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Short communication

Approach to the large-scale preparation of highly pure phosphatidylserine from bovine brain

Su Chen[a,*], Magda Claeys[b]

[a]*Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK*
[b]*Department of Pharmaceutical Sciences, University of Antwerp (UIA), B-2610 Antwerp, Belgium*

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Abstract

An approach to the large-scale preparation of highly pure phosphatidylserine from bovine brain is described in this paper. The method is based on (i) the separation of phosphatidylserine from phosphatidylinositol in bovine brain extract by preparative aminopropyl normal-phase high-performance liquid chromatography using methanol–1 *M* phosphoric acid (90:10, v/v) as mobile phase and (ii) further purification of phosphatidylserine by anion-exchange chromatography. The main advantage of this approach is that polyunsaturated acid-containing molecular species of brain phosphatidylserine are not lost in the preparation procedure.

1. Introduction

The biochemical and biophysical effects of phosphatidylserine (PS) in a number of biological processes are well documented and appear to be determined by the composition of the fatty acyl chains, and, more specifically, the nature of the polyunsaturated fatty acid esterified at the *sn*-2 position of the glycerol backbone [1]. For example, 1-stearoyl-2-docosahexanoyl-glycero-*sn*-3-phosphoserine (PS 18:0–22:6), which is fairly rich in the brain [2] and in fish liver [3], has been shown to be important for the cell membrane fluidity [1].

Due to the difficulty of preparing PS molecular species containing polyunsaturated fatty acid by chemical synthesis, the preparation of highly pure PS from various natural resources is a main route chosen in lipid and pharmacological research laboratories. Most methods are based on silica-gel column chromatography [4], DEAE ion-exchange chromatography [5], silica thin-layer chromatography (TLC) [6] and normal-phase high-performance liquid chromatography (HPLC) [7]. However, it has been found that phosphatidylinositol (PI) species are also present as major impurities in purified PS [8] since both have a similar chromatographic behaviour upon preparative silica or anion-exchange chromatography [9]. Although pure PS can be obtained by these methods, loss of polyunsaturated fatty acid-containing molecular species of PS often occurs. For example, the PS 18:0–22:6 species may partly overlap with the PI molecular species when using silica column chromatography; a complete separation between PI and PS can only be achieved when loading low quantities of

* Corresponding author.

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sample and, therefore, this approach is only suitable for a small-scale preparation.

Since we are interested in the metabolism of natural PS liposomes containing polyunsaturated fatty acid molecular species in vivo, a method was worked out for this purpose. In this paper, an approach to the large-scale preparation of PS from bovine brain by using aminopropyl normal-phase HPLC and anion-exchange chromatography is described.

2. Experimental

2.1. Chemicals

Analytical-grade chloroform and methanol, purchased from Fisons Company (Loughborough, UK), were used in this study. Phosphoric acid, Q-Sepharose anion-exchange resin, bovine brain extract III (containing mainly 80–85% PS and 15–20% PI as well as other phospholipids) were obtained from Sigma (Poole, Dorset, UK). Aminopropyl HPLC stationary phase (APS-2, 2 μm) was purchased from Shandon Scientific (Cheshire, UK). Diethanolamine and *m*-nitrobenzyl alcohol were obtained from Fluka (Buchs, Switzerland).

2.2. Preparation of phosphatidylserine from bovine brain extract

Small-scale preparation of bovine brain PS

Bovine brain extract III (1 mg) was dissolved in tetrahydrofuran–water (70:30; v/v) and was loaded on an aminopropyl analytical column (5 μm, 15 cm × 4.6 mm I.D., Sigma-Aldrich, Sigma, St. Louis, MO, USA), equipped on a Perkin Elmer HPLC facility with a Series 2 pump and a L75 UV detector. The HPLC fractions were monitored at a UV wavelength of 205 nm, using methanol–1 *M* phosphoric acid (90:10, v/v) as the mobile phase and a flow-rate of 0.9 ml/min. The fractions eluting between 4 and 12 min (fraction 1) and, those eluting between 13 and 17 min (fraction 2) were collected. The phosphoric acid present in the fractions was removed by the method Folch [10].

After evaporating the solvent the residue of fraction 1 was redissolved in chloroform–methanol (60:40, v/v) and applied onto a short column (10 × 0.5 cm) containing anion-exchange resin (Q-Sepharose Fast Flow) [11]. The resin of this column was washed with (i) 2 × 5 bed volumes of chloroform–methanol–1 *M* sodium acetate (30:60:8, v/v); (ii) 2 × 5 bed volumes of chloroform–methanol–water (30:60:8, v/v); and (iii) 2 × 10 volumes of chloroform–methanol (60:40, v/v). The non-acidic phospholipids were eluted with 2 bed volumes of chloroform–methanol (60:40, v/v), followed by 1 bed volume of chloroform–methanol (40:60, v/v). Finally, the PS molecular species were eluted with 3 bed volumes of acetic acid–chloroform (5:1, v/v). Acid-free powder of PS was obtained by lyophilization.

Large-scale preparation of bovine brain PS

A 50-mg quantity of the same brain extract, dissolved in 300 ml of tetrahydrofuran–water (70:30, v/v), was applied on a home-made preparative aminopropyl HPLC column (5 μm, 30 × 1 cm). The monitoring conditions and the mobile phase used were the same as those given above. The flow-rate was 9 ml/min. The fractions eluting between 6 and 23 min (fraction I) and between 24 and 37 min (fraction II) were collected and further processed as described above. The PS was then purified on a column (20 × 1 cm) containing Q-Sepharose resin. The mobile phase used was identical to that as mentioned above.

2.3. Analysis of the purified PS and PS-derived fatty acids by mass spectrometry

Liquid secondary-ion (LSI) mass spectrometric analysis of the purified PS and PS-derived methylated fatty acids was performed on a VG70SEQ (Fisons Instruments, Manchester, UK) mass spectrometer of EBQ1Q2 design, equipped with a cesium ion source. A sample of purified PS was dissolved in chloroform; diethanolamine was applied on the probe tip, and, thereafter, 2 μl of the solution (containing approximately 5 μg of PS) was loaded on the

surface of the diethanolamine matrix without mixing [12], and analyzed in the negative-ion mode. The methylated fatty acid mixture, prepared by hydrolysis of PS followed by methylation of the free fatty acids, was mixed with 2 μl of *m*-nitrobenzyl alcohol saturated with LiI, and analyzed in the positive-ion mode.

2.4. Analysis of PS-derived fatty acids by gas chromatography

Gas chromatographic analysis of fatty acid methyl esters was performed with a Pye Unicam gas chromatographic system (Model Series 204, UK) with a packed column (200 × 0.3 cm I.D.) coated with Silar 10C, using a temperature program from 170 to 210°C at a rate of 1°C/min. A flame ionization detector was used, and the carrier gas was nitrogen. The area under each peak was determined using a Shimazu C-R5A integrator (Kyoto, Japan), and the PS and brain extract-derived fatty acids were identified by comparing retention time values with those of known methylated fatty acid standards (PUFA II, Sigma, St. Louis, MO, USA).

2.5. Analysis of purified PS by TLC

The purity of PS, obtained by preparative HPLC followed by anion-exchange chromatography, was monitored by TLC. The plate (Merck, Frankfurt, Germany) was developed with a mixture of chloroform–ethyl acetate–propanol–methanol–water (25:25:25:10:9, v/v) as described previously [13]. A 200-μg quantity of the sample was loaded on a TLC plate in order to detect minor components (phosphatidic acid and lysophosphatidylserine).

The serine phospholipids were identified by pink and blue colouring after spraying with ninhydrin and phosphomolybdic acid reagents (Sigma), respectively.

3. Results

Fig. 1 shows the HPLC separation of brain extract III chromatographed on an analytical (a) and a preparative (b) aminopropyl column, respectively. The fractions eluting between 4 and 12 min (fraction 1) and between 13 and 17 min (fraction 2) from the analytical column, as well as between 6 and 23 min (fraction I) and between 24 and 37 min (fraction II) from the preparative column were collected. Based on the results described previously [14], fractions 1 and I contain mainly phosphatidic acid, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, lysophosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylserine; fractions 2 and II contain inositol phospholipids.

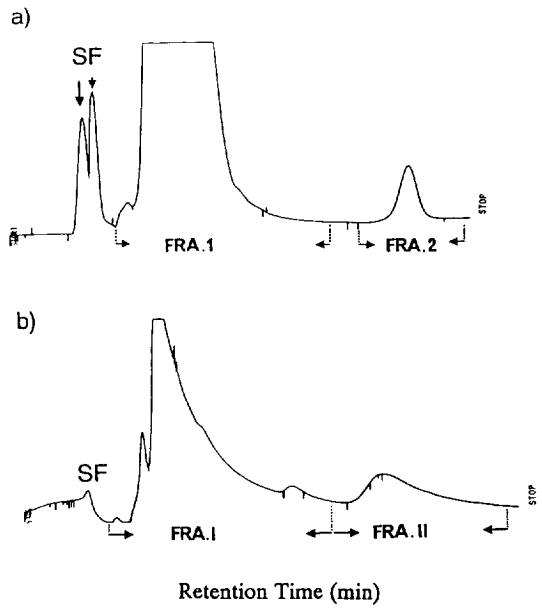

Fig. 1. (a) Normal-phase HPLC separation of phospholipids from the bovine brain extract III on an analytical aminopropyl column. The mobile phase is methanol–1 *M* phosphoric acid (90:10, v/v). The flow-rate is 0.9 ml/min. The chart speed is 1 cm/min. SF relates to the solvent front. (b) Normal-phase HPLC separation of phospholipids from the bovine brain extract III on a preparative aminopropyl column. The mobile phase is methanol–1 *M* phosphoric acid (90:10, v/v). The flow-rate is 9 ml/min. The chart speed is 0.5 cm/min. SF is present as the solvent front.

After further purification by Q-Sepharose anion-exchange chromatography, 40.7 mg of PS was obtained. TLC analysis indicates that there is a small amount of lysophosphatidylserine (less

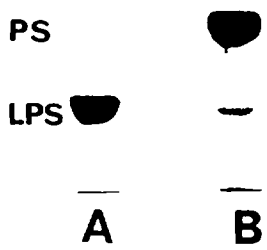

Fig. 2. Thin-layer chromatography of lysophosphatidylserine (LPS) standard (A) and the PS sample (B) prepared by the preparative aminopropyl column and ion-exchange chromatography.

than 1%) present in the sample (Fig. 2) and that no phosphatidic acid can be detected. This sample was also characterized by negative-ion LSI mass spectrometry (not shown). Ions at *m/z* 788, 810, 816 and 834 correspond to the deprotonated molecules of the PS 18:0–18:1, PS 18:0–20:4, PS 18:0–20:1 and PS 18:0–22:6 species, respectively. Peaks at *m/z* 701, 437 and 419, due to $[788 - \text{serine}]^-$, $[701 - R_2CO\text{-}H]^-$ and $[701 - R_2COOH]^-$ anions [11], correspond to the fragment ions derived from PS 18:0–18:1, a major molecular species in this class. PI 18:0–20:4 was identified as a major species in fraction II (not shown).

The PS-derived fatty acids were analyzed as their methyl ester derivatives by positive-ion LSI mass spectrometry (not shown). Peaks at *m/z* 303, 305, 325, 331 and 349 corresponding to $[M + Li]^+$ ions of 18:1, 18:0, 20:4, 20:1 and 22:6 fatty acids were found. The methyl esters of the PS-derived fatty acids (PS was separated on both an analytical and preparative HPLC column followed by anion-exchange chromatography) were also quantitatively analyzed by gas chromatography. These results are in agreement with those obtained by direct mass spectrometric analysis of the fatty acid derivatives. The percentages of the PS-derived fatty acids are listed in Table 1.

In order to compare the fatty acid composition in PS before and after purification, the ratio of the gas chromatographic peak area of the methyl ester of 22:6 fatty acid versus that of 18:1 fatty acid, which are mainly derived from PS 18:0–18:1 and PS 18:0–22:6 rather than the PI species, was calculated. Values of 0.39 and 0.37 were obtained for the extract and the purified sample, respectively.

Table 1
Percentage of the major fatty acids of phosphatidylserine purified on an analytical and preparative aminopropyl HPLC column followed by anion-exchange chromatography

Fatty acid	Percentage fatty acids	
	Analytical column	Preparative column
18:0	45.1	46.5
18:1	32.4	32.8
20:1	5.8	5.5
20:4	3.3	3.0
22:6	12.3	12.1

4. Discussion

The use of ultraviolet detection does not allow to quantitatively monitor the HPLC fractions, because there is no common absorption group in the phospholipid species; the absorption wavelength in the 200–210 nm range reflects the number of double bonds of the fatty acid residues rather than the number of lipid residues. The main reason for losing polyunsaturated fatty acid-containing molecular species of PS, more specifically PS 18:0–22:6, when using silica or anion-exchange chromatography, is due to overlapping of the molecular species containing polyunsaturated fatty acid residues with PI species. This was evidenced indirectly by the analysis of both bovine brain PS extract and brain PS purified on a preparative silica column by negative-ion fast atom bombardment mass spectrometry. The intensity ratio of the ions at *m/z* 834 (PS 18:0–22:6) to *m/z* 788 (PS 18:0–18:1) in the spectrum of the PS extract is higher than that obtained for purified PS (not shown).

The most critical step in the large-scale preparation procedure relates to the complete separation of PS and PI with aminopropyl normal-phase HPLC. The separation of the seven phospholipid classes on an aminopropyl HPLC could be achieved with ethanol–1 *M* phosphoric acid as

mobile phase [11]. The advantage of the use of a mobile phase of methanol–1 *M* phosphoric acid is that the phosphoric acid present in the HPLC fractions can easily be removed by the method of Folch after separation. Although PS is coeluting mainly with phosphatidylethanolamine and phosphatidylcholine, the purpose of the aminopropyl HPLC step is to isolate PS from PI in the extract rather than to separate the classes of phospholipid. The elution time of PS and PI from the aminopropyl HPLC column can be altered by changing the methanol to phosphoric acid ratio in the mobile phase or adjusting the flow-rate of the mobile phase. The quantitative results obtained by gas chromatographic analysis of bovine brain PS- and purified PS-derived fatty acids indicates that there is no significant loss of 22:6 fatty acid-containing molecular species in the preparation procedure. A method for rapidly purifying phospholipids from rat liver microsomes and mitochondria by silica preparative normal-phase HPLC has been reported [7]. The approach described in the present study, however, is more suitable for the preparation of highly pure PS from bovine brain.

In conclusion, this procedure, based on aminopropyl HPLC followed by anion-exchange chromatography, is useful for the large-scale preparation of PS from bovine brain, without loss of polyunsaturated fatty acid-containing molecular species. The availability of natural polyunsaturated fatty acid-containing PS should provide biological scientists with the necessary material to perform further studies on its therapeutic use and metabolism.

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