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

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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) 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-
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 egg lecithin. Reagent-grade chemicals and HPLC-grade solvents were purchased from Wako Pure Chemicals (Osaka, Japan).

Materials

A male Wistar 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/acetate acid (82:17:1, v/v) with 0.08% triethylamine (TEA), and mobile phase B was 2-propanol/water/acetate 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.
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

![HPLC chromatograms](image)

**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.

![HPLC chromatograms](image)

**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.
plasmalogens, 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.

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

<table>
<thead>
<tr>
<th></th>
<th>Control (%)</th>
<th>tBHP (%)</th>
</tr>
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<tbody>
<tr>
<td>pl-PE</td>
<td>14.5 ± 0.5</td>
<td>11.3 ± 0.2</td>
</tr>
<tr>
<td>PE</td>
<td>15.9 ± 0.4</td>
<td>14.6 ± 0.3</td>
</tr>
<tr>
<td>PC</td>
<td>33.4 ± 1.5</td>
<td>36.4 ± 0.7</td>
</tr>
<tr>
<td>PS</td>
<td>6.5 ± 0.3</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>SM</td>
<td>29.5 ± 2.5</td>
<td>31.5 ± 1.6</td>
</tr>
</tbody>
</table>

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


