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Short communication

Quantification of phosphatidylserine, phosphatidic acid and free fatty acids in an ultrasound contrast agent by normal-phase high-performance liquid chromatography with evaporative light scattering detection

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Abstract

Sonazoid™ is a new contrast agent for ultrasound imaging. The product is an aqueous suspension of perfluorobutane microbubbles coated with phospholipids obtained from hydrogenated egg phosphatidylserine (H-EPS). A normal-phase high-performance liquid chromatographic (HPLC) method with evaporative light scattering detection was developed for quantification of free fatty acids, phosphatidylserine and phosphatidic acid in H-EPS and Sonazoid™. Separation of the lipids was carried out on an HPLC diol column and a gradient of chloroform and methanol with 0.2% formic acid titrated to pH 7.5 with ammonia. The calibration standards contained stearic acid, distearoyl-phosphatidic acid (DSPA) and distearoyl-phosphatidylserine (DSPS) in the concentration range of 0.016–1.0 mg/ml (0.4–25 μg injected). The method was validated with a limit of quantification of the three lipids set to 0.4 μg (approximately 20–60 μM). The best fit of the three calibration curves were obtained when the logarithmic transformed theoretical lipid concentration was plotted against the logarithmic transformed area under the peak and fitted to a second order polynomial equation. Stearic acid, DSPA and DSPS were analysed with an intermediate precision ranging from 4.4% to 5.3% R.S.D. and they were extracted from an aqueous suspension with a recovery ranging from 103.3% to 113.3%. The sum of total phospholipid concentration determined in H-EPS ranged from 96.4% to 103.2% of the theoretical values. The lipids in the ultrasound product were quantitated with a repeatability ranging from 6.2% to 11.7% R.S.D.

Keywords: HPLC; Evaporative light scattering detector; Phosphatidylserine; Phosphatidic acid; Free fatty acids

1. Introduction

Phospholipids are the main structural and functional compounds of cellular membranes and due to their emulsifying properties they are used commercially in different type of products. Phospholipid vesicles, i.e. liposomes, have become important as drug delivery systems in general and as drug targeting applications in particular. Liposomes can be made from a variety of materials, which make them versatile as drug carrier systems.

With the increasing use of liposomes as drug carriers it is important to have proper analytical techniques to quantitate and characterise the composition of the liposomes and their raw material. Previous quantification of phospholipids has been obtained with thin-layer chromatography [1]. In recent years, application of high-performance liquid chromatography (HPLC) has become more important and numerous HPLC methods have been described for the separation of phospholipids (for review, see [2]). Separation of the different phos-

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pholipid classes is obtained with normal-phase chromatography with silica as the most frequently used stationary phase [3–7]. In addition, modified silica, particularly diol [8–12], cyanopropyl [13,14] and aminopropyl [15–17] have also been used. With respect to the mobile phase different solvent mixtures have been used; *n*-hexane–2-propanol–water/acids/bases [8,11,17], acetonitrile–methanol–water/acids/bases [11,15,16] or chloroform–methanol–ammonium hydroxide [4–6,12].

The major difficulty in phospholipid analysis has been the detection of the substance. Only phospholipids with unsaturated fatty acids (one or more double bonds) have some absorption in the low UV range, i.e. at or below 210 nm. The problem with UV detection is that the response depends on the degree of unsaturation of the phospholipid fatty acid chain and makes the choice of standards difficult. In addition, this detection method requires solvents with high spectral transparency. Other detection principles that may be used are fluorescence detection (by post-column formation of mixed micelles) [18], mass spectrometry (MS) [19,20], flame ionisation detection (FID) [21,22] and refractive index detection (RI) [23–25]. MS represents a very sensitive detection method that is very useful for qualitative or semiquantitative analyses of phospholipids; it is however, more complicated to use this detector for exact quantitative analyses of mixtures of phospholipids/lipids [19,20]. FID is not at present commercially available in combination with HPLC and RI is not compatible with gradient elution. Evaporative light scattering (ELS) detection, on the other hand, enables gradient elution and can be used with all volatile solvents as mobile phases. The ELS detector response is primarily caused by the mass of the analyte [26,27] and has a reasonable sensitivity as phospholipids have been reported to be quantitated down to 0.1 μg [5,28].

A new contrast agent for ultrasound imaging has been developed by GE Healthcare. This ultrasound product (Sonazoid™) is an aqueous suspension of perfluorobutane (PFB) microbubbles coated with phospholipids obtained from hydrogenated egg phosphatidylserine (H-EPS). The main phospholipids in H-EPS are phosphatidylserine (PS) and phosphatidic acid (PA).

We have earlier presented a normal-phase HPLC method using a narrow-bore diol column with mass spectrometric detection for specific quantification of one molecular species of PS from human blood, i.e. palmitoyl-stearoyl-phosphatidylserine [29]. The main objective of the present work was to develop a normal-phase HPLC method with ELS detection for the separation and quantification of both phospholipids and breakdown products of phospholipids in H-EPS and Sonazoid™. The separation of free fatty acids (FFA), PA and PS were optimised and the three lipid classes were quantitated using external standard curves. In addition, the lysoforms of PA and PS were separated from their parent phospholipid and determined qualitatively. The validation of the method is presented in this paper. To our knowledge this is the first paper describing a quantitative method for analysis of the lipid content of an ultrasound contrast agent. We show that by using this method it is possible to obtain good quantitative data of these lipid classes in a short runtime (20 min only) with only one species from each lipid class as standard. The method would be expected to be useful also for analysis of other lipid mixtures, e.g. liposomes.

2. Experimental

2.1. Materials

Sonazoid™ was from GE Healthcare Bio-Sciences, Oslo, Norway. Chloroform (stabilised with amylene (2-methyl-2-butene)) was either LiChrosolv grade from Merck or HiPersolv grade from BdH. Methanol was LiChrosolv grade from Merck. Formic acid (98–100%), ammonia (25%) and hydrochloric acid were pro analysis grade from Merck. Stearic acid and palmitic acid were from Sigma Chemical Company, St. Louis, MO, USA. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-distearoyl-*sn*-glycero-3-phosphate (DSPA), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] (DPPS), 1,2-distearoyl-*sn*-glycero-3-[phospho-L-serine] (DSPS), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid (lyso-PA), hydrogenated plant L-α-phosphatidylinositol (PI) were from Avanti Polar Lipids Inc., Alabaster, Alabama, USA. Monoacyl-*sn*-glycero-3-phospho-L-serine (lyso-PS) containing primarily octadecanoic acid, sphingomyelin (Sm), L-α-lysophosphatidylethanolamine palmitoyl (lyso-PE) and L-α-lysophosphatidylcholine palmitoyl (lyso-PC) were from Sigma Chemical Company. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were from Sygena Inc., Cambridge, MA, USA. The purity of the standard compounds was approximately 99%. Hydrogenated egg phosphatidylserine (H-EPS) was from NOF Corporation, Amagasaki-Shi, Hyogo, Japan.

2.2. Chromatographic conditions

The chromatographic method is based on a method developed in our laboratory for LC–MS analyses [29]. The chromatographic system consisted of a Spectra-Physics SP 8800 pump connected to a Spectra-Physics SP 8880 autosampler, equipped with a 100 μl sample loop (Rheodyne). A Sedex 55 ELS detector, SEDERE, Alfortville, France, was used for detection of the lipids. The lipids were separated on a LiChroCART, LiChrospher 100 Diol, 250 mm × 4 mm (5 μm) column (Merck) with a LiChrospher 100 Diol, 4 mm × 4 mm (5 μm) precolumn (Merck). The mobile phase consisted of chloroform (mobile phase A) and methanol with 0.2% (v/v) formic acid titrated to pH 7.5 (if not otherwise stated) with ammonia (mobile phase B). The lipids in the samples were separated by running a gradient 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. Total run time for each sample was set to 30 min and the flow rate was 1.3 ml/min. The analyses were performed at ambient temperature. The samples were kept at room temperature and 25 μl was injected for each analysis. One injection per vial was performed. The ELS detector drift-tube temperature was set to 40 °C, the nitrogen gas pressure was set to 2.0 bar and gain was set to 6 if not otherwise stated.

2.3. Preparation of standards

The calibration standards were prepared by dissolving approximately equal amounts of stearic acid, DSPA and DSPS

in chloroform/methanol (75:25, v/v) and further dilution of the standards to the following concentrations (in mg/ml): 0.016, 0.031, 0.063, 0.13, 0.25, 0.50 and 1.0 of stearic acid; 0.016, 0.032, 0.064, 0.13, 0.25, 0.51 and 1.0 of DSPA; and 0.016, 0.031, 0.062, 0.12, 0.25, 0.50 and 1.0 of DSPS. The calibration standards were then distributed to HPLC-vials, dried by evaporation under nitrogen and kept at −20 °C. Prior to analysis the standards were redissolved in chloroform/methanol/water (65:25:4, v/v/v).

The control samples containing stearic acid, DSPA, DSPS, lyso-PS and lyso-PA (approximately 0.2 mg/ml) and other standard lipid solutions were prepared similarly to the calibration standards.

2.4. Lipid extraction of drug product

Drug product samples were prepared by reconstitution of the ultrasound drug product in sterile water and dilution in sucrose (92 mg/ml). Immediately after reconstitution, the lipids in the product were extracted essentially as described by Jääskeläinen [16]. Briefly, 2 ml of reconstituted Sonazoid™ was transferred to a separation funnel. The lipids in the product were extracted by adding 7.5 ml of methanol/chloroform (2:1, v/v), mixed thoroughly, followed by 2.5 ml of 0.1 M hydrochloric acid and 2.5 ml of chloroform. The solution was mixed thoroughly before the lower chloroform phase was sampled. The extraction procedure was repeated once and the pooled chloroform phase was evaporated to dryness under a stream of nitrogen gas. The residues were dissolved in 500 μl of chloroform/methanol/water (65:25:4, v/v/v).

2.5. Sample analysis and validation parameters

The samples were analysed in sequences together with calibration standards, control samples and injection blanks. The calibration 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 three standard curves were evaluated from three calibration graphs prepared and run on three different days. Repeatability was evaluated by analysing six or seven sample replicates at medium and high concentration of stearic acid, DSPA and DSPS. Intermediate precision was evaluated by analysing three sample replicates of the control sample on three different days. The efficiency of the extraction was examined in samples prepared by dissolving a known concentration of DSPA and DSPS in a 10% (w/v) sucrose solution. The solution was lyophilised and stearic acid was added. The dry mixture was dissolved in 2 ml water prior to extraction and analysis as described.

The repeatability and accuracy of analysing lipids in H-EPS were evaluated by analysing three different batches of approximately 0.5 mg/ml of H-EPS dissolved in chloroform/methanol/water (65:25:4, v/v/v). The repeatability of analysing lipids in Sonazoid™ was evaluated by extracting 10 vials of lyophilised Sonazoid™ from 1 batch.

2.6. Data handling

PE Nelson ACCESS*CHROM GC/LC data sampling system, v. 1.9 was used for sampling and integration of the chromatograms. GraphPad™ Prism v. 2.0 was used for regression analysis and for calculating the sample concentration. Microsoft Excel, v. 5.0 was used for statistical calculation.

3. Results and discussion

3.1. Choice of calibration standards

H-EPS is composed of 85–90% (w/w) of PS and 10–15% (w/w) of PA. The fatty acid composition of the phospholipids (calculated as w/w) is approximately 30% of palmitic acid (C16:0), 60% of stearic acid (C18:0), 5% of arachidic acid (C20:0) and 5% of behenic acid (C22:0). The species distribution of PS and PA in H-EPS was determined by negative electrospray ionisation (ESI) tandem quadrupole mass spectrometry (MS/MS) essentially as previously described [30]. Briefly, negative ESI-MS of H-EPS yielded mass spectra, which displayed deprotonated molecules representing the various species of PS and PA in H-EPS. The identity confirmation of the species was obtained by MS/MS experiments were the deprotonated molecules were selected by MS1 and fragmented by collision-induced dissociation. The resulting product ion spectra displayed carboxylate anion fragments, which identified the PS and PA species in H-EPS. Calculated from the intensities of the deprotonated molecules, the MS data showed that the main molecular species of PS were palmitoyl-stearoyl-phosphatidylserine (PSPS), accounting for approximately 60% (w/w), and distearoyl-phosphatidylserine (DSPS), accounting for approximately 30% (w/w). Corresponding molecular species were also found in PA. For calibration it was decided to use pure lipid standards for each lipid class to represent this mixture, i.e. stearic acid, DSPA and DSPS.

3.2. Method development and optimisation of the mobile phase

Initially, mobile phase B 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 the mobile phase B was then adjusted to 7.5 with formic acid, which was more suitable for the column and also increased the ELS detector response for PA and PS. 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 7.5 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. With a pH below 7.5 in mobile phase B the fatty acid peak was split in two (not shown). This is probably due to a gradual change in polarity of the fatty acid due to a higher degree of protonation at lower pH. Mobile phase B at pH 7.5 was therefore routinely used for all analysis. Previously, we have used similar chromatographic conditions with the mobile

phase at pH 5.3 for quantification of PSPS in human blood using MS detection [29].

3.3. Optimisation of the detector temperature

The ELS detector response is dependent on the temperature of the drift-tube [31]. When using the present method the response of the lipids gradually decreased at a temperature increment from 40 to 90 °C. When the drift-tube temperature was increased from 40 to 60 °C, a two- to three-fold decrease in the response was observed. A further temperature increase of 10 °C led to an even more dramatically drop in response, as only approximately 5% of the response at 40 °C was observed at 70 °C. From 70 to 90 °C only small changes in the response was observed. Thus a drift-tube temperature of 40 °C was chosen. A similar drift-tube temperature has previously been reported for determination of phospholipids with HPLC and ELS detection [32,33], while others have reported an optimal drift-tube temperature of 85 °C [34]. The most favourable drift-tube temperature of the ELS detector for phospholipid determination will most probably vary depending on the make of the ELS detector.

3.4. Separation and specificity

Separation of up to nine lipid classes was achieved by the HPLC method (Fig. 1). As shown in Fig. 1A, PE and Sm co-eluted with mobile phase at pH 7.5, but it was possible to obtain some separation between the two lipids by reducing the pH of mobile phase B to 5.3 (Fig. 1B). When each phospholipid class was analysed separately, Sm eluted as two peaks (not shown) as reported by others [4–6,32]. Fig. 2A shows that FFA, PA and PS were well separated with a resolution factor between DSPS and DSPA of; $R_S = 2.94 \pm 0.03$ (mean ± S.D., $n = 3$). Fig. 2B shows that there was little difference in retention times when comparing the pure calibration standards with PA and PS from H-EPS. The two lysophospholipids, lyso-PS and lyso-PA, eluted essentially as one single peak (Fig. 2A). Since the lysophospholipids were only qualitatively determined, no attempt was made in improving the separation between lyso-PS and lyso-PA.

3.5. 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% relative standard deviation (R.S.D.) of the mean [35]. Based on the prevalidation work, the lowest calibration standard at 0.016 mg/ml (corresponding to 0.4 μg of lipid injected) was chosen as the LOQ of the method. Stearic acid and DSPS were analysed at 0.4 μg with a repeatability of 6.2% R.S.D. ($n = 7$) and 10.4% R.S.D. ($n = 7$), respectively. The response of DSPA was slightly poorer than the two other lipids and the LOQ for DSPA was therefore at first set to 0.8 μg, but with time DSPA was repeatedly detected at 0.4 μg with a repeatability ranging from 11.2% to 14.1% R.S.D. (three independent analyses with two sample replicates for each analysis). The lipids have successfully been detected at lower concentra-

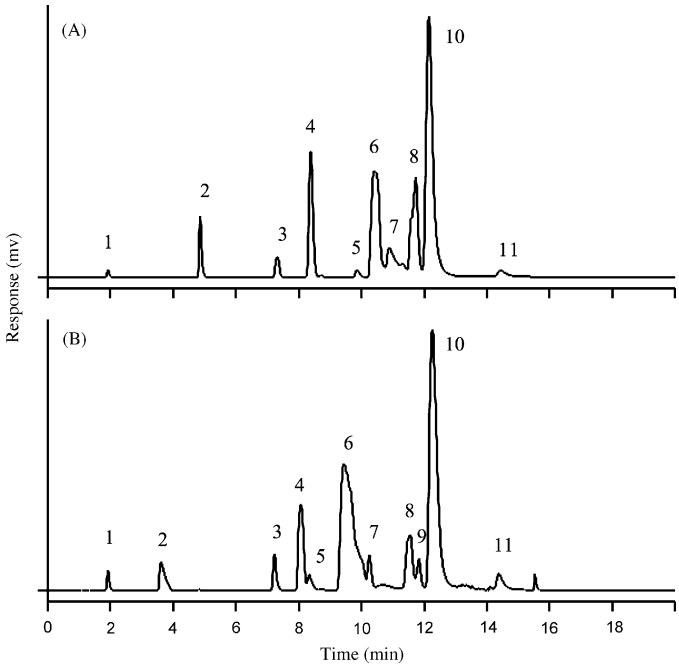

Fig. 1. (A) Typical chromatogram of a mixture of phospholipids analysed with mobile phase B at pH 7.5. Peaks: (1) unknown; (2) palmitic acid (0.25 mg/ml); (3) DSPC (0.13 mg/ml); (4) DSPE and Sm co-chromatographing (0.13 mg/ml of each); (5) lyso-PC (0.13 mg/ml); (6) DPPA (0.25 mg/ml); (7) lyso-PE (0.13 mg/ml); (8) PI and peak from chloroform co-chromatographing (0.13 mg/ml); (10) DPPS (0.25 mg/ml); (11) lyso-PS (0.22 mg/ml). (B) Typical chromatogram of a mixture of phospholipids analysed with mobile phase B at pH 5.3. Peaks: (1) unknown; (2) palmitic acid (0.25 mg/ml); (3) DSPC (0.13 mg/ml); (4) DSPE (0.13 mg/ml); (5) Sm (0.13 mg/ml); (6) DPPA and lyso-PC co-chromatographing (0.25 and 0.13 mg/ml); (7) lyso-PE (0.13 mg/ml); (8) PI (0.13 mg/ml); (9) peak from chloroform; (10) DPPS (0.25 mg/ml); (11) lyso-PS (0.22 mg/ml). Conditions are described in the text.

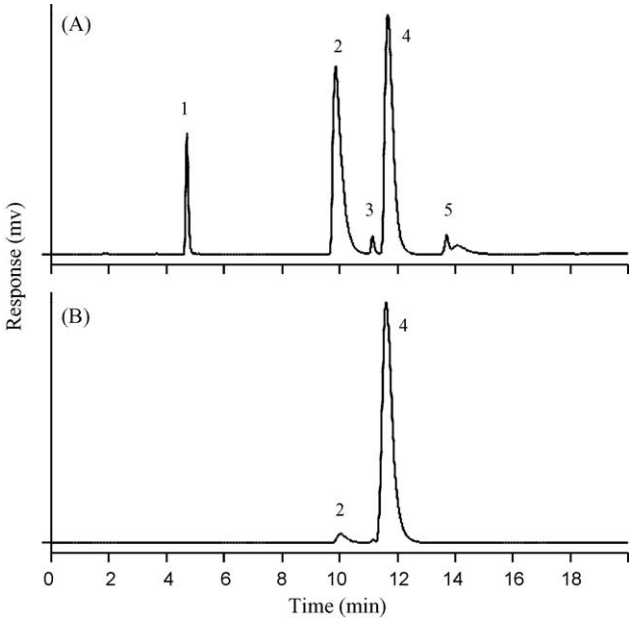

Fig. 2. (A) Typical chromatogram of a standard mixture of lipids. Peaks: (1) stearic acid (0.21 mg/ml); (2) DSPA (0.20 mg/ml); (3) peak from mobile phase; (4) DSPS (0.21 mg/ml); (5) lyso-PS (0.21 mg/ml) and lyso-PA (0.20 mg/ml). (B) Typical chromatogram of H-EPS (1.0 mg/ml). Peaks: (2) PA and (4) PS. Conditions are described in the text.

tions than 0.4 μg, e.g. stearic acid has been detected at 0.125 μg, but with varying response. Detection limits for different phospholipids after analysis with ELS detection have previously been reported to range between 0.25 and 0.5 μg [28,36] and even as low as 0.1 μg [5]. In addition, it has been found that neutral lipids elicit greater ELS responses than polar lipids [37].

The LOQ of an HPLC-method using the present type of ELS detector can be improved by increasing the ELS detector gain. Preliminary experiments showed that it was possible to increase the gain to 8–9 without a concomitant increase of the noise and thereby theoretically reducing the LOQ of the method (not shown). The LOQ of lyso-PS and lyso-PA was not determined but was approximately three times higher than for stearic acid, DSPA and DSPS (not shown). Generally, the detector response of the two lysophospholipids were much poorer compared to the other three lipids (Fig. 2).

3.6. Calibration

The concentration of the calibration standards ranged from 0.016 to 1.0 mg/ml for stearic acid and DSPS and, initially, from 0.032 to 1.0 mg/ml for DSPA. When the theoretical concentrations of the lipids were plotted against the calculated peak areas, the curve appearance was sigmoidal for DSPA and DSPS and exponential for stearic acid (Fig. 3A and B). Similar response curves have previously been reported for this type of detector [5,6,36,38]. This is believed to be due to the dependence of the light scattering mechanisms on the size of the particles formed by the non-volatile compounds and the particle size increases with increasing concentration of the compounds [27,39].

Due to the sigmoidal and exponential appearance of the three curves, the best fit of the calibration curves were obtained when the logarithmic transformed theoretical lipid concentration was plotted against the logarithmic transformed area under the peak. The linearity of a calibration curve can be described by the equation; $y = a + bx^m$ [40]. The m values for the three transformed curves of stearic acid, DSPA and DSPS were: $m = 1.25 \pm 0.20$ (mean ± S.D., $n = 3$), $m = 0.012 \pm 0.001$ (mean ± S.D., $n = 3$) and $m = 0.011 \pm 0.001$ (mean ± S.D., $n = 3$), respectively. In addition, an F-test to evaluate linear versus quadratic regression was performed. For DSPS and DSPA, the values from the F-test ranged from 116 to 2085, clearly showing that non-linear regression fits the data significantly better than linear regression. For stearic acid, the values from the F-test were 0.77, 9.9 and 32. Even if the results from the F-test are not conclusive, they indicate that non-linear regression fits the data better in two of three curves. In view of this it was decided to fit the three transformed calibration curves to a second-order polynomial equation: $y = a + bx + cx^2$ (Fig. 3C).

The estimated parameters of the calibration curves from three series of analysis are listed in Table 1 and shown to be reproducible. The reverse predicted standard points showed a deviation ranging from −3% to 6% of the nominal concentrations over the whole range of the three standard curves. This was reproducible in three analytical series (not shown) and shows that the calibration model chosen for the three curves gives a good fit of the standard points.

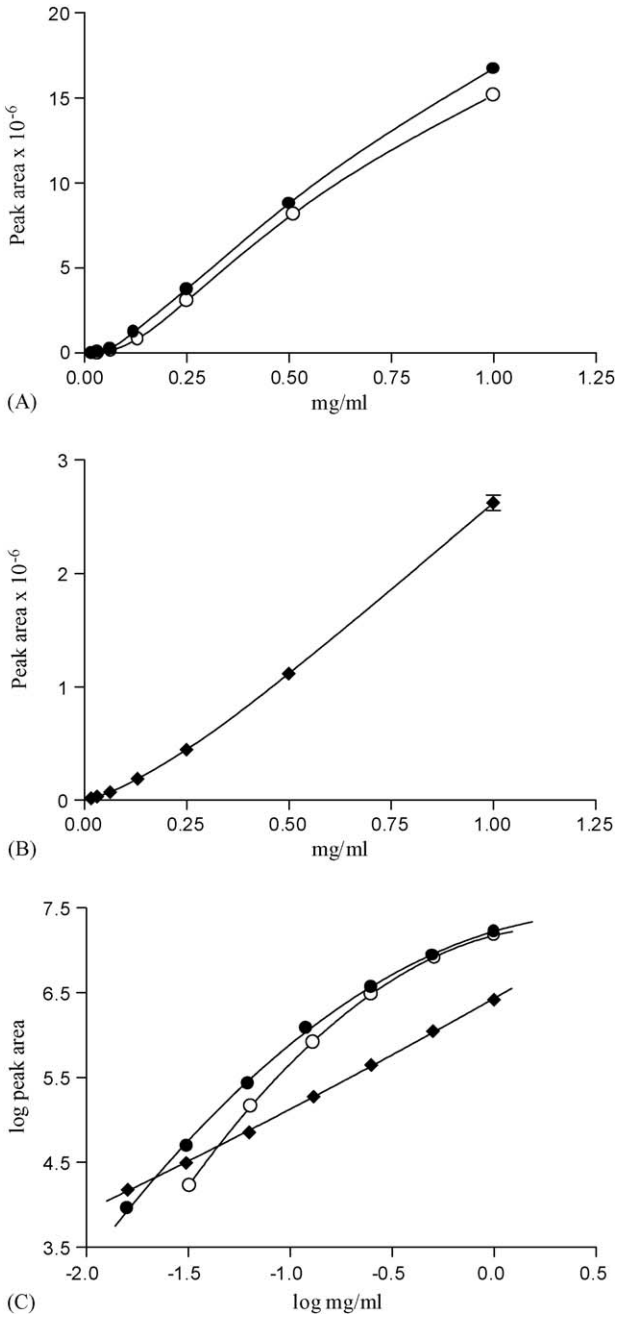

Fig. 3. Typical response curves where each point on the curves represent the mean of two parallels. (A) Response curves of DSPA (◯) and DSPS (●) and (B) response curve of stearic acid. The curves were fitted to a cubic spline equation. (C) Calibration curves of stearic acid (♦), DSPA (◯) and DSPS (●). The logarithmic transformed concentration of lipids were plotted against the logarithmic transformed area under the peak and fitted to a second order polynomial equation.

3.7. Repeatability, intermediate precision and accuracy

The repeatability and intermediate precision of the method were found to range from 0.9% to 3.3% R.S.D. and 4.4% to 5.3% R.S.D. (Table 2), respectively. The efficiency of extraction was determined as described in Section 2 and found to be (in % of theoretical concentration): 113.3 ± 5.8 (mean ± S.D., $n = 3$), 110.8 ± 4.1 (mean ± S.D., $n = 6$) and

Table 1
Parameters of the calibration curves

Calibration curves	a	b	c	r^2
Stearic acid	6.40 ± 0.03	1.32 ± 0.05	0.07 ± 0.05	0.9990 ± 0.0004
DSPA	7.16 ± 0.02	0.69 ± 0.16	−0.85 ± 0.08	0.9984 ± 0.0013
DSPS	7.19 ± 0.03	0.71 ± 0.02	−0.66 ± 0.06	0.9986 ± 0.0008

For the three standard curves the logarithmic transformed theoretical lipid concentration was plotted against the logarithmic transformed area under the peak and fitted to the equation: $y = a + bx + cx^2$. The regression parameters were estimated after analysing three calibration series with two parallels for each standard. The values are the mean ± S.D. ($n = 3$).

Table 2
The repeatability and intermediate precision of the method

	Stearic acid		DSPA		DSPS	
	Mean concentration (mg/ml)	R.S.D. (%)	Mean concentration (mg/ml)	R.S.D. (%)	Mean concentration (mg/ml)	R.S.D. (%)
Repeatability						
$n = 6$[a]	0.129 ± 0.003	2.0	0.133 ± 0.003	2.4	0.130 ± 0.003	2.3
$n = 7$[b]	0.982 ± 0.025	2.6	0.935 ± 0.008	0.9	0.979 ± 0.032	3.3
Intermediate precision						
$n = 9$[c]	0.226 ± 0.012	5.3	0.213 ± 0.011	5.0	0.235 ± 0.010	4.4

For the repeatability the values are expressed as the mean ± S.D. For the intermediate precision the values are expressed as the grand average of three series of analysis each with three sample replicates ± S.D.
[a] Theoretical concentrations: 0.126 mg/ml (stearic acid); 0.127 mg/ml (DSPA); 0.125 mg/ml (DSPS).
[b] Theoretical concentrations: 1.006 mg/ml (stearic acid); 1.016 mg/ml (DSPA); 0.998 mg/ml (DSPS).
[c] Theoretical concentrations: 0.21 mg/ml (stearic acid); 0.20 mg/ml (DSPA); 0.22 mg/ml (DSPS).

103.3 ± 2.1 (mean ± S.D., $n = 6$) for stearic acid, DSPA and DSPS, respectively.

3.8. Stability

The stability of stearic acid, DSPA and DSPS in chloroform/methanol/water (65:25:4, v/v/v) at both medium and high concentration, i.e. 0.13 and 1.0 mg/ml, respectively, was examined with the samples kept at room temperature. After 5 days, the mean percentage of the 0-time value at medium and high concentration for stearic acid, DSPA and DSPS were found to be ($n = 3$): 98.5 ± 4.5 and 101.9 ± 0.0; 101.4 ± 4.4 and 113.9 ± 6.2; 101.1 ± 0.0 and 102.9 ± 0.0, respectively. Thus these lipids were stable for at least 5 days at room temperature. Correspondingly, the stability of the phospholipids after extraction was also examined. Extracted DSPA and DSPS redissolved in chloroform/methanol/water (65:25:4, v/v/v) were found to be stable for at least 7 days at room temperature (not shown).

3.9. Repeatability and accuracy of quantitating lipids in H-EPS and Sonazoid™

Table 3 shows that the accuracy of estimating total phospholipids (PA and PS) in three H-EPS batches ranged from 96.4% to 103.2%. This indicates that DSPA and DSPS are useful calibration standards for quantification of PA and PS in H-EPS. Table 3 further shows that the repeatability of analysing PA and PS in H-EPS was below 2% R.S.D.

Table 3
Quantitation of free fatty acids (FFA), phosphatidic acid (PA) and phosphatidylserine (PS) in H-EPS and Sonazoid™

	FFA		PA		PS		Concentration as % of theoretical value
	Mean concentration (mg/ml)	R.S.D. (%)	Mean concentration (mg/ml)	R.S.D. (%)	Mean concentration (mg/ml)	R.S.D. (%)	
Batch of H-EPS							
1 ($n = 3$)[a]	<LOQ		0.075 ± 0.001	1.3	0.426 ± 0.005	1.2	96.4 ± 1.0
2 ($n = 3$)[b]	<LOQ		0.098 ± 0.001	1.0	0.358 ± 0.001	0.3	99.0 ± 0.2
3 ($n = 3$)[c]	<LOQ		0.095 ± 0.001	1.1	0.514 ± 0.006	1.2	103.2 ± 1.2
Sonazoid™							
$n = 10$	0.024 ± 0.0028	11.7	0.039 ± 0.0028	7.2	0.181 ± 0.011	6.2	

Three different batches of H-EPS were analysed. The values are the mean ± S.D. In addition, the sum of total phospholipid concentration as percentage (%) of theoretical value was calculated. Ten different glasses of Sonazoid™ from one batch were analysed. The values are the mean ± S.D. <LOQ = below limit of quantification.
[a] Theoretical concentration of H-EPS: 0.52 mg/ml.
[b] Theoretical concentration of H-EPS: 0.46 mg/ml.
[c] Theoretical concentration of H-EPS: 0.59 mg/ml.

The repeatability of lipid analysis in Sonazoid™ was found to be in the range of 6.2–11.7% R.S.D. (Table 3). Based on the previous precision data (Tables 2 and 3) these results indicate that in addition to the variations of the analysis there is also some variation in the glass to glass content of lipids in the Sonazoid™ product.

In conclusion, the present method was developed for quantification of FFA, PA and PS in H-EPS and in the ultrasound contrast agent Sonazoid™. The method has successfully been validated and the method provides a sufficiently sensitive, accurate and reproducible analytical procedure for analysis of these lipids in one run of 20 min with only one species from each lipid class as standard. The method is applicable for the analysis of lipid constituent in both H-EPS and Sonazoid™, and also for analysis of other lipid mixtures such as found in e.g. liposomes.

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