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Journal of Chromatography B, 716 (1998) 47–56

JOURNAL OF CHROMATOGRAPHY B

Specific detection and quantification of palmitoyl-stearoyl-phosphatidylserine in human blood using normal-phase liquid chromatography coupled with electrospray mass spectrometry

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Received 28 April 1998; received in revised form 11 June 1998; accepted 18 June 1998

Abstract

A narrow-bore normal-phase high-performance liquid chromatography (HPLC) method was developed for separation of phospholipid classes using an HPLC diol column and a gradient of chloroform and methanol with 0.2% formic acid titrated to pH 5.3 with ammonia. The HPLC system was coupled on-line with an electrospray mass spectrometry (ES-MS) or electrospray tandem mass spectrometry (ES-MS–MS) system and the separation of several major phospholipid classes was shown. The molecular species of some phospholipid classes in human blood were qualitatively determined. A method was further developed for specific determination of a molecular species from phosphatidylserine, palmitoyl-stearoyl-phosphatidylserine (PSPS), in human blood using HPLC–ES-MS. The analyses were performed by single ion monitoring of the $[M-H]^-$ molecular ions of PSPS and an internal standard, dipalmitoyl-phosphatidylserine. The limit of quantification of the method was 1.2 ng of PSPS. The calibration curve ranged from 0.12 to 5.81 μg/ml of PSPS dissolved in the mobile phase. The curve was fitted to a second-order polynomial equation and found to be highly reproducible. Analysis of control samples was found to be reproducible with a between-series precision below 9.2% R.S.D. The amount of endogenous PSPS in human blood was determined in 13 subjects and found to range from 1.73 to 3.09 μg/ml. The identity of endogenous PSPS was confirmed by HPLC–ES-MS–MS. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Palmitoyl-stearoyl-phosphatidylserine; Phospholipids

1. Introduction

Phospholipids are the main constituent of biological membranes and have important structural and functional properties. Phospholipids are made up of several distinct molecular classes which vary in the polar head group and are unevenly distributed in different organs. Each phospholipid class is further composed of a mixture of many molecular species containing different fatty acids. Recently, phospholipid vesicles, i.e., liposomes, have become commercially important as drug carrier systems and are extensively used in the pharmaceutical industry. The increasing commercial use of phospholipids as constituents of drugs requires specific analytical methods to separate and identify the added phospholipids from endogenous phospholipids.

Several analytical techniques for separation and quantification of phospholipids have been developed. Thin-layer chromatography and normal-phase high-

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PII: S0378-4347(98)00303-X

performance liquid chromatography (HPLC) have been extensively used to separate the phospholipids into different molecular classes (for review see Refs. [1,2]). The detection methods for normal-phase HPLC have been UV absorbance, fluorescence, refractive index, flame ionization and evaporative light scattering [3]. To increase the specificity of the phospholipid analysis, reversed-phase HPLC can be used. Reversed-phase HPLC separates the phospholipids into different molecular species [4], however, it often requires the use of ion-pair reagents which might limit the detectors that can be used [5,6]. Another approach to increase the specificity of the phospholipid analysis is to use mass spectrometry (MS).

Analysis with HPLC coupled to a mass spectrometer is now routinely applied for lipid analysis (for review see Ref. [7]). Several ionization techniques have been used, such as particle beam [8], thermospray [9–11], discharge assisted thermospray (plasmaspray) [12], atmospheric pressure chemical ionization (APCI) [13,14] and modern electrospray (ES) [8,14,15]. The soft atmospheric ionization techniques, APCI and ES, offer the possibility of accommodating chromatographic flow-rates up to 1.0 ml/min and have become increasingly popular. With these flow-rates, post-column splitting has become unnecessary and both narrow bore and standard columns can be used.

HPLC coupled with a mass specific detector makes it possible to directly quantitate specific phospholipid species of interest on-line. The present work describes the development of such an HPLC–ES-MS method for normal-phase separation of phospholipid classes and for specific quantification of one endogenous molecular species of phosphatidylserine (PS) from human blood, i.e., palmitoyl-stearoyl-phosphatidylserine (PSPS). The amount of PSPS in human blood was determined and the method was validated. The validation results are presented in this paper.

2. Experimental

2.1. Materials

Chloroform was stabilized with ethanol and was pro-analysis grade from Rathburn (Walkerburn, UK). Methanol was LiChrosolv grade and formic acid (98–100%), ammonia (25%) and hydrochloric acid were pro-analysis grade all from Merck (Darmstadt, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] (DPPS), palmitoyl-stearoyl-*sn*-glycero-3-[phospho-L-serine] (PSPS) and hydrogenated plant L-α-phosphatidylinositol (PI) were from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were from Sygena (Cambridge, MA, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG) was from Sigma (St. Louis, MO, USA). According to the manufacturers, the purity of the standard compounds were approximately 99%.

2.2. Standards and control samples

The standards contained PSPS in the concentration range of 0.12–5.81 μg/ml. In addition, the standards contained 2.0 μg/ml of the internal standard, DPPS. HPLC–MS analysis is suitable for the use of internal standard since the detection principle is very specific and very often deuterated standards of the lipids to be quantitated are used [7]. Deuterated standards of PS are, however, very expensive and difficult to obtain. DPPS was therefore chosen as the internal standard of the method, due to the assumed low endogenous level of the compound in blood.

The standards were dissolved in the injection solution, i.e. chloroform–methanol–water (65:25:4, v/v/v). To avoid evaporation during storage, the solvent was removed and the standards were stored dry at −20°C. Prior to analysis the standards were redissolved in the injection solution. The concentration of the control samples were: 0.23, 0.58 and 2.33 μg/ml of PSPS and 2.0 μg/ml of DPPS. They were treated and stored similarly to the standards. Blank samples were chloroform–methanol–water (65:25:4, v/v/v).

2.3. Sample preparation

Heparinised human blood was collected from fasting subjects. Prior to analysis, the lipids in the samples were extracted essentially as described by Eder et al. [16]. Briefly, the internal standard and 0.7 ml of water was added to 300 μl of the blood

sample; then 5 ml of methanol and 10 ml of chloroform was added and the solution was sonicated for 60 s both before and after adding chloroform. After sonication the solution was whirlmixed for 30 s and incubated for approximately 1 hour at room temperature. Finally, 5 ml of 0.1 *M* hydrochloric acid was added before the solution was mixed for 5 s and centrifuged at 2600 *g* for 10 min. The lower chloroform phase was sampled and dried by evaporation under nitrogen. Prior to analysis the extracted samples were redissolved in 300 μl of the injection solution.

2.4. Chromatographic conditions

The chromatographic system consisted of a Spectra-Physics SP 4000 mobile phase pump, equipped with a Spectra-Physics SCM 400 degassing unit; a Spectra-Physics AS300 autosampler, equipped with a 10 μl (Rheodyne) sample loop. The lipids were separated on a LiChroCART, LiChrospher 100 Diol, 250×2 mm I.D., (5 μm) column (Merck) with a LiChrospher 100 Diol, 4×4 mm I.D., (5 μm) precolumn (Merck). Mobile phase A was chloroform, while mobile phase B was prepared by adding ammonia (25%) to methanol with 0.2% (v/v) formic acid until the pH of the solution was 5.3. To separate the lipids in the sample a gradient was run starting at 100% mobile phase A, decreasing to 64% A in 9 min and further decreasing to 40% A in 4 min and then back to 100% A in 4 min. The total chromatographic run time was 30 min. For specific determination of PSPS in blood the gradient was slightly modified to starting with 90% mobile phase A and 10% mobile phase B, decreasing to 64% A in 6.5 min. The rest of the gradient was as above. The flow-rate was 0.3 ml/min and the analysis was performed with a column temperature at 30°C. The total chromatographic run time was 23.5 min. The samples and standards were kept at room temperature and 10 μl was injected for each analysis. Only one injection per vial was performed.

2.5. Mass spectrometry

The HPLC system described above was coupled on-line to a VG Platform single quadrupole mass spectrometer (Micromass, Altrincham, UK) equipped with pneumatically-assisted electrospray ionization source. The HPLC effluent entered the MS through an electrospray capillary set at 4.0 kV at a source temperature of 140°C. Nitrogen was used both as drying gas and nebulizing gas at flow-rates of approximately 400 l/h and approximately 10 l/h, respectively. The ion-source parameters were optimized with respect to the negative molecular ions of DPPS (m/z 734.7) and PSPS (m/z 762.7). The optimal settings of m/z for the two molecular ions were routinely checked by choosing multiple settings of m/z for PSPS and DPPS ranging from $m/z = \pm 1$ of the theoretical settings and the m/z settings that gave the highest response was chosen. The cone voltage was 50 V and the span (in Da) was zero giving unit mass resolution in single ion monitoring (SIM) mode. Each molecular ion was monitored with a dwell time of 0.25 s and an inter channel delay of 0.01 s. Dose response curves and quantification of PSPS in the different samples (control and blood) were conducted by SIM of the two selected ions. When the MS was operated in the scanning mode, the scan range was 600 to 1000 in the continuum mode and the scan speed was 800 amu/s with a mass resolution of 1.5 amu at half peak height.

2.6. Tandem mass spectrometry

Tandem mass spectrometry (MS–MS) analysis was carried out on a VG QUATTRO II mass spectrometer (Micromass) equipped with pneumatically-assisted electrospray ionization source. The HPLC system was a Varian Model 9012 gradient pump with a Varian autosampler (Varian Chromatography Systems, Walnut Creek, CA, USA). The column, mobile phase and gradient was the same as mentioned above. The HPLC system was coupled on-line to the MS–MS and the product ion spectra of negative precursor ions were obtained with MS–MS parameters as described by Karlsson et al. [14].

2.7. Sample analysis and validation parameters

The samples were analysed by negative-ion HPLC–ES-MS (SIM of m/z 734.7 and of m/z 762.7) in sequences together with standards, control samples and injection blanks. The standards were positioned at the beginning and at the end of each

sequence, while the control samples and the injection blanks were randomly placed in the sequence together with the samples.

The standard curve was evaluated from six calibration graphs analysed on six different days. The precision was evaluated by analysing the three control samples (0.23, 0.58 and 2.33 μg/ml of PSPS) in duplicate in a total of eight analytical series. After analysis the following was calculated; the mean of the within-series means, the standard deviation ($S.D._x$) of this mean and the pooled within-series standard deviation of the daily mean [$S.D._w(p)$]. The standard deviations were determined from the mean square values of an ANOVA single factor calculation of the results where; $S.D._w(p)^2=$ within-group mean square and $nS.D._x^2=$between-group mean square (where n is the number of replicates per analysis).

Repeatability and accuracy of estimating PSPS in human blood was evaluated by adding either 0 or 50 μl of a phospholipid mixture containing 0.48 μg PSPS to 0.3 ml heparinised blood. The samples were extracted and analysed with six sample replicates in one analytical series.

2.8. Data handling

A Micromass MassLynx data sampling system, v. 2.0, was used for sampling and integration of the SIM chromatograms. GraphPad PRISM, v. 2.0, was used for preparation of calibration curves and estimation of concentrations. Microsoft Excel, v. 5.0, was used for the ANOVA calculations and the other statistical calculations.

3. Results and discussion

3.1. Method development and optimization of the mobile phase

Initially, mobile phase B (see Section 2.4) consisted of a mixture of methanol and 1.25% (v/v) ammonia, giving a relative rapid deterioration of the column due to the high pH. The pH of mobile phase B was therefore adjusted to 5.3 with formic acid which was more suitable for the column. The maximum amount of formic acid and ammonia that could be added to the methanol was 0.2% (v/v) formic acid adjusted to pH 5.3 with ammonia. At higher ammonium formate concentration the HPLC system was repeatedly blocked when running the gradient, probably because the chloroform precipitated the ammonium formate above a critical concentration.

3.2. Separation and specificity of the method

Our main goal was to specifically determine PSPS in blood and since mass spectra of different phospholipids often overlap, it is important to have class separation of the phospholipids. Separation of the major phospholipid classes was achieved by using the present HPLC method and as shown in Fig. 1, only PI eluted close to the phospholipid class of interest, i.e., PS. For routine analysis of human blood the gradient used in Fig. 1 was slightly changed to

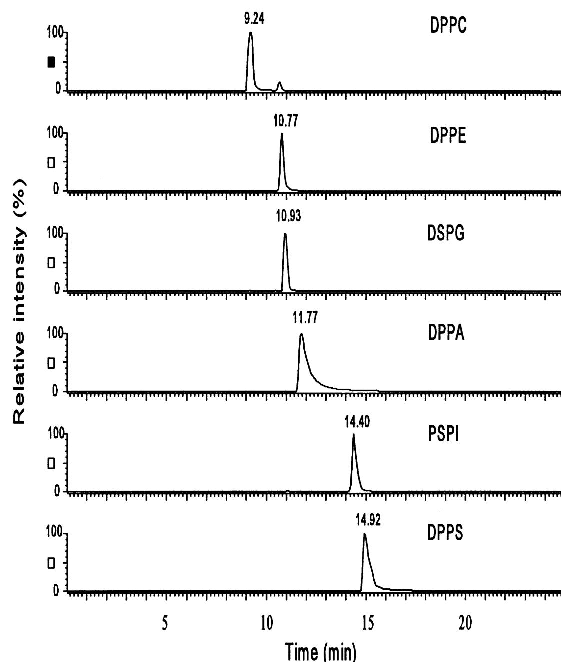

Fig. 1. Negative-ion HPLC–ES-MS analysis of a phospholipid mixture with the MS set to SIM of the following phospholipids (approximately 8 μg/ml of each); DPPC, DPPE, DSPG, DPPA, palmitoyl-stearoyl-phosphatidylinositol (PSPI) and DPPS. For compound abbreviations, see Section 2.1. Mobile phase as described in Section 2.4 and the gradient starting with 100% chloroform.

shorten the chromatographic run time (see Section 2.4). After the normal-phase HPLC method was established, the specificity of the method for PSPS in blood was further determined. Extracted human blood was analysed by negative-ion HPLC–ES-MS in the scanning mode. Fig. 2 shows that a major peak in blood eluted with approximately the same retention time as synthetic PSPS. As expected, the mass spectrum under this peak showed $[M-H]^-$ ions that either corresponded to molecular species of PS or PI (Fig. 2C and Table 1). The MS software offers the possibility of making chromatograms of individual molecular ions that are found in a spectrum. The molecular ion under the endogenous peak in Fig. 2 that was assigned to PI was found to elute before the molecular ions that were assigned to PS at 11.80 min and 12.32 min, respectively. As expected, the different PS species were also found to have slightly different retention times (not shown). Thus only molecular species of PS will elute close to PSPS under the present conditions.

The molecular ion at m/z 762.7 that was found under the PS peak from blood (Fig. 2), indicated a certain amount of endogenous PSPS. The identity and purity of this ion was further determined by negative-ion HPLC–ES-MS–MS. The product ion spectra of the precursor PSPS related ion from both extracted human blood and standard PSPS were compared and the same carboxylate anion fragments were found (Fig. 3). The two molecular ions detected, i.e., m/z 255 and m/z 283, correspond to the $[M-H]^-$ ions of C16:0 and C18:0 fatty acids, respectively (Fig. 3). Fig. 3 shows, however, that the relative abundance of the fatty acid fragments were different in the two samples. The precision of the abundance ratios was below 7% R.S.D. for both synthetic PSPS ($n=7$) and endogenous PSPS ($n=2$) and the abundance ratio difference was therefore highly significant. We believe that the difference in abundance ratio indicates a difference in the position of the fatty acids on the glycerol backbone of the two PS species. Preliminary experiments in our laboratory indicate that the sn-1 fatty acid fragment appears as the most intense peak in the product ion spectra of PS at the given experimental conditions. Probably because they have stronger affinity for the negative charge compared to the *sn*-2 fatty acid fragment. However, in corresponding experiments looking at

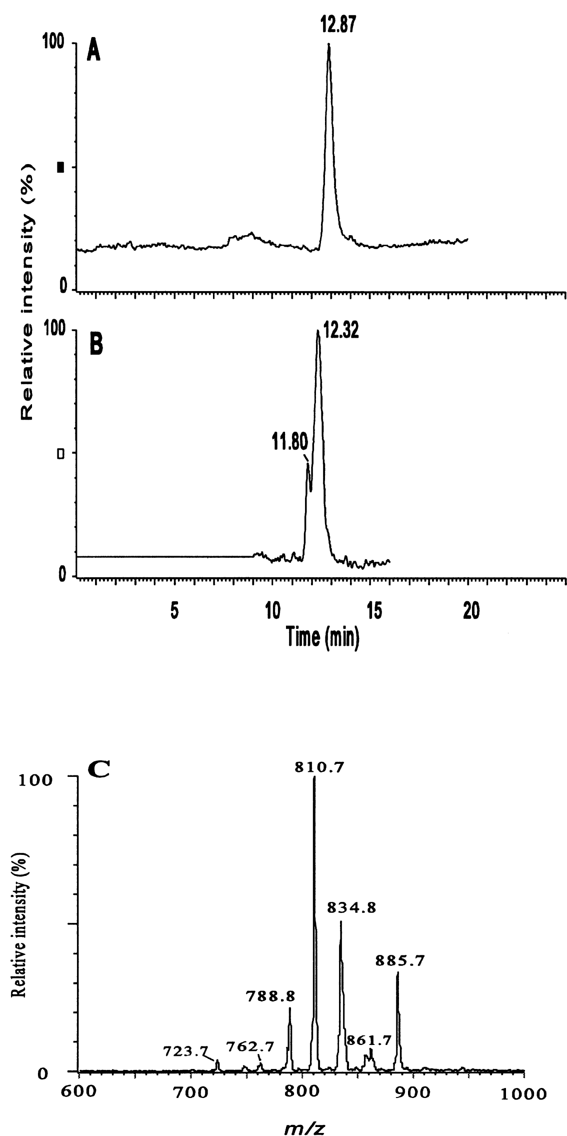

Fig. 2. Negative-ion HPLC–ES-MS analysis of (A) control sample with 0.58 μg/ml of PSPS with MS operated in SIM (m/z 762.7); (B) extracted human blood (0.3 ml) with MS operated in the scanning mode; (C) the mass spectrum under the peak in (B), i.e., the spectrum is obtained from 11.5 min to 13.5 min in (B). Mobile phase as described in Section 2.4 and the gradient starting with 90% chloroform.

product ion spectra of PE, one report states that the intensity of the fatty acid fragment in the *sn*-2 position was approximately twice that of the fatty acid in the *sn*-1 position [17] and another report states that there was no preferential loss of the fatty

Table 1
Major molecular ion peaks from the mass spectrum in Fig. 2C are listed and the possible theoretical combinations of phospholipid species that correspond to each $[M-H]^-$ ion are given

	Possible combinations of molecular species	
$[M-H]^-$ ions (m/z)	PI species	PS species
885.7	C18:0/C20:4 C16:0/C22:4	n.p.
834.8	n.p.	C18:0/C22:6 C18:1/C22:5 C18:2/C22:4
810.7	n.p.	C18:0/C20:4 C16:0/C22:4
788.8	n.p.	C18:0/C18:1
786.8	n.p.	C18:0/C18:2 C18:1/C18:1
762.7	n.p.	C16:0/C18:0

n.p.=Theoretically not possible.

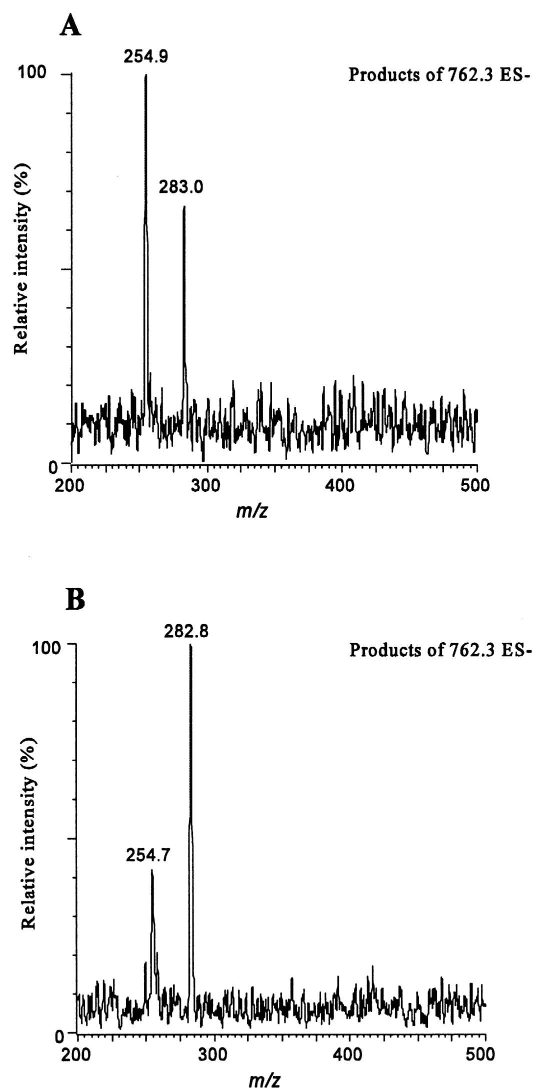

Fig. 3. Negative-ion HPLC–ES-MS–MS product ion spectra of the precursor PSPS related ion from (A) control sample with 0.58 μg/ml of PSPS and 2.0 μg/ml of DPPS; (B) extracted human blood sample.

acid moiety from either the *sn*-1 or *sn*-2 position [18].

Standard and endogenous (from extracted blood) PSPS were found to have the same retention times (Fig. 4) and in one analytical series the retention times were shown to be (in min); 13.02±0.02 (mean±S.D., $n=6$) and 13.02±0.02 (mean±S.D., $n=5$), respectively.

Human blood was also examined for the presence of the internal standard (DPPS). The full scan mass spectrum under the endogenous human blood PS peak did not show any molecular ion at m/z 734.7, i.e., the $[M-H]^-$ ion of DPPS (Fig. 2). However, HPLC analysis with the MS set to the scanning mode is not as sensitive as SIM and when extracted human blood was analysed with SIM at m/z 734.7, a small peak with the same retention time as synthetic DPPS was found (Fig. 4).

The possible species of PI and PS that are listed in Table 1 have not yet been confirmed by MS–MS experiments, except for m/z 762.7 (PSPS). Previously published reports, however, have shown that the major molecular ions of PS in human plasma are m/z 788 and 810, i.e., C18:0/C18:1 PS and C18:0/C20:4 PS, respectively [19]. Furthermore, the fatty acid profile of human erythrocyte PS has shown that the main fatty acids are C18:0 and C20:4 [20].

3.3. Quantitation

To specifically determine PSPS in biological matrixes it is important to have standards made from PSPS. We found in our system a slight response

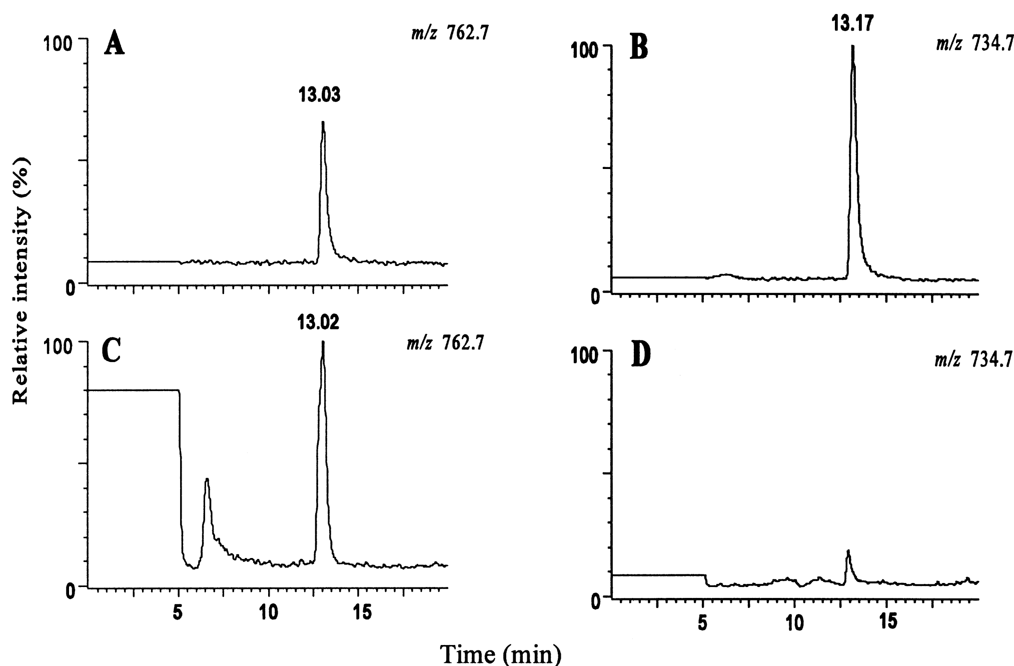

Fig. 4. Typical chromatograms after LC–MS analysis with the MS set to SIM of m/z 734.7 (DPPS) and m/z 762.7 (PSPS). (A, B) Control sample containing 0.58 μg/ml of PSPS and 2.0 μg/ml of DPPS; (C, D) extracted human blood with no internal standard added. The ordinates are shown with arbitrary units where (A) and (C) are comparable and (B) and (D) are comparable. Mobile phase as described in Section 2.4 and the gradient starting with 90% chloroform.

difference between closely related phosphatidylserine species, namely DSPS and DPPS. The area ratio between DSPS and DPPS was determined after analysing 2.0 μg/ml of each standard phospholipid on HPLC–ES-MS and found to be 1.16±0.07 (mean±S.D., $n=4$). This in contrast to earlier reports that found similar peak intensities with ES-MS between phospholipid species of the same molecular class and subclass [21,22]. This was, however, with direct infusion of the phospholipid mixture into the ES-MS system, i.e., without a previous chromatographic separation.

The standards containing different concentration of PSPS and one concentration of the internal standard, DPPS, prepared as described in Section 2.2, were analysed in duplicate in each analytical sequences and a calibration curve was made by plotting the peak area ratio of PSPS to DPPS against the theoretical concentration of PSPS. The curvature was estimated from the following equation; $y=a+bx^m$ and the parameter m was found to be; $m=0.91\pm0.037$ (mean±S.D., $n=6$). The calculated m value indicated that the calibration curve deviated slightly from linearity. It was therefore decided to fit the curvature to a second-order polynomial equation; $y=a+bx+cx^2$. A calibration curve fitted to this equation is shown in Fig. 5. The estimated parameters of the calibration curve from six series of analysis are listed in Table 2 and shown to be highly reproducible. The goodness of fit of the calibration points to the calibration curve was also estimated and the largest deviation from the theoretical concentration was 4.0% at the lowest standard (not shown).

3.4. Limit of quantification

According to published recommendations the limit of quantification (LOQ) of a method can be set to a specific concentration provided that the repeatability of analysing at this concentration is below 20% R.S.D. of the mean [23]. Based on the prevalidation work, the lowest standard at 0.12 μg/ml of PSPS

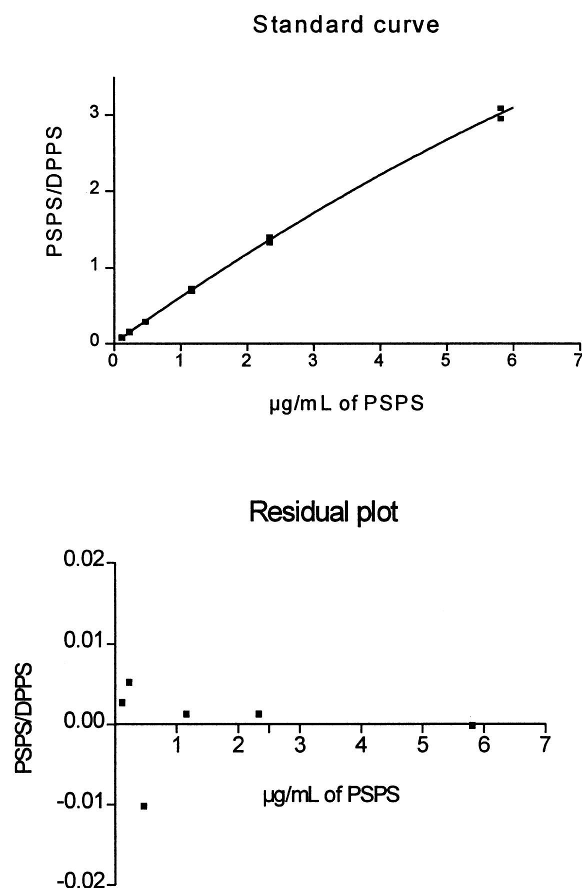

Fig. 5. Typical calibration curve fitted to the equation; $y=a+bx+cx^2$ and the residual plot of the curve. The ordinate in the two figures is the calculated peak area ratio of PSPS to DPPS. Two sample replicates were analysed for each standard and both values are given for the calibration curve and for the residuals it is the mean distance of the two points from the curve.

Table 2
Parameters of the calibration curve

Regression coefficient	Mean	R.S.D. (%)
a	0.0005±0.0104	n.a.[a]
b	0.61±0.024	3.9
c	−0.013±0.0053	40.8
r^2	0.9983±0.0009	0.09

[a] n.a.=Not applicable.
The standard curve was fitted to the equation; $y=a+bx+cx^2$. The standards were analysed in six series with two parallels for each standard. The values are the mean±S.D. ($n=6$).

(corresponding to 1.2 ng injected) was chosen as the LOQ of the method and the repeatability of analysing this was found to be 10.8% R.S.D. ($n=6$). However, as low as 0.5 ng of DPPS can be detected in the system.

3.5. The precision of the method

The precision of the method was calculated as described in Section 2.7. The between-day coefficient of variation of the control samples was found to be below 9.2% (Table 3), showing that the analysis of the control samples were reproducible. The within-day coefficient of variation was calculated as the pooled within-series standard deviation of the daily mean [$S.D._w(p)$] and found to be below 4.8%. (Table 3), showing that the daily precision of analysing the control samples was acceptable.

3.6. Repeatability and accuracy of estimating PSPS in human blood

The repeatability of estimating PSPS in blood was determined as described in Section 2.7 and found to be 2.9% and 8.3% R.S.D. in blood and in blood spiked with PSPS, respectively (Table 4). The actual amounts of PSPS estimated in blood and in blood spiked with PSPS were 1.49 and 3.06 µg/ml, respectively (Table 4). The difference between the two concentrations accounts for the net amount of PSPS added to blood, i.e., 1.57 µg/ml or 0. 47 µg PSPS in 0.3 ml of blood. The extraction efficiency at this concentration of PSPS in blood was therefore 97.9%. The results indicate that the small amount of internal standard (DPPS) present in human blood does not affect the quantitation of PSPS.

Theoretically, the amount of DPPS in human blood might show both individual variations and daily variations. The ratio of endogenous PSPS to endogenous DPPS in human blood was estimated in four subjects. The individual variation of the mean ratio was relatively small and found to be 28.4% R.S.D. ($n=4$). The actual ratio varied from 11.8 to 18.1 indicating that the amount of endogenous DPPS is small. The daily variation was also insignificant (not shown). The endogenous concentration of PSPS in blood from 13 subjects was estimated and Table 5

Table 3
Precision of the method

	Mean (μg/ml PSPS)	$S.D._x$	$S.D._w(p)$	$R.S.D._x$ (%)	$R.S.D._w(p)$ (%)
Control 1[a]	0.24	0.022	0.010	9.2	4.2
Control 2[b]	0.56	0.013	0.021	2.3	3.8
Control 3[c]	2.25	0.079	0.108	3.5	4.8

Theoretical concentration: [a] 0.23 μg/ml of PSPS; [b] 0.58 μg/ml of PSPS; [c] 2.33 μg/ml of PSPS.
Three control samples were analysed in duplicate in each of eight series. The following were calculated; the mean of the within-series means, the standard deviation ($S.D._x$) of these means and the pooled within-series standard deviation of the daily mean [$S.D._w(p)$].

Table 4
Repeatability and accuracy of estimating PSPS in blood

Samples	Concentration of PSPS (μg/ml of PSPS)	R.S.D. (%)
Blood	1.49±0.043	2.9
Blood added PSPS[a]	3.06±0.254	8.3

[a] 0.48 μg PSPS to 0.3 ml blood.
A 0.3-ml volume of blood was spiked with either 0 or 50 μl of a phospholipid mixture containing 0.48 μg PSPS and subsequently extracted and analysed. The values are the mean±S.D. (n=6).

Table 5
Concentration of PSPS in blood from 13 individuals

Subject	Concentration of PSPS (μg/ml) of PSPS
1	2.02/1.97
2	2.45/1.96
3	1.83/1.98
4	1.97/2.06
5	3.09/2.75
6	2.28/2.24
7	2.18/2.10
8	1.75/1.73
9	2.08/2.43
10	1.92/2.41
11	2.78/2.96
12	2.43/2.54
13	2.39/2.26

The blood from 13 individuals was measured with two sample replicates each. Both values are given.

shows some individual variations ranging from 1.73 to 3.09 μg/ml.

3.7. Sample stability

PSPS dissolved in the injection solution was found to be stable for at least 24 h at room temperature (not shown). PSPS in blood and extracted blood was found to be stable for at least 42 days at −70°C (Table 6). PSPS in blood was found to be stable for at least three freeze–thaw cycles (not shown).

4. Conclusions

PSPS was specifically determined in human blood by normal-phase HPLC coupled on-line with negative-ion ES-MS. Sequence of samples were run unattended over night and several hundred blood samples were analysed with seemingly little loss of response. A small but significant response difference was found between different phospholipid species of PS showing that for specific determination of PSPS in biological matrices, standards prepared with PSPS are necessary. The validated analytical method was found to be suitable for specific quantitation of PSPS in human blood.

Table 6
Stability of PSPS in blood and extracted blood samples stored at −70°C

Storage period (days)	Blood (%)	Blood added PSPS (%)	Extracted blood (%)	Extracted blood added PSPS (%)
0 (n=6)	100.0[a]±2.9	100.0[b]±8.3	100.0[a]±2.9	100.0[b]±8.3
7 (n=3)	111.4±4.5	101.6±6.7	98.0±4.0	92.5±7.0
42 (n=3)	112.8±5.5	106.2±7.0	116.1±7.8	103.9±11.6

Measured concentration of PSPS: [a] 1.49 μg/ml of PSPS; [b] 3.06 μg/ml of PSPS.
The values are the mean±S.D. (%) of the concentration measured at zero-time. The number of sample replicates is given in parentheses.

Acknowledgements

The expert technical assistance of Inger Oulie is appreciated.

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