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Short communication

Separation and identification of phosphatidylserine molecular species using reversed-phase high-performance liquid chromatography with evaporative light scattering and mass spectrometric detection

Åsmund Larsen[a,*], Eva Mokastet[a], Elsa Lundanes[a], Erlend Hvattum[b]

[a]*Department of Chemistry, University of Oslo, PO Box 1033, Blindern, 0315 Oslo, Norway*
[b]*Department of Chemistry and Biochemistry, Agricultural University of Norway, PO Box 5026, N-1432 Ås, Norway*

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Abstract

A reversed-phase HPLC method compatible with evaporative light scattering (ELS) and electrospray mass spectrometric (ES-MS) detection was developed for separation of phosphatidylserine (PS) molecular species. The method was optimised for separation of three disaturated synthetic species: dipalmitoyl glycerophosphoserine, palmitoyl-stearoyl glycerophosphoserine and distearoyl glycerophosphoserine using isocratic elution with a mixture of 2-propanol, tetrahydrofuran and ammonium formate. Baseline separation was obtained on three different columns: one polystyrene/divinylbenzene (PS/DVB) column and two silica based C_{18} and C_{30} columns. The best chromatographic resolution was achieved with the C_{30} column. The limit of detection for DPPS was 5 μg/ml ($S/N=3$) with ELS detection and 0.1 μg/ml ($S/N=3$) with negative ion ES-MS in the single ion monitoring mode. Baseline separation of the five main species in a biological PS sample, bovine brain PS, was obtained with the PS/DVB column. Species identification was done by using the retention times of the intact PS species and their corresponding carboxylate anion fragments obtained by in-source fragmentation. Data have shown that individual PS species can be identified by their retention times using direct ELS detection in a mixture of disaturated PS species. However, for the bovine brain PS electrospray-MS detection was necessary for species identification due to the many possible fatty acid combinations in biological PS.

Keywords: Evaporative light scattering detection; Phosphatidylserines

1. Introduction

Phospholipids are the main constituents in biological membranes. The different phospholipid classes have important structural and functional properties, which are essential for cell life [1]. One of the important cell membrane phospholipids, phosphatidylserine (PS), occurs in most mammalian cells and is known to be involved in a number of biological processes [1,2]. Hence, characterisation of endogenous PS is important to understand biological functions. The PS in mammalian tissues and cells has a diacyl glycerol backbone structure with fatty acid residues in *sn*-1 and *sn*-2 positions and with

*Corresponding author. Tel.: +47-2285-5535; fax: +47-2285-5441.
E-mail address: aasmunl@kjemi.uio.no (Å. Larsen).

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phosphate ester attached to the *sn*-3 position. The most commonly found fatty acid chain lengths are C_{14}–C_{24} and the degree of unsaturation varies from 0 to 6 [1]. The fatty acids are generally more saturated in the *sn*-1 position than in the *sn*-2 position [1,2]. However, the fatty acid combination in *sn*-1 and *sn*-2 positions can be different in different tissues [3]. The biological importance and the general pharmaceutical interest in phospholipids have led to increased focus on analytical methods for characterisation of the individual phospholipid species.

Species determination of phospholipid fractions can be performed in many ways. One of the more common approaches has been hydrolysis of the *sn*-3-phosphoryl moiety, conversion to non-polar derivatives followed by HPLC, TLC or GLC [4]. This is a very sensitive and accurate method, but involves a rather time consuming sample preparation procedure. Another approach has been to use normal-phase HPLC separation of intact phospholipid classes combined with collision-induced dissociation and product ion scan MS detection for individual species determination [5–8]. This approach, however, requires electrospray tandem MS capabilities which is still not standard equipment in many laboratories. In this study, we have used standard electrospray single MS and ELS detection in combination with reversed-phase HPLC. Methods for separation of PS species have previously been described [9–12]. However, these methods were either based on derivatisation for UV detection or on mobile phases that contain involatile buffers, ion-pairs or salts which would impair detection with electrospray MS or ELS detector.

The aim of this study was to develop a method for separation and direct detection of synthetic and biological PS species. Three different reversed-phase columns were evaluated, one polymer based column and two silica based columns.

2. Experimental

2.1. Instrumentation

Two different high-performance liquid chromatographic systems were used: HP1050 and HP1090 (Hewlett-Packard, Germany). The evaporative light scattering detector (ELSD) was a Sedex 55 (S.E. D.E.R.E., Alfortville, France) and the mass spectrometer was a Quattro II (Micromass, Manchester, UK) equipped with a standard electrospray ionisation interface. The HP1090 HPLC system was coupled on-line to the ELSD and the HP1050 HPLC system was coupled to the Quattro II mass spectrometer. Due to incompatibility between tetrahydrofuran and PEEK tubing, 180-μm I.D. stainless steel tubing was used between the pump and autosampler and between autosampler and analytical column. The HPLC columns evaluated were: PLRP-S (PS/DVB), 150×4.6 mm, 100-Å pore size, 5-μm particles (Polymer Laboratories, Shropshire, UK), Supelcosil LC-18-DB (C_{18}), 250×4.6 mm, 5-μm particles (Supelco, Bellefonte, PA, USA) and Carotenoid (C_{30}), 250×4.6 mm, 5-μm particles (YMC, Wilmington, NC, USA). The injection volumes were 2–50 μl. Access*Chrom GC/LC datasystem was used to collect data from the ELSD (Perkin-Elmer Nelson, Cupertino, CA, USA) whereas MassLynx software (Micromass) was used for collecting data with MS detection.

2.2. Reagents and solutions

The phospholipid standards, dipalmitoyl glycerophosphoserine (DPPS), palmityl-stearoyl-glycerophosphoserine (PSPS) and distearoyl glycerophosphoserine (DSPS) were all from Avanti Polar Lipids (Michigan, USA) while bovine brain PS (L-α-phosphatidyl-L-serine) was from Sigma–Aldrich (St. Louis, MO, USA). Hexane, 2-propanol and tetrahydrofuran were LiChrosolv grade while ammonia (25%) was analytical grade from Merck (Darmstadt, Germany). The formic acid was from Rathburn (Walkerburn, UK) and the water quality was Milli-Q grade.

2.3. Preparation of buffer solution and standard solutions

The ammonium formate buffer (10 m*M*) was prepared by adding 0.37 ml ammonia (25%) to 0.45 l water and adding formic acid to pH 5.5 (the final method). The total volume was adjusted to 500 ml. The ammonium acetate buffer was made in the same

way as the ammonium formate buffer. The phospholipid standards were dissolved in 2-propanol/hexane/water, 20/10/4 (v/v/v) and diluted with the mobile phase 1:9 (v/v) prior to analysis. The saturated PS species were heated to ~40 °C for ~5 min while no heating was necessary to dissolve the bovine brain PS.

2.4. Evaporative light scattering and mass spectrometric detection

The ELSD drift tube temperature was set to 54 °C. Nitrogen (99.99%, Aga, Oslo, Norway) was used as nebulizing gas at a pressure of 1.8 bar. For electrospray MS detection, a post column splitting device (1:6) was made by using a Valco T-piece and 250-μm I.D. fused-silica tubing. The electrospray capillary was set to 3.0 kV (negative) and the source temperature was set to 80 °C. Nitrogen was used both as drying gas and nebulizing gas at flow rates of approximately 300 and 10 l/h, respectively. Masses for selected ion monitoring were *m/z* 834.5, 816.5, 788.5, 786.5, 760.5, 327.2, 309.2, 283.2, 281.2 and 255.2, each ion with a dwell time of 0.3 s. The cone voltage was set to 40 V for the parent ions (low cone voltage) and optimised to 120 V for in-source fragmentation with maximum abundance of the fatty acid carboxylate anions (high cone voltage). Full scan analysis was done with a scan speed of 300 a.m.u./s in the *m/z* 200–900 mass range. The mass axis was previously calibrated with NaI and operated at unit mass resolution.

3. Results and discussion

3.1. Separation of synthetic disaturated PS species

The saturated PS species were baseline separated on all three columns (Fig. 1): the optimised mobile phase for the three columns are given in Table 1. Preliminary experiments showed that dilution of the sample 1:9 (v/v) with the mobile phase improved peak shape and increased the maximum injected volume on the three columns. The maximum injected volume was found to be 50, 30 and 20 μl on the PS/DVB column, the C_{30} column and the C_{18} column, respectively, without peak deterioration (not shown) using the mobile phases listed in Table 1. Due to the poorer loading capacity no further work was done on the C_{18} column.

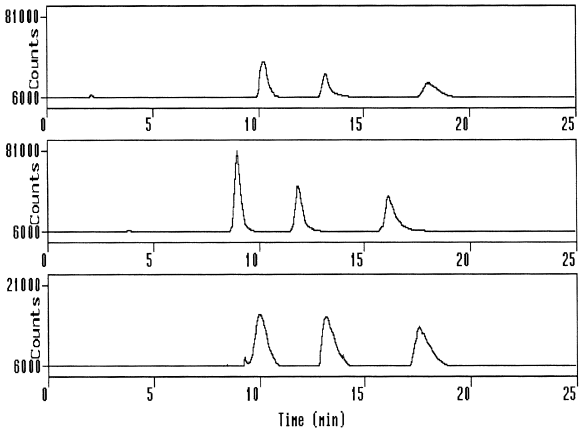

Fig. 1. HPLC–ELSD: separation of DPPS (first peak), PSPS (second peak) and DSPS (last peak) on the PS/DVB column (upper trace), the C_{30} column (middle trace) and the C_{18} column (lower trace) using the mobile phase given in Table 1. The concentration was 0.1 mg/ml of the individual species and the injection volume was 20 μl.

Volatile buffers are preferred when using ELS- and ES-MS detectors. The buffers tested were ammonium acetate and ammonium formate at a concentration of 10 and 50 m*M*, pH 6 for both. The type of buffer had a minor effect on the retention time and peak shape. However, the ammonium formate buffer gave the highest signal-to-noise ratio (*S/N*) with the ELS detector for the same buffer concentration. Furthermore, when the ammonium formate buffer concentration was reduced from 50 to 10 m*M* the background noise decreased from 4 to 2 μV (ELS detector) while the retention time and response signal remained the same. With no mobile phase buffer, however, no retention was obtained.

Due to the zwitter-ionic nature of PS, the effect of mobile phase pH was examined in the pH 3–9 range (pH 3–6 for the C_{30} column and pH 3–9 for the PS/DVB column). The mobile phase pH was adjusted by adding different amounts of formic acid to the 10 m*M* ammonia solution. The retention increased with increasing pH on both columns (not shown). However, at pH 6 and higher, peak splitting occurred for DPPS on the C_{30} column. In the pH 4–6 region the change in retention time was rela-

Table 1
Mobile phase composition and column temperatures for the separation of DPPS, PSPS and DSPS species on three different HPLC columns

Column	Ammonium formate (vol.%)	2-Propanol (vol.%)	Tetrahydrofuran (vol.%)	Column temp. (°C)
PS/DVB	50	35	15	50
C_{30}	30	55	15	40
C_{18}	35	50	15	40

tively small while the peakshape was the same, i.e. with an asymmetry factor of $A_s < 1.6$. The relatively little effect of pH and asymmetry factor in the pH 4–6 region could be explained by the fact that $pK_{a(COOH)} = 2.2$ and $pK_{a(NH_2)} = 9.2$ for serine [13]. However, the background noise with ELS detection increased with decreasing pH and a pH of 5.5 was therefore chosen for further studies. To our knowledge this is the first report on PS species separation combined with direct detection using ELS detector.

Using the optimised mobile phase given in Table 1, the C_{30} column had the highest column efficiency ($n = 6100$ for DSPS) compared to the PS/DVB column ($n = 3400$ for DSPS). Using 20-μl injections, the limit of detection (LOD) was found to be 5 μg/ml for DPPS with ELS detection ($S/N = 3$). Using ES-MS detection in the selected ion mode, however, the sensitivity was improved and the LOD was found to be 0.1 μg/ml ($S/N = 3$), which is in the same LOD range as previously reported for PSPS using normal-phase HPLC–MS [14].

3.2. Individual species determination in bovine brain PS

Fig. 2A,B shows the full scan mass spectra of bovine brain L-α-phosphatidyl-L-serine obtained with direct injection into the electrospray mass spectrometer with no prior chromatographic separation. The MS was operated at either low cone voltage (Fig. 2A) or high cone voltage (Fig. 2B). With low cone voltage deprotonated molecular ions $[M–H]^-$ of PS were mainly obtained. However, with in-source fragmentation at high cone voltage, signals were obtained for the fatty acid carboxylate anions. The carboxylate anions found were C16:0 (m/z 255.2), C18:1 (m/z 281.3), C18:0 (m/z 283.3), C20:1 (m/z 309.3) and C22:6 (m/z 327.3) (Fig. 2B). Together with the molecular ions obtained at low cone voltage, the carboxylate anions indicate which species are present. However, the different carboxylate anion combinations in the *sn*-1 and *sn*-2 positions make

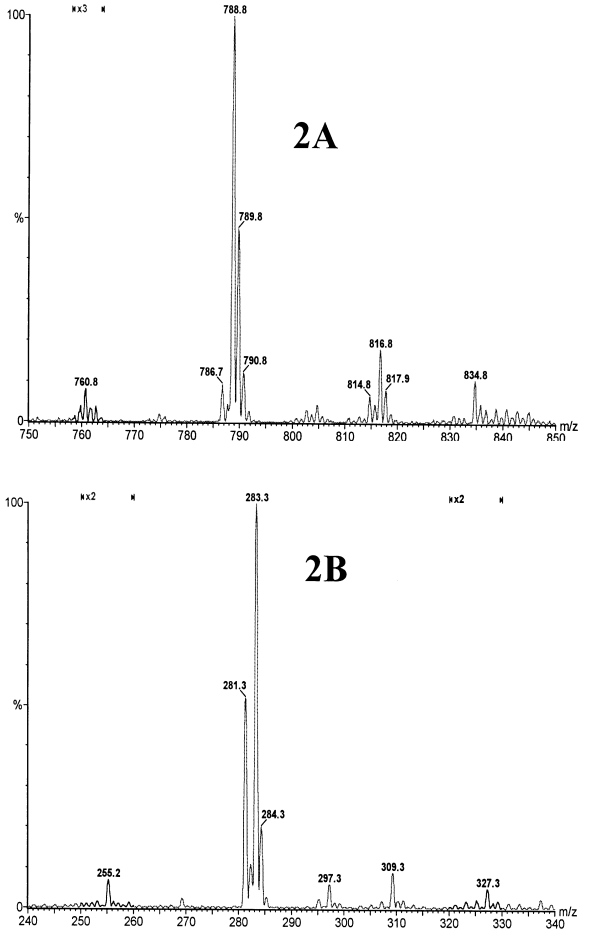

Fig. 2. Loop injection of bovine brain PS. The negative ion electrospray mass spectrum of the molecular ion region (A) and the fatty acid carboxylate anions obtained by in-source fragmentation (B) is shown.

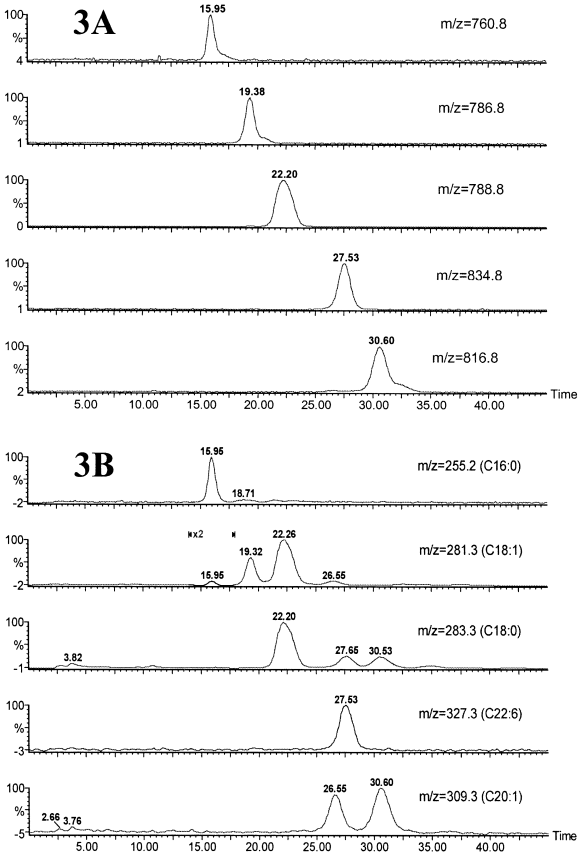

Fig. 3. HPLC–MS of bovine brain PS using the PS/DVB column and the mobile phase listed in Table 1. Traces in (A): mass chromatograms of molecular ions (low cone voltage). Traces in (B): mass chromatograms of the carboxylate anion fragments (high cone voltage). (A) and (B) were obtained in the same run alternating between high and low cone voltages. Other conditions are given in the Experimental section.

chromatographic separation necessary for species identification.

Due to different selectivity towards the unsaturated PS species, the C_{30} column separated three species only (not shown) as compared to the PS/DVB column which baseline separated the five main PS species (Fig. 3A). Fig. 3B shows the mass chromatogram obtained with in-source fragmentation at high cone voltage. Species identification was done by comparing retention times between parent ions and their corresponding carboxylate anion fragments (Fig. 3A,B). For instance, the molecular ion at m/z 788.8 could theoretically be both 16:0/20:1 PS and/or 18:0/18:1 PS. However, the parent ion at m/z 788.8 has the same retention time as the fragments at m/z 281.3 and m/z 283.3 identifying the species as 18:0/18:1 PS. A summary of species identification in L-α-phosphatidyl-L-serine is given in Table 2. The identification was verified using product ion scan (MS–MS) of each molecular ion (results not shown). Although the retention time of the parent molecular ion was not confirmed, Fig. 3B also indicates the presence of a 18:1/20:1 species at 26.55 min. This is supported by the molecular ion at m/z 814.8 found by full scan loop injection (Fig. 2A).

As far as we know this is the first report on how PS species separation can be carried out using reversed-phase HPLC in combination with ELS- and single ES-MS detection. Furthermore, with single ES-MS detection, a complete chromatographic separation of all species is not necessarily needed for identification as long as isobaric species are separated. Separation of phosphatidylcholine (PC) species has previously been reported on the same

Table 2
Summary of species identification in bovine brain PS found by reversed-phase HPLC using the PS/DVB column with negative ion electrospray single MS detection

Retention time (min)	Molecular ion (m/z)	Carboxylate anions (m/z)	PS species	Relative amount (area %)[a]
15.95	760.8	255.2, 281.3	16:0/18:1	2
19.38	786.8	281.3, 281.3	18:1/18:1	7
22.20	788.8	281.3, 283.3	18:1/18:0	69
27.53	816.8	283.3, 309.3	18:0/20:1	11
30.60	834.8	283.3, 327.3	18:0/22:6	10

The identification was verified using product ion scan (MS–MS) of each molecular ion. Other conditions are given in the Experimental section.

[a] Based on the molecular ion signal.

PS/DVB column using MS compatible mobile phase [15]. Hence, the same identification principle could be used for PC species. However, in a crude biological extract, other phospholipid classes may give isobaric overlap and hence, the present method could be limited to pure PS fractions. Normal phase chromatography, on the other hand, has the advantage of resolving the classes and should be included for samples from natural sources of phospholipids containing several classes.

4. Conclusions

The present work has shown baseline separation between disaturated DPPS, PSPS and DSPS as well as between the unsaturated main species in bovine brain PS. Separation of disaturated PS species was obtained on three different HPLC columns using isocratic elution. The best chromatographic resolution between disaturated PS species was obtained on the C_{30} column. The limit of detection for DPPS was found to be 5 μg/ml ($S/N=3$) with ELS detection and 0.1 μg/ml ($S/N=3$) with ES-MS detection in the single ion monitoring mode.

The polymer based column (PS/DVB) gave the best chromatographic resolution between the five unsaturated main species in bovine brain PS. Species identification was done by using the retention times of the intact PS species and their corresponding carboxylate anion fragments obtained by in-source fragmentation. Data have shown that individual PS species can be identified by their retention times using direct ELS detection in a mixture of disaturated PS species. However, for the bovine brain PS electrospray-MS detection was necessary for species identification due to the many possible fatty acid combinations in biological PS.

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