to HCl fumes [9]. Thus, complete separation of PL from PE was achieved after a brief derivatization of samples with HCl (Fig. 2a and b), without affecting the resolution times or the analyses of the phospholipids (Fig. 2c). The optimum resolution of the major phospholipid classes was achieved within 8 min in a single run. Retention times for PS, PE, PL, PC and SM were 3.11, 3.85, 4.87, 5.90 and 7.62 min, respectively (Fig. 2c).

A successful resolution of PS, PE, PL, PC and SM was also achieved in *ca.* 25 min without derivatization of the samples with the alternative method II, using a mobile phase acetonitrile-methanol–85% phosphoric acid (100:40:0.4, v/v). As

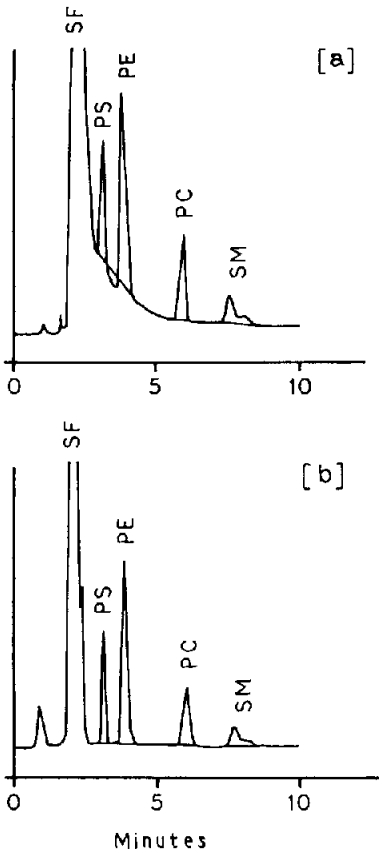

Fig. 1. Chromatograms illustrating the separation of commercial phospholipids (not to scale). (a) Injection of sample in 10 μl of chloroform–methanol (2:1) produced a tailing SF peak on which PS and PE were eluted subsequently; (b) optimum baseline separation of the phospholipids was achieved by redissolving the dried sample in 10 μl of *n*-hexane–2-propanol (3:1).

shown in Fig. 3, the phospholipid concentrations and the peak-area values exhibited a linear relationship in the range 0.01–1 μg per 10 μl (also tested up to 20 μg per 10 μl). The peak-area values of different phospholipids are distributed in the following manner: PE > PS > PC > SM. The calibration graph for PL was not prepared, but the percentage of PL in PE was determined from the equation:

$$\% \text{ PL} = [\,(\text{PL})_{\text{pac}} / \textstyle\sum (\text{PE} + \text{PL})_{\text{pac}}\,] \times 100$$

where pac is the peak-area count. Similarly, the percentage of PE in PE consisting PL was evaluated.

A 10-μl extract of the erythrocyte ghost membranes. corresponding to 30–40

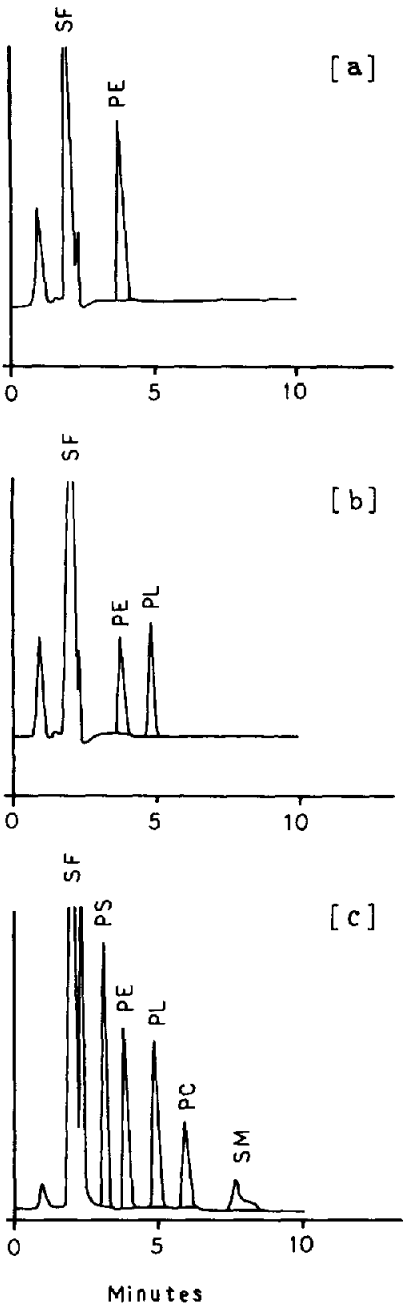

Fig. 2. Chromatograms showing the separation of plasmalogen (PL) from phosphatidylethanolamine (PE). (a) Peak of commercial PE (50 ng per 10 μl); (b) PL separated from PE after derivatization of PE (50 ng) with HCl as described in Experimental; (c) elution patterns of the phospholipids after HCl derivatization of erythrocyte ghost membrane extract (10 μl). The retention times of other phospholipids were not affected by derivatization of the phospholipids or by elution of PL.

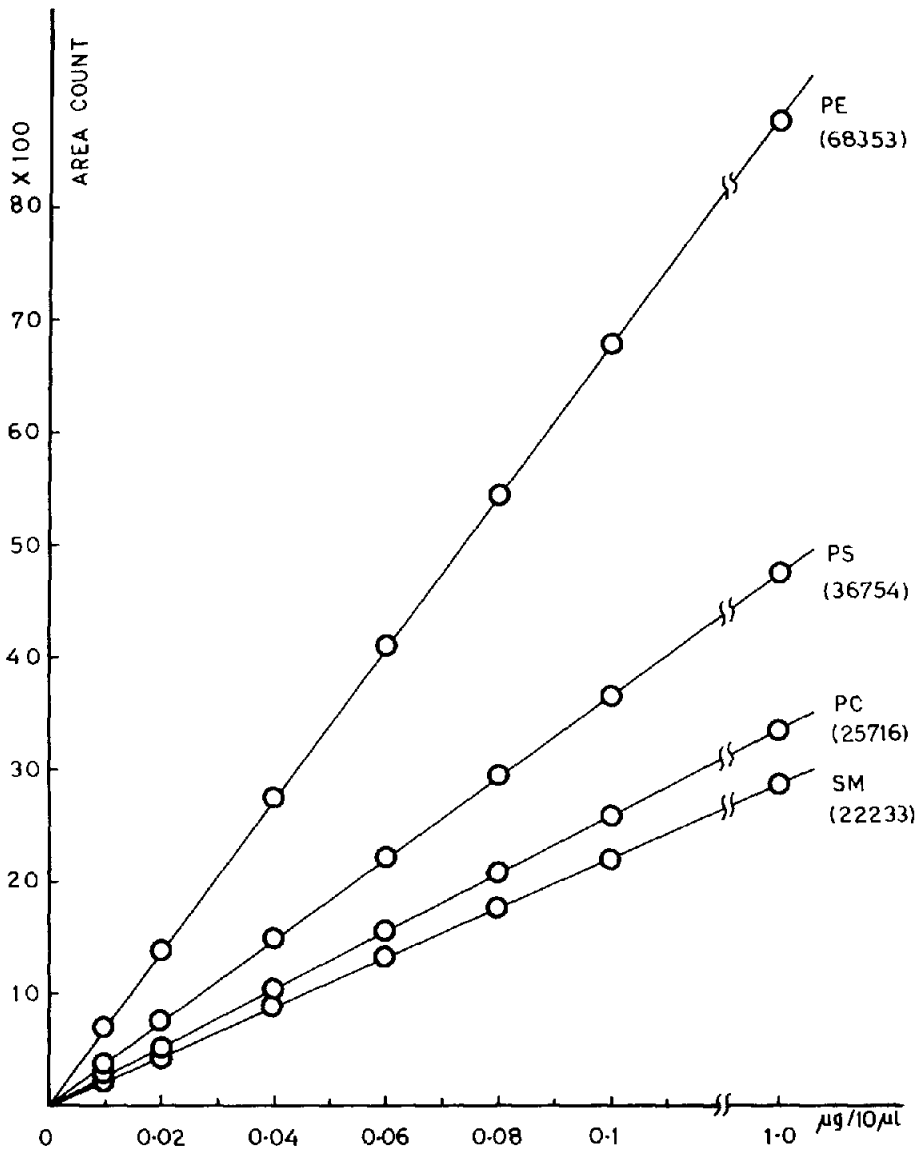

Fig. 3. Standard calibration graphs of different phospholipids. Various amounts of each phospholipid were analysed in 10 μl of *n*-hexane–2-propanol (3:1). Each point represents the mean value of three measurements. Figures in parentheses represent area counts at 1 μg. The linearity was tested up to 20 μg.

μg of membrane protein, was analysed for phospholipids (Fig. 4); a smaller volume of 2 μl was also tested successfully. The composition of the major phospholipid classes was investigated in the erythrocyte ghost membranes of fourteen healthy human blood samples, and the compositions of thrombocytes and lym-

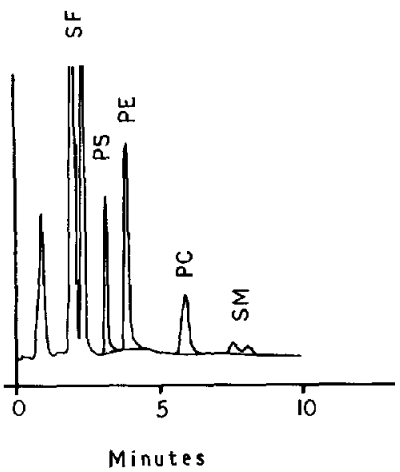

Fig. 4. Chromatogram showing complete resolution of the phospholipids from a 10-μl extract of human erythrocyte ghost membranes, corresponding to 35–40 μg of membrane protein. Peaks: SF = solvent front; PS = phosphatidylserine; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin.

phocytes were also studied. The results are summarized in Table I for preliminary information and comparision. The distribution patterns of the phospholipids in the erythrocyte ghosts, lymphocytes and thrombocytes of healthy humans follow an identical sequence: PC > PE > PS > SM, although the percentage amounts of particular phospholipids differ in the different blood constituents.

TABLE I

PHOSPHOLIPID COMPOSITION OF HUMAN ERYTHROCYTE GHOST MEMBRANES, LYMPHOCYTES AND THROMBOCYTES

Mean values (± standard error) of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) of normal healthy human erythrocyte ghost membranes, lymphocytes and thrombocytes are shown. Values were calculated by peak-area integration for different phospholipids; n = number of samples.

Human blood elements	n	Composition (%)			
		PS	PE	PC	SM
Erythrocyte ghost membranes	14	26.03 ± 0.48	26.88 ± 0.30	31.96 ± 0.55	15.11 ± 0.26
Lymphocytes	14	10.51 ± 0.26	29.69 ± 0.26	51.01 ± 0.49	8.80 ± 0.27
Thrombocytes	12	13.17 ± 0.57	27.77 ± 0.53	46.89 ± 0.76	12.15 ± 0.68

DISCUSSION

The optimum analysis of the phospholipids extracted from human erythrocyte ghost membranes, thrombocytes and lymphocytes was achieved with a mobile phase of acetonitrile–methanol–85% phosphoric acid (100:10:1.8, v/v) on a Beckman Ultrasphere SI 5-μm column (250 mm × 4.6 mm I.D.) and a Waters HPLC system with UV detection at 203 nm within 8 min (the main method I). The separation of PS, PE, PC and SM, including PL, was achieved also with a mobile phase ratio of 100:40:0.4 (v/v) within 25 min (the alternative method II). When the amount of phosphoric acid in the mobile phase was increased, the retention times of all the phospholipids were considerably reduced. Moreover, the sensitivity of the phospholipids, including the SM, is greatly increased, making this method suitable for smaller samples. Also, the acidic environment of the eluting solvent does not cause degradation of PL. Hence, the separation of PL from PE is achieved after derivatization of the samples with HCl, without affecting the analysis of other phospholipids.

Many neurological and neuromuscular disorders are generalized membrane defects and thus can be detected in a variety of tissues, including the blood elements [10]. We have attempted to evaluate the membrane defects of phospholipids in schizophrenia [11] with the present HPLC method, which has been shown to be extremely sensitive, for SM also, when compared with those of others [1,12], including Chen and Kou [3] and Kaduce *et al.* [4]. Under the conditions used by Chen and Kou [3] 400% more SM (2.5 μg) than PS, PE or PC (0.5 μg) was required to appear on the chromatograms. Kaduce *et al.* [4], on the other hand, used smaller amounts of lipid extracts of the endothelial cells, corresponding to 90–100 μg of cell protein, for the analysis of PI, PS, PE and PC. They were able to resolve SM from the extract containing 300 μg of cell protein. With the present method, the complete and successful separation of the phospholipids (PS, PE, PC and SM) in the extract of erythrocyte ghost membranes, corresponding to 10–20 μg of membrane protein, was achieved. Also, a mixture of standard phospholipids consisting of smaller amounts of 5–10 ng per 2 μl each of PS, PE, PC and SM was analysed. The recoveries of the phospholipids were found to be: PS, 98 ± 1%; PE, 99 ± 1%; PC, 100 ± 2% and SM, 89 ± 4% (relative standard deviation).

CONCLUSION

The HPLC method described here offers great advantages in terms of high speed, sensitivity and simplicity. It allows the reproducible identification and quantitative analyses of all the major phospholipids, thus making it suitable for routine clinical studies of membrane disorders in health, toxicity and disease, as well as in research.

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