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Simultaneous Preparation of Purified Plasmalogens and Sphingomyelin in Human Erythrocytes with Phospholipase A_1 from *Aspergillus orizae*

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A method for the simultaneous purification of plasmalogens and sphingomyelin (SM) in human erythrocytes is described. Treatment of total lipids with *n*-hexane/acetone (1:1 v/v) resulted in selective precipitation of SM. Both the supernatant and the precipitate fractions were incubated with a phospholipase A_1 (PLA1) from *Aspergillus orizae* for 3.5 h. The PLA1-treated lipids were extracted with *n*-hexane/isopropanol, the hexane layer was obtained using a Na_2SO_4 solution, and the hexane layer was further washed with water. At this step, the relative concentration of the plasmalogens was 92% of the total phospholipids in the supernatant fraction, and that of SM was 97.7% in the precipitate fraction. Each fraction was applied to high performance liquid chromatography (HPLC) for further purification. The plasmalogen and SM obtained were almost free of the other lipids. The purity of the plasmalogens and SM was monitored by HPLC, which can separate intact plasmalogens from their diacyl analogs.

Key words: human erythrocytes; plasmalogens; sphingomyelin; phospholipase A_1

Plasmalogens are glycerophospholipids containing a vinyl ether bond at the *sn*-1 position of the glycerol backbone. They are found in all animal tissues.[1)] They are not only structural membrane components and a reservoir for second messengers, but also can be involved in membrane fusion, ion transport, and cholesterol efflux.[1,2)] The vinyl ether bond at the *sn*-1 position makes plasmalogens more susceptible to oxidative stress than the corresponding ester glycerophospholipids,[3)] and hence plasmalogens can also act as antioxidants, protecting cells from oxidative stress.[4–10)] A causal relation of ethanolamine plasmalogen (pl-PE) to Alzheimer type dementia is also possible.[11–13)]

Sphingomyelin (SM) is another structural cell membrane component and a reservoir of second messengers. It is known to form a "lipid raft" together with cholesterol in the membrane. Lipid rafts are thought to be "hubs of signal transduction" in membranes.[14–16)]

Highly purified plasmalogens are still difficult to obtain commercially. The structures of plasmalogens and SM can differ among the animal species, and may not be same even in different tissues of the same animal. Therefore, in some studies of phospholipids, there is a need to prepare purified plasmlogens or SM from each tissue as a standard or a substrate for some enzymes. The ether bond at the *sn*-1 position of plasmalogens is resistant to phospholipase A_1 for diacylphospholipids. Many attempts have been made to purify plasmalogens using various phospholipases: snake venom phospholipase A_2,[17)] purified phospholipase from porcine pancreas,[18)] phosphlipase A_1 from guinea pig pancreas,[19)] *Rhizoups delemar* lipase,[20)] and a combination of *Rhizoups delemar* lipase and *N. naja naja* phospholipase A_2.[21)] To our knowledge, the most recent study on the purification of plasmalogen using lipases was done with commercially available phosphatidylcholine and phophatidylethanolamine from porcine heart.[21)] Hydrolysis of diacylphospholipids with *R. delemar* lipase took 18 h,[21)] and thin-layer chromatography (TLC) was used to monitor purity and in the quantification of plasmalogens.[21)]

The present method includes *n*-hexane/acetone treatment of total lipids for selective precipitation of SM and hydrolysis of diacylphospholipids with phospholipase A_1 from *Aspergillus orizae* (PLA1). PLA1 is commercially available, and the hydrolysis of diacyl phospholipids with PLA1 took less than 4 h. Plasmalogen was collected from an HPLC with ultraviolet (UV) detection. The purity of the plasmalogens and SM was monitored by HPLC with evaporative light scattering detection (ELSD), which can separate intact plasmalogens from their diacyl analogs.[22)] Most of the procedures presented here have never been used in the purification of plasmalogens from biological tissues. The procedure is much simpler and requires less time than the reported procedures.

Materials and Methods

Chemicals. Phospholipase A_1 (EC. 3.1.1.32), from *Aspergillus orizae*, was purchased from Mitsui Chemicals and Foods (Tokyo). The

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Abbreviations: ELSD, evaporative light scattering detector; HPLC, high performance liquid chromatography; pl-PE, ethanolamine plasmalogen; pl-PC, choline plasmalogen; LPE, lysophophatidylethanolamine; LPC, lysophospahtidylcholine; PE, diacylphosphatidylethanolamine; PC, diacylphosphatidylcholine; PS, diacylphosphatidylserine; PLA1, phospholipase A_1; SM, sphingomyelin; UV, ultraviolet light; Hct, hematocrit

brochure of the manufacturer stated that the specific activity of PLA1 was 10.000 to 13.000 units/g. Authentic free fatty acids and phospholipids were purchased from Sigma (Tokyo). 9-Anthrildiazomethane was from Funakoshi (Tokyo). Reagent grade chemicals and HPLC grade solvents were from Wako Pure Chemical Industries (Osaka).

HPLC system. The HPLC system used was an Agilent 1100 system (Agilent Technologies, Tokyo) 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 ChemiStation (Agilent Technologies) for the control and analysis of chromatograms.

Monitoring of the purity of the plasmalogens. Monitoring of the relative concentrations of the phospholipid classes, including plasmalogen, was done by the HPLC method, which was developed recently by us,[22] with minor modifications. The HPLC column was a Lichrosphere Diol, 250 × 4 mm, 5 µm (Agilent). Mobile phase A was *n*-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 63% in 21 min. The gradient continued from 63% to 15% A in 4 min, and 15% A was maintained for 1 min. Mobile phase A was increased to 95% in 3 min, then 95% A was maintained for 5 min for pre-conditioning to the next injection. The column temperature was 50 °C and the flow rate was 1 ml/min. The phospholipid class was detected by ELSD, which was set at 50 °C as the evaporation temperature, at gain 6 as to sensitivity, and at 2.3 bars as to air pressure.

Preparation of human erythrocytes. Human venous blood of healthy volunteers was drawn into a tube containing heparin. Preparation of washed erythrocytes was done as described previously.[22,23] The erythrocyte suspension was finally adjusted to 10% Hct. Hct value was determined using a cell counter (Sysmex 800, Sysmex, Kobe, Japan).

Extraction of total lipids. Extraction of total lipids from the erythrocytes was done by the *n*-hexane/2-propanol method,[24] with modifications. Eight ml of erythrocytes suspension (10% Hct) was centrifuged at 1000 *g* for 5 min, and 6 ml of *n*-hexane/2-propanol (3:2 v/v) was added to the packed erythrocytes. After 20 min at room temperature, the *n*-hexane/2-propanol layer was separated by centrifugation and transferred to another tube. The hexane layer was separated by the addition of 4 ml of Na_2SO_4 solution (1 g of anhydrous Na_2SO_4 in 15 ml of water). The hexane layer was collected and dried under N_2 gas.

Treatment of total lipids with n-*hexane/acetone.* The weights of the total lipids were 4.5 mg and 4.9 mg respectively for the two determinations. One ml of *n*-hexane/acetone (1:1 v/v) per 1 mg of total lipids was added to each total lipids. After mixing, the solution was kept in an ice bath for 30 min, and then centrifuged at 1000 *g* for 10 min at 4 °C. Both the supernatant and the precipitate were collected, and they were dried under N_2 gas.

Treatment of lipids with phospholipase A_1 (PLA1). Ten mg/ml of enzyme preparation containing 25% PLA1 was prepared with 0.1 M citrate buffer (pH 4.5), and then 1 mg of total lipids/2 mg of material containing PLA1 (equivalent to 20 to 26 units of PLA1) was added. The suspension was well emulsified by ultrasonic bath, and then incubated at 50 °C for 3.5 h.

After treatment with PLA1, the lipids were extracted with 6 ml of *n*-hexane/2-propanol (3:2 v/v), and the hexane layer was separated by the addition of 4 ml of the Na_2SO_4 solution, as described above. The hexane layer was washed with 2 ml of water (one-third of the *n*-hexane/acetone solution) by vigorous mixing. Then, the hexane layer was collected and dried under N_2 gas.

Collection of plasmalogens from HPLC. The HPLC method for the separation of phospholipids with 205 nm UV detection has been described by us.[25] Briefly, the column was a Wakosil 5 NH_4, 150 × 4.6 mm (Wako Pure Chemical, Osaka), and the guard column was a μ-Bondapak NH_4, 3.9 × 20 mm (Waters, Tokyo). The mobile phase was composed of acetonitrile/methanol/0.15% phosphoric acid with 0.3% TEA (68:21:11 v/v). The flow rate was 1 ml/min, and the column temperature was 40 °C. The peak of pl-PE and SM was collected and dried under N_2 gas.

Acid hydrolysis of plasmalogen. Acid hydrolysis of plasmalogens in the total lipids was done by a method reported previously.[26] The hydrolyzed lipids were re-suspended in 0.5 ml of *n*-hexane/2-propanol (3:2 v/v). After filtration through a 0.45-µm filter, an aliquot of the suspension (usually 10 µl) was injected into the HPLC system. Each phospholipid class was collected from the HPLC with 205 nm detection and was dried under N_2 gas.

Fatty acid analysis of the phospholipid class. The fatty acid compositions of the phospholipids were analyzed by an HPLC method described previously.[23,25] Briefly, each phospholipid class was dried under N_2 gas and hydrolyzed with 1 ml of 0.5 M KOH in 70% methanol at 75 °C for 60 min. After the addition of 0.3 ml of 2 M HCl, the fatty acids were extracted with *n*-hexane. The fatty acids in *n*-hexane were dried under N_2 gas and labeled with 9-anthryldiazomethane. After filtration through a 0.45-µm filter, an aliquot of the labeled fatty acid was injected into the HPLC system.

Results and Discussion

A diagram of the procedure for the preparation of purified plasmalogens and purified SM of human erythrocytes is shown in Fig. 1. The steps of the procedure were named tentatively A, B-1, C-1, B-2, C-2, B-3, and C-3. These names correspond to those in figures and tables.

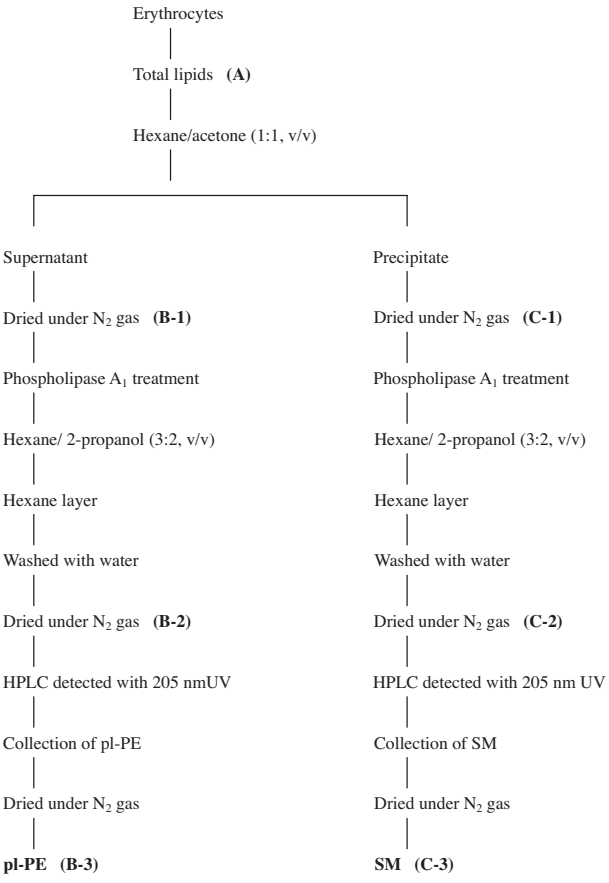

Fig. 1. Diagram of Procedure for Purification Plasmalogens and Sphingomyelin of the Human Erythrocyte Membrane.

The purification steps were named as A, B-1, B-2, B-3, C-1, C-2, and C-3, which correspond to those in the figures and Table 1. pl-PE, ethanolamine plasmalogen; SM, sphingomyelin.

It is known that the addition of acetone to total lipid extract precipitates all kinds of phospholipids, but to our knowledge, it has not been reported that treatment of total lipids with *n*-hexane/acetone (4:6 to 6:4 v/v) causes selective precipitation of SM. The relative concentration of SM after treatment with *n*-hexane/acetone (1:1 v/v) was about 93% of the total phospholipids in the precipitate (C-1 in Fig. 2 and Table 1). The precipitate of SM by hexane/acetone (1:1) is accompanied by 5 to 11% of other phospholipids (mainly PC) in our experience. The amount of SM in the human erythrocyte membranes was about one-third of the phospholipids on the chromatogram as detected by ELSD (A in Table 1 and Fig. 2). SM is not prone to decomposition by phospholipase A_1, and hence the precipitate fraction containing SM was also applied to the PLA1 treatment for further purification of SM. On the other hand, other phospholipids, including plasmalogens, were found in the supernatant of *n*-hexane/acetone treatment (B-1 in Fig. 2 and Table 1). Hence, purification of plasmalogens with PLA1 was started from this fraction.

Table 1. Changes in Relative Composition of Phospholipids of Human Erythrocyte Membrane Due to Treatment with Hexane/Acetone and Phospholipase A_1

	A	B-1	B-2	B-3	C-1	C-2	C-3
pl-PE	17.8	23.7	91.2	100	1.6	0.6	0
PE	17.9	24.3	0.5	0	1.4	0.3	0
pl-PC		0.4	0.8	0	0	0	0
PC	31.2	39.2	0.4	0	3.8	1.4	0
SM	28.5	5.9	7.1	0	93.2	97.7	100
PS	4.1	6.3	0	0	0	0	0

Values are the means of two determinations and are expressed as % of the phospholipids detected by HPLC with ELSD detection. Fractions A to C-3 correspond to Fig. 1. A, total phospholipids; B-1 and C-1, after treatment with hexane/acetone (1:1, v/v); B-2 and C-2, after phospholipase A_1 treatment; B-3 and C-3, collected from HPLC with 205 nm UV detection. pl-PE, ethanolamine plasmalogen; PE, diacylphosphatidylethanolamine; pl-PC, choline plasamlogen; PC, diacylphosphatidylcholine; SM, sphingomyelin; PS, diacylphosphatidylserine

To our knowledge, phospholipase A_1 from *Aspergillus orizae* has not been used for the purification of plasmalogens hitherto. The enzyme preparation consisted of 25% of PLA1 and 75% of food materials, and is commercially available. In this study, 2 mg of material/1 mg of total lipids of erythrocytes was used, which was equivalent to 20 to 26 units/mg of the lipids. The diacylphospholipids in the supernatant fraction were almost completely destroyed within 4 h at 50 °C, and all of the plasmalogens appeared to be preserved. The shapes of pl-PE on the chromatogram did not change after treatment with PLA1 (B-2 and C-2 in Fig. 2). Hydrolysis of bovine heart phosphatidylcholine and phosphatidylethanolamine by *R. delemar* lipase took about 18 h.[21] The hydrolysis time with lipase by the present method was only one-fourth of that by the reported method.[21]

Alkylacyl analogs of phospholipids might not be destroyed by PLA1 from *Aspergillus orizae*, although this was not confirmed in the present study, but the content of alkylacyl analogs of ethanolamine phospholipids in the human erythrocytes has been found to be only 0.6% of total phsopholiplipids.[27]

The method for removing lysophospholipids after PLA1 treatment is important, because large amounts of lysophospholipids from diacylphospholipids are generated by PLA1. We adapted the lipid extraction method reported by Hara and Radin,[24] in which Na_2SO_4 solution is used in partition of the *n*-hexane layer from *n*-hexane/2-propanol (3:2 v/v) solution. The method using Na_2SO_4 solution effectively removed lysophospholipids as well as other substances, including nonlipid substances, but lysophospholipids remained. Hence, we further washed the hexane layer with water. The washing resulted in almost complete removal of lysophospholipids (B-2 in Fig. 2 and Fig. 3).

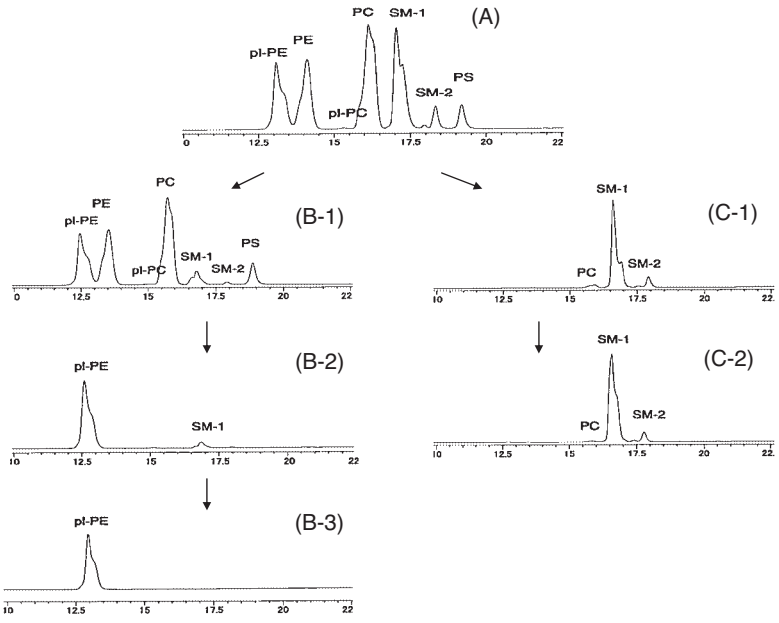

Fig. 2. HPLC Chromatograms with ELSD Detection for the Phospholipids in the Human Erythrocyte Membranes.

The HPLC method is described in "Materials and Methods." A, B-1, B-2, B-3, C-1 and C-2 correspond to Fig. 1. pl-PE, ethanolamine plasmalogen; PE, diacylphosphatidylethanolamine; pl-PC, choline plasmalogen; PC, diacylphosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine

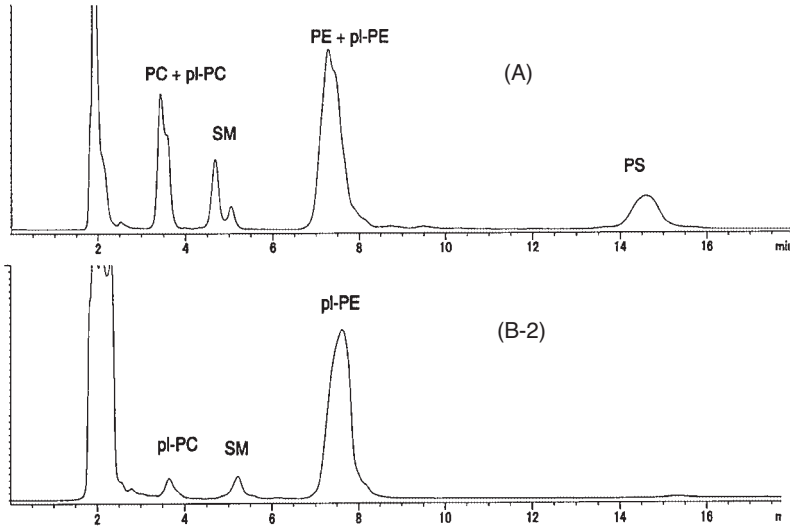

Fig. 3. HPLC Chromatograms with 205 nm UV Detection for the Phospholipids in Human Erythrocyte Membrane.
The method is described in "Materials and Methods." A and B-2 correspond to Fig. 1. Ethanolamine plasmalogen (pl-PE) was collected by this HPLC. For abbreviations, see Figs. 1 and 2.

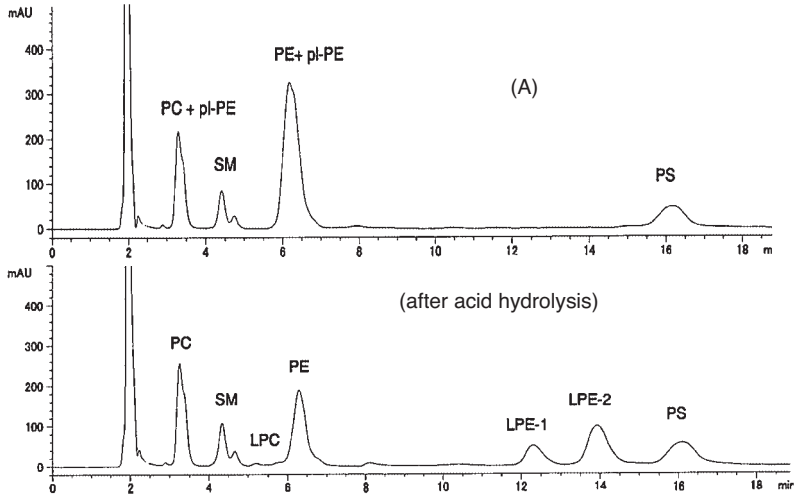

Fig. 4. HPLC Chromatograms with 205 nm UV Detection for the Phospholipids in Human Erythrocyte Membrane before and after Acid Hydrolysis.
The method described in "Materials and Methods." After acid hydrolysis of the total lipids, two peaks of the lysophospholipid of pl-PE (LPE-1 and LPE-2) and a small peak of the lysophospholipid of pl-PC (LPC) were newly seen. Each phospholipid class was collected and was subjected to fatty acid analysis. For other abbreviations, see Figs. 1 and 2.

The plasmalogen content (the sum of pl-PE and pl-PC) after treatment with PLA1 reached 92% of phospholipids, but the fraction still contained SM (7.1% of phospholipids), and other phospholipids. When this fraction (B-2) was further washed with *n*-hexane/acetone (1:1) to remove SM, the purity of pl-PE was increased to about 97% of phospholipids. Contamination by small amount of free fatty acids and cholesterol was reduced by further washing with acetone to precipitate the plasmalogens, but these washings with organic solvents caused large decreases in recovery of the plasmalogens. Therefore, instead of these washing with organic solvents, we used HPLC for further purification of pl-PE (Fig. 3). On the other hand, the SM fraction (C-2) contained little cholesterol or free fatty acids, and the purity of SM had already reached 97.7% at this step. If needed, 100% pure SM can be obtained by collection of the SM peak after applying the C-2 fraction (or total lipids) to HPLC with UV detection, as was done with pl-PE (Fig. 3).

The HPLC method with ELSD detection can separate intact plasmalogens from its diacyl analogs. However, individual peaks could not be collected from the HPLC method, because all the substances were evaporated for detection with ELSD. On the other hand, the HPLC method with UV detection does not destroy phospholipids. Because the diacylphospholipids are completely removed after PLA1 treatment, the HPLC peaks of PE + pl-PE and PC + pl-PC of the fraction indicate pl-PE and pl-PC respectively, even if HPLC is not able to separate the plasmalogens from the diacylphospholipids. The HPLC method with UV detection clearly separated the phospholipid classes (Figs. 3 and 4). Thus plasmalogens can be collected by the HPLC with UV detection. The pl-pE collected from the HPLC was almost free of the other lipids. Because the size of the column used was for analytical use, only a small part of the pl-PE fraction was applied to the HPLC, and the recovery of pl-pE was not calculated. However, even a single chromatographic run provided enough pl-PE for further use for such fatty acid analysis.

Acid hydrolysis of total lipids selectively destroys the vinyl ether bond at the *sn*-1 position of plasmalogens

Table 2. Fatty Acid Compositions of Glycerophospholipids in Human Erythrocyte Membrane

	pl-PE (%)	LPE-1 (%)	LPE-2 (%)	PE (%)	PC (%)	PS (%)	PE + pl-PE (%)
16:0	3.5	0	0	26.0	37.4	0.6	17.3
18:0	2.1	0	0	9.9	12.0	44.8	5.9
18:1	9.2	9.0	7.9	25.4	20.2	2.5	21.0
18:2	10.2	7.3	8.7	7.4	17.9	1.4	5.2
20:4	32.7	34.9	36.6	14.6	6.2	21.3	21.6
20:5	2.3	1.1	0.4	2.1	1.2	1.6	3.9
22:4	7.5	7.3	8.5	2.3	0	2.2	4.4
22:5	10.2	10.5	12.0	2.7	0.7	5.7	5.4
22:6	24.6	28.5	25.9	9.6	4.5	19.8	15.2

Each phospholipids class was collected by HPLC with 205 nm UV detection (Figs. 3 and 4). Values are the means of two determinations. Analysis of fatty acid composition was done by the HPLC method described in "Method and Materials." LPE-1 and LPE-2 were lysophospholipids after acid hydrolysis of pl-PE and showed fatty acids in the *n*-2 position of pl-PE. PE, PC, and PS were diacylphospholipids after acid hydrolysis of plasmalogens. PE + pl-PE indicate total ethanolamine phospholipids.

and generates lysophospholipids of plasmalogens, which contain fatty acids at the *sn*-2 position.[26] The treatment did not alter the fatty acid compositions of phospholipids.[26] The HPLC method with UV detection showed baseline separation of the lysophosholipids of pl-PE and of other major phospholipids composed of the erythrocyte membrane (Fig. 4). Because acid hydrolysis of plasmalogens can be done with total lipid extract, the total lipid fraction was subjected to acid hydrolysis. Thus pure diacylphosphatidylethanolamine (PE), pure diacylphosphatidylcholine (PC) and lysophospholipids of pl-PE were obtained simultaneously. Two large peaks of the lysophospholipids of pl-PE on the HPLC chromatogram were observed after acid hydrolysis of pl-PE (LPE-1 and LPE-2 in Fig. 4). We cannot explain why lysophospholipids from pl-PE showed two peaks after acid hydrolysis. Detection of a phospholipid class with 205 nm UV depends primarily on the double bond in fatty acids of the phospholipid, and thus a large peak of LPE indicates the presence of large amounts polyunsaturated fatty acids at the *sn*-2 position pl-PE. Fatty acid analysis showed that both LPE-1 and LPE-2 were composed of long chain polyunsaturated fatty acids (Table 2). The differences in fatty acid composition between pl-PE and LPE indicate the presence of fatty acids of the *sn*-1 position in pl-PE. It has been reported that the fatty acids at the *sn*-2 position of plasmalogens in pig and rat hearts, human neutrophils and brain tissues preferably consist of long-chain polyunsaturated fatty acids.[1,2,28–31] This study confirmed this with human erythrocyte membranes. Because mature human erythrocytes do not have any intracellular organelles, lipids are present only in the cell membranes.

In summary, a new method for the purification of plasmalogens and SM in human erythrocyte membranes is described. The purity of the plasmalogens and SM was monitored by the HPLC with ELSD detection, which can separate intact plasmalogens from their diacyl analogs. Treatment of total lipids with *n*-hexane/acetone (1:1 v/v) precipitated SM selectively. The PLA1 from *Aspegillus orizae* appeared to be specific to the ester bond at the *sn*-1 position of the diacylglycerophospholipids. Washing of the hexane layer with water was efficient in removing lysophospholipids. The plasmalogen (pl-PE) collected by HPLC with 205 nm UV detection was almost free of other lipids. The present method can be applied to total lipids from other tissues, and should offer highly pure plasmalogens and SM standard of each tissue. The method can also be applied in large-scale preparation of plasmalogens or SM.

Acknowledgments

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