Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 370 (2007) 54–59
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Separation of intact plasmalogens and all other phospholipids by a single run of high-performance liquid chromatography

Shiro Mawatari *, Yumika Okuma, Takehiko Fujino

Institute of Rheological Function of Food, Hisayama-chou, Kasuya-gun, Fukuoka 811-2501, Japan

Received 20 April 2007
Available online 26 May 2007

Abstract

Plasmalogens are a unique subclass of glycerophospholipids characterized by the presence of a vinyl ether bond at the *sn*-1 position of the glycerol backbone, and they are found in high concentration in cellular membranes of many mammalian tissues. However, separation of plasmalogens as intact phospholipids has not been reported. This article describes a high-performance liquid chromatographic method that can separate intact ethanolamine plasmalogens (pl-PEs) and choline plasmalogens (pl-PCs) as well as all other phospholipid classes usually found in mammalian tissues by a single chromatographic run. The separation was obtained using an HPLC diol column and a gradient of a hexane/isopropanol/water system containing 1% acetic acid and 0.08% triethylamine. The HPLC method allowed a clear separation of plasmalogens from their diacyl analogues. The HPLC method, as applied to the study of peroxidation in human erythrocytes by a hydroperoxide, demonstrated that pl-PEs were targeted twice as much as their diacyl analogues.

Keywords: Plasmalogens; Phospholipids; High-performance liquid chromatography; Peroxidation of lipids; Erythrocytes

Plasmalogens are glycerophospholipids containing a vinyl ether (enol ether) bond at the *sn*-1 position of the glycerol backbone. They are found in nearly all animal tissues, with ethanolamine plasmalogens (pl-PEs)[1] much more abundant than choline plasmalogens (pl-PCs) except in muscles [1]. Plasmalogens not only are structural membrane components and a reservoir for second messengers but also may be involved in membrane fusion, ion transport, and cholesterol efflux [1]. The vinyl ether bond at the *sn*-1 position makes plasmalogens more susceptible to oxidative stress than the corresponding ester bonded glycerophospholipids [1,2]; therefore, plasmalogens may also act as antioxidants, protecting cells from oxidative stress [3–7].

Current methods for the detection and quantification of plasmalogens seem to depend on the susceptibility of the vinyl ether bond at the *sn*-1 position to acid-catalyzed hydrolysis. Acidic hydrolysis of plasmalogens produces a lysophospholipid and a fatty aldehyde. Then the measurement of plasmalogens is carried out by quantifying one of the two products. The separation of lysophospholipids can be done by a thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) method [8,9], and the measurement of fatty aldehydes usually is done with gas chromatography [10–12].

HPLC methods for separation of plasmalogens have been reported after derivatization with acetic anhydride [13,14], benzoate [15], or radioactive iodine [16]. However, to our knowledge, an HPLC method for separation of plasmalogens as intact phospholipids from their diacyl ana-

* Corresponding author. Fax: +81 92 976 2899.
E-mail address: mawatari@rheology.po-jp.com (S. Mawatari).

[1] *Abbreviations used:* pl-PE, ethanolamine plasmalogen; pl-PC, choline plasmalogen; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; CER, cerebroside; DPG, diphosphoglyceride; PA, phosphatidic acid; EDTA–2Na, ethylenediaminetetraacetic acid, disodium salt; PBS, phosphate-buffered saline; Hct, hematocrit value; tBHP, *tert*-butylhydroperoxide; BHT, butylhydroxytoluene; ELSD, evaporative light scattering detector; TEA, triethylamine; PUFA, polyunsaturated fatty acid.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.05.020

logues has not been reported. In this article, we present an HPLC method that can separate pl-PEs and pl-PCs as well as all other phospholipid classes usually found in animal tissues by a single chromatographic run. Because pl-PEs show nearly baseline resolution by the current method, we applied this method to the examination for peroxidation of human erythrocytes by a hydroperoxide.

Materials and methods

Chemicals

Authentic phospholipids were purchased from Serdary Research Laboratories (Englewood Cliffs, NJ, USA) as a phospholipid kit (cat. no. A-481). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), and lysophosphatidylethanolamine (LPE) were obtained from pig liver. Phosphatidylserine (PS), sphingomyelin (SM), and cerebroside (CER) were obtained from beef brain. Diphosphoglyceride (DPG) was obtained from beef heart, and phosphatidic acid (PA) was obtained from egg lecithin. Reagent-grade chemicals and HPLC-grade solvents were purchased from Wako Pure Chemicals (Osaka, Japan).

Materials

A male Wister rat (12 weeks of age) was anesthetized with ether, blood was collected from an abdominal artery into citrated syringes, and then femoral muscle, heart, and cerebellum were obtained. Human venous blood from a healthy adult volunteer was drawn into a tube containing ethylenediaminetetraacetic acid, disodium salt (EDTA–2Na). Chicken breast muscle and bovine heart muscle were obtained at a marketplace. Preparation of washed erythrocytes from human and rat was done as described previously [17].

Peroxidation of human erythrocytes

The washed human erythrocytes were suspended in phosphate-buffered saline (PBS) to make a 10% hematocrit value (Hct). Hct was determined by using a cell counter (Sysmex 800, Sysmex, Kobe, Japan). The total volume of the reaction mixture was brought up to 3 ml by the addition of PBS. The final concentration of the erythrocyte was 5% Hct. The final concentration of *tert*-butylhydroperoxide (tBHP) was 1 mM when it was added. After preincubation for 5 min, the reaction at 37 °C for 10 min was started by the addition of tBHP. The reaction was terminated by cooling in an ice bath, and then the erythrocytes were pelleted by centrifugation at 4 °C. The packed erythrocytes were subjected to lipid extraction.

Lipid extraction

Extraction of total lipid from the human and rat erythrocytes was accorded to the method of Dise and coworkers [18] with minor modification. After 3 ml of the erythrocyte suspension (5% Hct) was centrifuged at 1000 *g* for 10 min, the packed erythrocytes were hemolyzed with 0.5 ml of 10 mM ascorbic acid solution, followed by the addition of 4 ml of methanol. After 30 min at room temperature, 4 ml of chloroform was added and left for 20 min at room temperature. After brief centrifugation, the chloroform–methanol layer was transferred to another tube. The residues were washed by 4 ml of chloroform–methanol (1:1, v/v). To the combined chloroform–methanol solution, 10 ml of 0.88% KCl was added and mixed. The lower layer (chloroform layer) was collected and dried under a stream of nitrogen gas. The total lipid of the other tissues was extracted by the method of Bligh and Dyer [19]. Briefly, a 1-g portion of the tissues was homogenized with a Polytron homogenizer in 3 ml of methanol–chloroform (2:1, v/v), and then 1 ml of chloroform was added. After the addition of 2 ml of 0.88% KCl and a brief centrifugation, the lower chloroform layer was collected. The residues were washed with 1 ml of chloroform. The combined chloroform layer was dried under a stream of nitrogen gas. All solvents used for lipid extraction contained 1.2 mM butylhydroxytoluene (BHT).

Acidic hydrolysis of plasmalogens was done by the method described by Murphy and coworkers [9] except that reextraction of lipids after the acid hydrolysis was done with chloroform and methanol. Briefly, the lipids were extracted with 2 ml of chloroform–methanol (2:1, v/v), 0.5 ml of 0.88% KCl was added, and then the chloroform layer was dried under nitrogen gas.

HPLC system

The HPLC system used was an Agilent 1100 system (Agilent Technologies, Tokyo, Japan) equipped with a four-solvent delivery system, a degasser, and a UV detector. The system was connected to an evaporative light scattering detector (ELSD, Sedex-55, Sedere, Vitry sur Seine, France). The system was also connected to an HPLC ChemStation (Agilent Technologies) for control and analysis of chromatograms.

HPLC method

The method for separation of phospholipid classes was a modification of the method described by Silversand and Haux [20]. The HPLC column was a Lichrosphere 100 Diol, (250 × 4 mm, 5 μm, Agilent Technologies). Mobile phase A was hexane/2-propanol/acetic acid (82:17:1, v/v) with 0.08% triethylamine (TEA), and mobile phase B was 2-propanol/water/acetic acid (85:14:1, v/v) with 0.08% TEA. Mobile phase A was 95% at 0 min and decreased linearly to 60% in 23 min. The gradient continued from 60% A to 15% A in 4 min, and 15% A was maintained for 1 min. Then mobile phase A was increased to 95% in 4 min, and 95% A was maintained for 5 min. The turnaround time was 37 min. The flow rate was 1 ml/min,

and the column temperature was 50 °C. Each phospholipid class was detected by an ELSD, which was set at 50 °C for evaporation temperature, at gain 6 for sensitivity, and at 2.4 bars for compressed air.

Results and discussion

The HPLC method described by Silversand and Haux clearly separated diacylphospholipids usually found in animal tissues, but detection of plasmalogens was not done [20]. In our experience, their method showed an important drawback, namely that the backpressure of column reached nearly 300 bars during elution. Therefore, we changed the program for gradient elution and column temperature. The modification resulted in a decrease of the uppermost backpressure from 300 to 200 bars and a decrease of the turnaround time from 44 to 37 min. The chromatogram for the authentic phospholipid classes was nearly identical to that shown by Silversand and Haux [20]. We currently use a column resulting in more than 150 separations without any decay of column functions.

We first applied the HPLC method to a total lipid extract from human erythrocyte, and we observed two peaks with mobility higher than PC that were named PE-1 and PE-2 (Fig. 1). PE-2 was identified as PE by comparison with a pure standard. The unknown PE-1 was tentatively identified as pl-PE due to the fact that, following acid hydrolysis, its disappearance from the chromatogram was counterbalanced by the appearance of an LPE peak. On the other hand, the analysis of the same lipid extract by our HPLC method with UV detection for phospholipid analysis [17, 21] did not reveal any unknown peak (Fig. 1). Total lipids of various tissues from the rat were examined by this HPLC method, and it was confirmed that PE-1 is pl-PE (Figs. 2 and 3). PC of rat heart showed two peaks, but both peaks did not react to acidic hydrolysis. We expected the presence of pl-PC in some rat tissues, but only a trace of pl-PC was observed in the striated muscle (Fig. 3). Therefore, we examined the total lipid of bovine heart muscle and chicken breast muscle (Fig. 4), and we observed that the peak PC-1 in both tissues is pl-PC (Fig. 4). Thus, the HPLC method presented here clearly can separate both pl-PEs and pl-PCs as well as all other diacyl phospholipids usually present in normal animal tissues. Cerebroside of rat cerebellum was detected (Fig. 2). LPC and LPE can be detected with this HPLC method; however, LPE shows overlap with PI when both are present (Figs. 2–4). The HPLC method showed excellent reproducibility in separation and in retention time of each lipid.

It has been suggested that plasmalogens may play a role in antioxidant defense as a biological function [3–7],

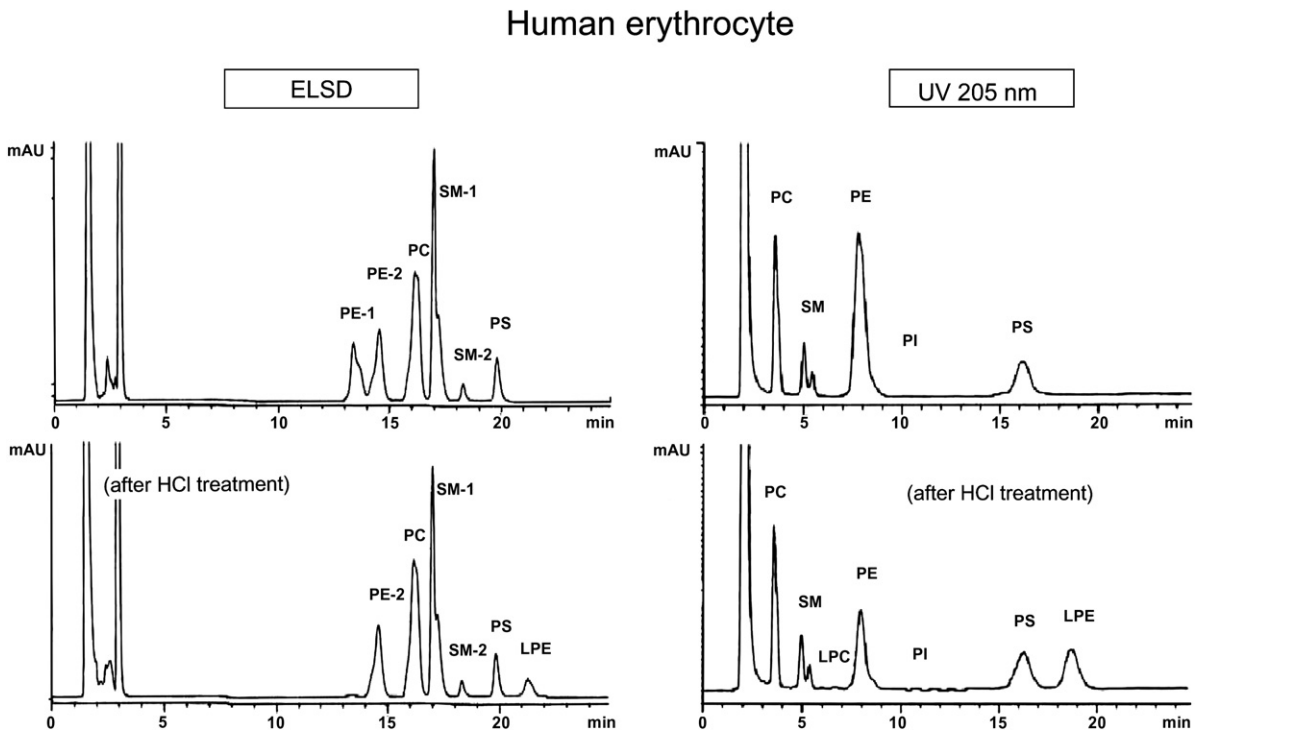

Fig. 1. Chromatograms of total lipids from human erythrocytes. "ELSD" indicates the HPLC method with ELSD detection, and "UV 205 nm" indicates another HPLC method with UV detection at 205 nm. PE-1 in the ELSD method disappeared after acidic hydrolysis of the total lipid and LPE appeared newly (after HCl treatment). When the same sample was applied to the UV 205 nm method, PE showed a single peak, and after acid hydrolysis of the lipid, PE decreased markedly and LPE appeared newly (after HCl treatment). The HPLC method with ELSD detection is described in the "HPLC method" section. The HPLC method with UV detection was done with a Wakosil 5 NH2 (150 × 4 mm column, Wako Pure Chemicals) at 40 °C, and the mobile phase was acetonitrile/methanol/0.25% TEA (pH 3.5 with phosphoric acid) (68:21:11, v/v). Isocratic elution at 1 ml/min was done.

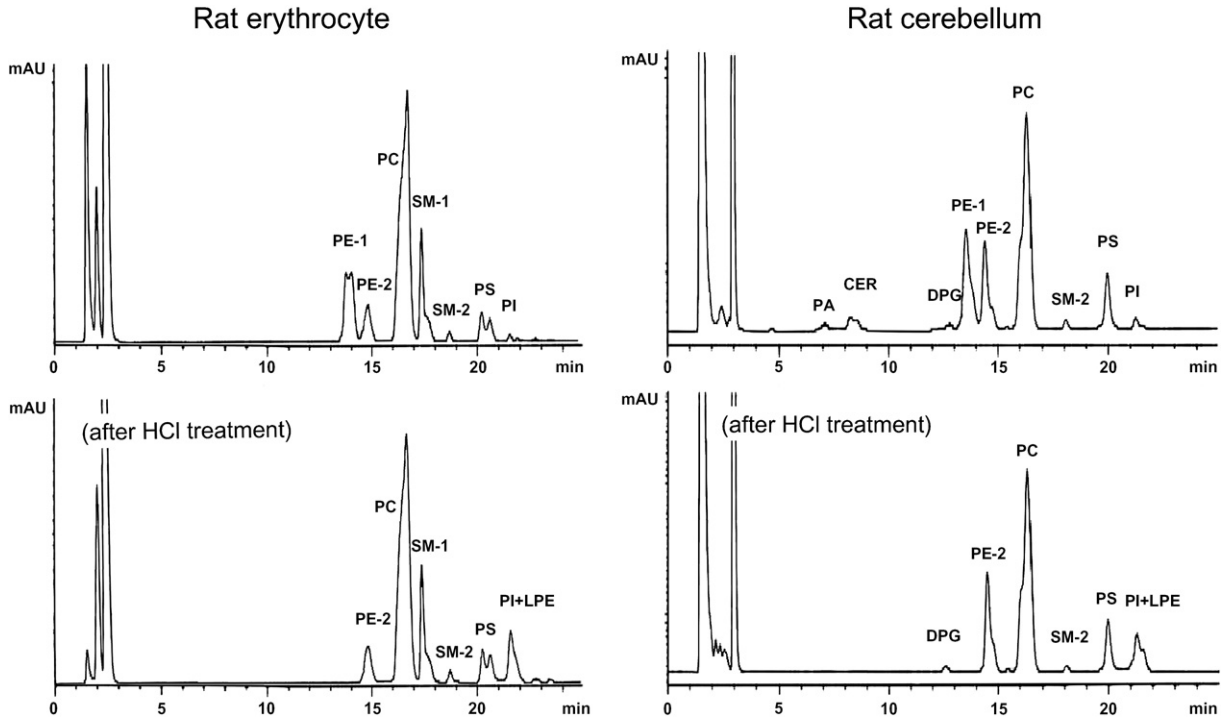

Fig. 2. HPLC chromatograms of total lipids from rat erythrocytes and rat cerebellum. PE-1 is clearly seen in total lipids of both tissues. After acidic hydrolysis of the lipids, PE-1 disappeared and LPE appeared newly (after HCl treatment). LPE overlapped on PI.

although antioxidant properties of plasmalogens sometimes are questioned [22–25]. We applied the HPLC method to peroxidation of the erythrocytes by a hydroperoxide, namely tBHP. Living human erythrocytes incubated with 1 mM tBHP for 10 min at 37 °C showed apparent changes in phospholipid composition of the erythrocyte membranes (Table 1). Because SM usually contains very few polyunsaturated fatty acids (PUFAs) and no

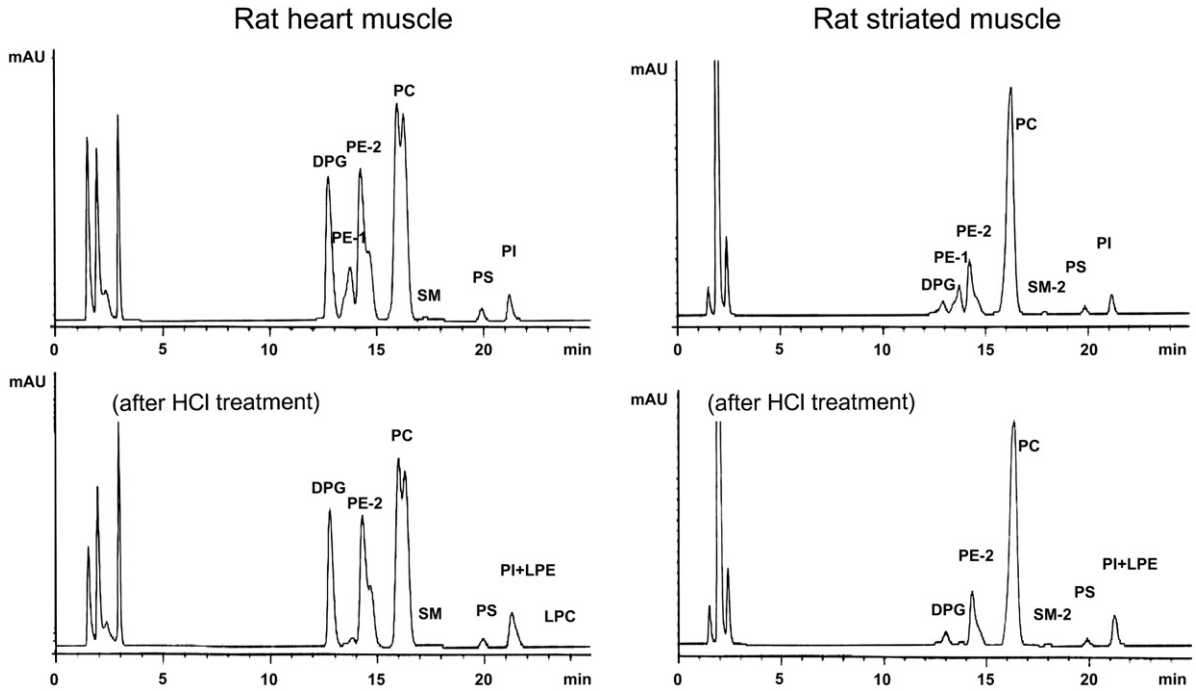

Fig. 3. HPLC chromatograms of total lipids from rat heart muscle and rat striated muscle. Relatively small but clear peaks of PE-1 present in the total lipids of both tissues. After acidic hydrolysis of the lipids, PE-1 nearly disappeared and LPE appeared newly (after HCl treatment). PC of rat heart shows two peaks, but both PC peaks did not react to acidic hydrolysis.

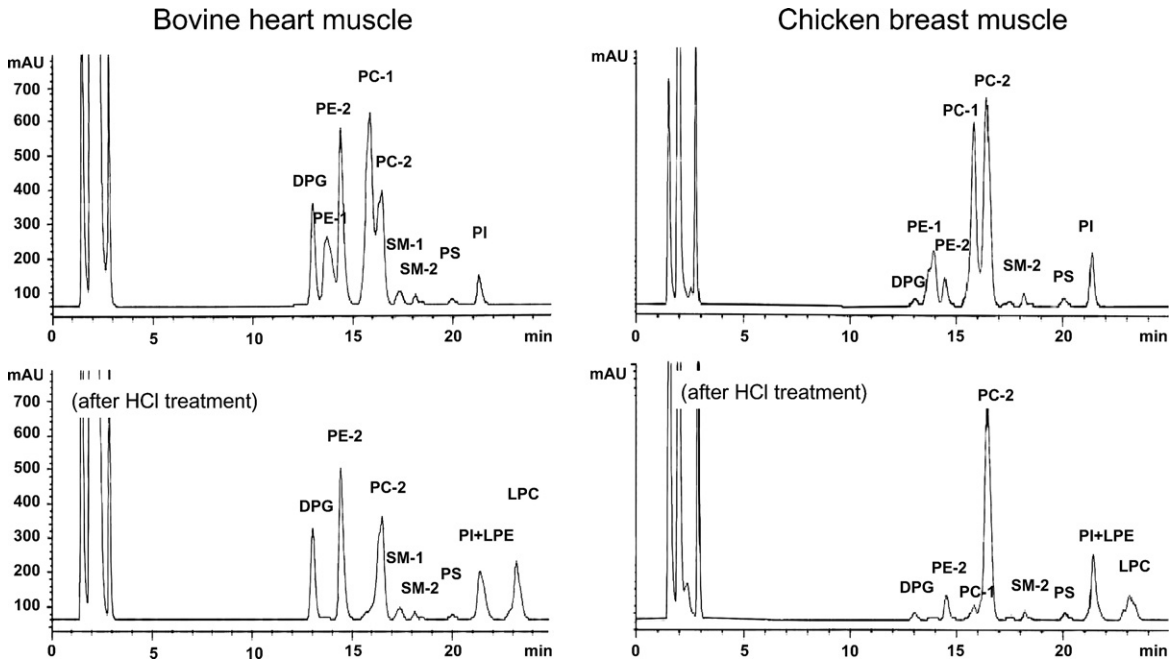

Fig. 4. HPLC chromatograms of total lipids from bovine heart muscle and chicken breast muscle. PE-1 and PC-1 were clearly seen in the total lipids. After acidic hydrolysis of the lipids, both PE-1 and PC-1 disappeared and LPE and LPC appeared newly. Small amounts of PE-1 and PC-1 are seen in the chromatogram of chicken muscle after acidic hydrolysis (after HCl treatment), indicating that plasmalogens were not completely hydrolyzed because the amount of lipids was too high for the acidic hydrolysis.

plasmalogen, SM is not prone to peroxidation as are other phospholipids. Therefore, a change in the relative amount of each phospholipid to SM after peroxidation reflects the degree of peroxidative change of each phospholipid (Fig. 5). Compared with control values, pl-PE decreased 28.1% and PE decreased 14.4%. The results indicate that pl-PE is much more vulnerable to peroxidation than are PE, PS, and PC. However, LPE was not detected after the peroxidation with tBHP, indicating that LPE, if generated, is rapidly metabolized to some other substance after peroxidation. It is also probable that the amount of LPE was too small to detect with the ELSD. Oxidative stress may attack not only the vinyl ether bond but also PUFAs of phospholipids [26]. PE and PS decreased by nearly same degree in the current study.

In summary, this article has described an HPLC method that can separate pl-PEs and pl-PCs as well as all other phospholipids usually found in mammalian tissues by a single chromatographic run. With the current HPLC method, pl-PE showed nearly baseline separation from PE in many tissues. The separation of pl-PC from PC was not baseline, but it showed a clear peak. The HPLC method showed excellent reproducibility in retention time of each phospholipid and chromatographic pattern of similar samples. Simultaneous detection of intact plasmalogens together with other phospholipid classes by HPLC may offer a valuable tool for the study of phospholipids.

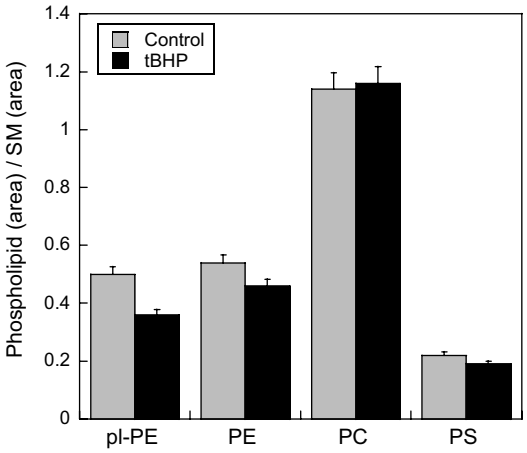

Fig. 5. Changes in phospholipid classes after peroxidation of human erythrocytes with tBHP. Because SM is not prone to peroxidation, the ratio of each phospholipid to SM was calculated. The data are the means ± SDs of four experiments.

Table 1
Changes in phospholipid classes after peroxidation of human erythrocytes with tBHP

	Control (%)	tBHP (%)
pl-PE	14.5 ± 0.5	11.3 ± 0.2
PE	15.9 ± 0.4	14.6 ± 0.3
PC	33.4 ± 1.5	36.4 ± 0.7
PS	6.5 ± 0.3	5.9 ± 0.1
SM	29.5 ± 2.5	31.5 ± 1.6

Note. The human erythrocytes were incubated with and without 1 mM tBHP for 10 min at 37 °C, and the total lipids of the erythrocytes were applied to the HPLC method described in the current article. The values are the means ± SDs of four experiments.

References

[1] K.A. Farooqui, L.A. Horrocks, Plasmalogens: Workhorse lipids of membranes in normal and injured neurons and glia, Neuroscientist 7 (2001) 232–245.

[2] E. Yabin, S. Gatt, Oxygen-dependent cleavage of he vinyl–ether linkage of plasmalogens: II. Identification of the low molecular weight active component and reaction mechanism, Eur. J. Biochem. 25 (1972) 437–446.

[3] R.A. Zoeller, O.H. Morand, C.R.H. Raetz, A possible role for plasmalogens in protecting animal cells against photosensitized killing, J. Biol. Chem. 263 (1988) 11590–11596.

[4] D. Hanel, K. Beyer, B. Engelmann, Inhibition of peroxyl radical-mediated lipid oxidation by plasmalogen phospholipids and α-tocopherol, Free Radic. Biol. Med. 27 (1999) 1987–1994.

[5] N. Khaselev, R.C. Murphy, Susceptibility of plasmamenyl glycerophosphoethanolamine lipids containing arachidonate to oxidative degradation, Free Radic. Biol. Med. 26 (1999) 275–284.

[6] B. Engelmann, Plasmalogens: Targets for oxidative stress and major lipophilic antioxidants, Biochem. Soc. Trans. 32 (2004) 147–150.

[7] R. Maeba, N. Ueta, Ethanolamine plasmalogen and cholesterol reduce the total membrane oxidizability measured by the oxygen uptake method, Biochem. Biophys. Res. Commun. 302 (2003) 265–270.

[8] G.Y. Sun, L.A. Horrocks, Acyl and alk-1-enyl group compositions of the alk-1′-enyl acyl and the diacyl glycerophosphoryl ethanolamines of mouse and ox brain, J. Lipid Res. 10 (1969) 153–157.

[9] E.J. Murphy, R. Stephens, M. Jurkowitz-Alexander, L.A. Horrocks, Acidic hydrolysis of plasmalogens followed by high-performance liquid chromatography, Lipids 28 (1993) 565–568.

[10] N. Maulik, D. Bagchi, R. Jones, G. Cordis, D.K. Das, Identification and characterization of plasmalogen fatty acids in swine heart, J. Pharm. Biomed. Anal. 11 (1993) 1151–1156.

[11] G. Dacremont, G. Vincent, Assay of plasmalogens and polyunsaturated fatty acids (PUFA) in erythrocytes and fibroblasts, J. Inherit. Metab. Dis. 18 (1995) 84–89.

[12] S. Stadelmann-Ingrand, A. Wahl, S. Favreliere, F. Barbot, C. Tallineau, Quantification of long-chain aldehyde by gas chromatography coupled to mass spectrometry as a tool for simultaneous measurement of plasmalogens and their aldehydic breakdown products, Anal. Biochem. 280 (2000) 65–72.

[13] Y. Nakagawa, L.J. Horrocks, Separation of alkenylacyl, alkylacyl, and diacyl analogues and their molecular species by high performance liquid chromatography, J. Lipid Res. 24 (1983) 1268–1275.

[14] Z. Guan, J. Grunler, S. Piao, P.J. Sindelar, Separation and quantitation of phospholipids and their ether analogues by high-performance liquid chromatography, Anal. Biochem. 297 (2001) 137–143.

[15] R. Schulz, K.D. Strynadka, D.L. Panas, P.M. Olley, G.D. Lapaschuk, Analysis of myocardial plasmalogens and diacyl phospholipids and their arachidonic acid content using high-performance liquid chromatography, Anal. Biochem. 213 (1993) 140–146.

[16] R. Maeba, N. Ueta, Determination of choline and ethanolamine plasmalogens in human plasma by HPLC using radioactive triiodide(1-) ion (125I3-), Anal. Biochem. 331 (2004) 169–176.

[17] S. Mawatari, K. Murakami, Effects of ascorbate on membrane phospholipids and tocopherol of intact erythrocytes during peroxidation by *t*-butylhydroperoxide: Comparison with effect of dithiothreitol, Lipids 36 (2001) 57–65.

[18] D.A. Dise, D.B.P. Goodman, H. Rasmussen, Definition of the pathway for membrane phospholipids fatty acid turnover in human erythrocytes, J. Lipid Res. 21 (1980) 292–300.

[19] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.

[20] C. Silversand, C. Haux, Improved high-performance liquid chromatographic method for the separation and quantification of lipid classes: Application to fish lipids, J. Chromatogr. B 703 (1997) 7–14.

[21] S. Mawatari, K. Murakami, Analysis of membrane phospholipid peroxidation by isocratic high-performance liquid chromatography with ultraviolet detection, Anal. Biochem. 264 (1998) 118–123.

[22] R. Felde, G. Spiteler, Plasmalogen oxidation in human serum lipoproteins, Chem. Phys. Lipids 76 (1995) 259–267.

[23] G.A. Jansen, R.J.A. Wanders, Plasmalogens and stress: Evidence against a major role of plasmalogens in protection against the superoxide anion radical, J. Inherit. Metab. Dis. 20 (1997) 85–94.

[24] B. Fauconneau, S. Stadelman-Ingrand, S. Favreliere, J. Baudin, L. Renaud, A. Priou, C. Tallineau, Evidence against a major role of plasmalogens in the resistance of astrocytes in lactic acid-induced oxidative stress in vitro, Arch. Toxicol. 74 (2001) 695–701.

[25] S. Stadelmann-Ingrand, R. Pontcharraud, B. Fauconneau, Evidence for the reactivity of fatty aldehydes released from oxidized plasmalogens with phosphatidylethanolamine to form Schiff base adducts in rat brain homogenates, Chem. Phys. Lipids 131 (2004) 93–105.

[26] N. Khaselev, R.C. Murphy, Structural characterization of oxidized phospholipid products derived from arachidonate-containing plasmenyl glycerophosphocholine, J. Lipid Res. 41 (2003) 564–572.