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Research Article

Simple Chromatographic Method for Simultaneous Analyses of Phosphatidylcholine, Lysophosphatidylcholine, and Free Fatty Acids

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Abstract. This study describes a simple chromatographic method for the simultaneous analyses of phosphatidylcholine (PC) and its hydrolytic degradation products: lysophosphatidylcholine (LPC) and free fatty acids (FFA). Quantitative determination of PC, LPC, and FFA is essential in order to assure safety and to accurately assess the shelf life of phospholipid-containing products. A single-run normal-phase high-performance liquid chromatography (HPLC) with evaporative light scattering detector has been developed. The method utilizes an Allsphere silica analytical column and a gradient elution with mobile phases consisting of chloroform: chloroform–methanol (70:30%, *v/v*) and chloroform–methanol–water–ammonia (45:45:9.5:0.5%, *v/v/v/v*). The method adequately resolves PC, LPC, and FFA within a run time of 25 min. The quantitative analysis of PC and LPC has been achieved with external standard method. The free fatty acids were analyzed as a group using linoleic acid as representative standard. Linear calibration curves were obtained for PC (1.64–16.3 μg, r^2=0.9991) and LPC (0.6–5.0 μg, r^2= 0.9966), while a logarithmic calibration curve was obtained for linoleic acid (1.1–5.8 μg, r^2=0.9967). The detection and quantification limits of LPC and FFA were 0.04 and 0.1 μg, respectively. As a means of validating the applicability of the assay to pharmaceutical products, PC liposome was subjected to alkaline hydrolytic degradation. Quantitative HPLC analysis showed that 97% of the total mass balance for PC could be accounted for in liposome formulation. The overall results show that the HPLC method could be a useful tool for chromatographic analysis, stability studies, and formulation characterization of phospholipid-based pharmaceuticals.

KEY WORDS: evaporative light scattering detection; free fatty acid; lysophosphatidylcholine; phosphatidylcholine.

INTRODUCTION

Phospholipids undergo oxidative and hydrolytic degradation reactions (Fig. 1) that limit the stability and useful shelf life of phospholipid-based pharmaceutical products such a liposomes, emulsions, and microemulsions (1–4). The hydrolytic pathway of phosphatidylcholine (PC) involves base-catalyzed breakdown of two carboxy ester bonds. Lysophosphatidylcholine (LPC) is produced as the first intermediate product along with a free fatty acid (FFA) (2). A subsequent hydrolytic event releases glycerophosphorylcholine and a second fatty acid (Fig. 1). The amounts of lysophospholipid and fatty acid generated are critical parameters for the suitability of pharmaceutical formulations. For example, the molecular organization within liposomes will shift from lamellar to micellar, with a concomitant change in drug release patterns (5,6) in proportion to the extent of phospholipid hydrolysis. Formation of substantial amounts of LPC has been reported to increase the size of the oil droplet, leading to coalescence and cracking of phospholipid-stabilized parenteral emulsions (7) and resulting in toxic effects (8–11).

The FDA has recently published a draft guidance for the pharmaceutical industry on establishing appropriate storage conditions for liposome drug products and recommends validating an accurate lipid composition assay with which to carry out stressed stability studies (12). Appropriate methods require both adequate resolution of the various molecular entities as well as quantification. A well-designed high-performance liquid chromatography (HPLC) technique exhibiting good resolution is the preferred method. For detection purposes, UV and fluorescence methods are often not suitable for the direct analyses of phospholipids as these molecules tend to lack adequate chromophores. Derivatization of the phospholipids have been proposed to enhance detectability and quantification, but at the cost of complicating development and validation (13–15). Chromophore-independent methods of detection such as refractive index (2), light scattering (3,16–18), mass spectrometry (19–21), and charged aerosol (22,23) have also been proposed.

Evaporative light scattering detection ("ELSD) (3,16–18) has been studied as a rapid and sensitive means for the direct detection of phospholipids without the need for derivatization. ELSD measures the intensity of light scattered

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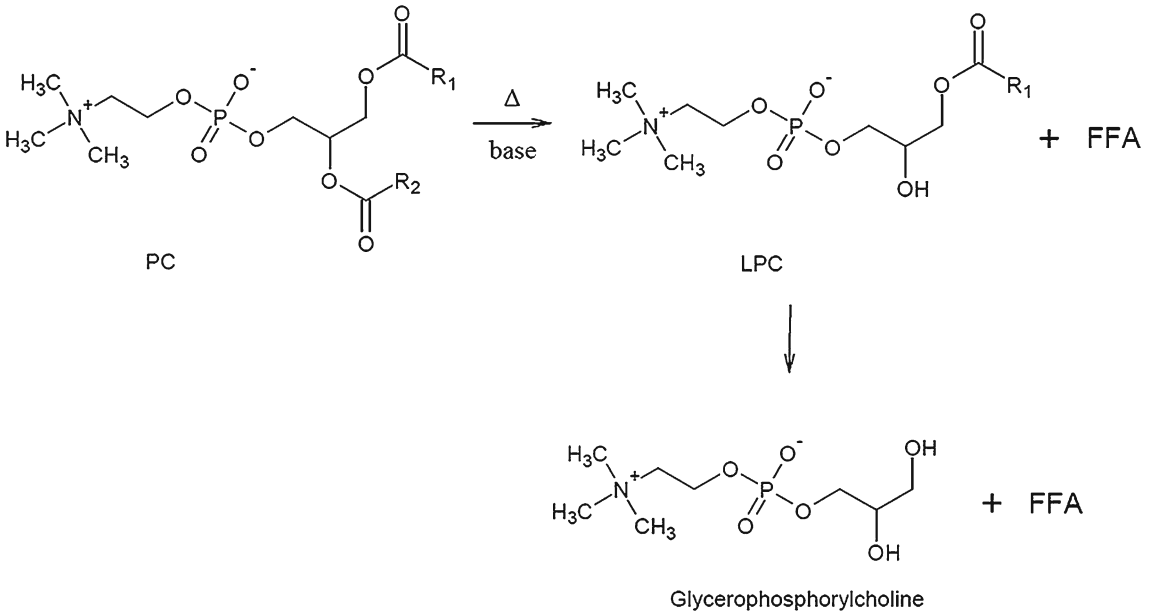

Fig. 1. Hydrolytic pathway of phosphatidylcholine (*PC*) into glycerophosphorylcholine and free fatty acids (*FFA*). Lysophosphatidylcholine (*LPC*) is produced as an intermediate product. LPC with R_2 is not shown in the figure. R_1 and R_2 are variable fatty acid chain on PC and LPC (Table II)

by analyte that remains after the solvent has been evaporated. Of particular advantage to HPLC-based studies is that ELSD allows gradient elution and is often not sensitive to solvent flow rate (24). In addition, ELSD detectors are often less expensive than mass spectrometric detectors. Lucena *et al.* (25) recently reviewed the instrumental innovations and new trends in the application of ELSD.

At present, there is no ELSD-based assay that may be applied to quantify simultaneously both phospholipids and degradation products LPC and FFA. The aim of this work was to develop a rapid and convenient HPLC-based method with evaporative light scattering detection suitable for the separation and quantification of soy L-α-PC and its degradation products: lysophosphatidylcholine and free fatty acids simultaneously in a single run. The proposed method avoids time-consuming and costly sample preparation steps prior to injection. As a means to validate the assay, a PC-based liposome suspension is subjected to hydrolytic conditions and the amounts of PC, LPC, and FFA determined. Mass balance is observed between these degradation products, and the original mass of PC verifying the assay is quantitative for all three components.

MATERIALS AND METHODS

Materials

L-α-phosphatidylcholine (soy, 95% purity) and L-α-lysophosphatidylcholine (soy, >99% purity) were purchased form Avanti Polar Lipids, Inc. (Alabaster, AL). Linoleic acid (99% purity) was purchased from Acros Organics (Morris Plains, NJ), and oleic acid (98%, purity) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Palmitic acid (92% purity) and stearic acid (min 40% purity; stearic acid + palmitic acid, 90% purity) were purchased from Spectrum Chemical (New Brunswick, NJ). HPLC-grade chloroform and HPLC-grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Analytical grade ammonium hydroxide (NF) was obtained from Spectrum Chemical. HPLC-grade water (specific resistance=18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Billerica, MA). All other reagents were of pharmaceutical grade and used as received.

Equipment

The chromatographic system included a Walters 2695 Separations Module equipped with an autosampler (Waters, Milford, MA) and a Sedex 85 low-temperature evaporative light scattering detector (SEDERE, France). The separations were performed on a 5-μm Allsphere Silica analytical column (150×4.6 mm i.d.; Alltech, Deerfield, IL). The signal was acquired and processed with Millennium^{32} software (Waters).

Methods

Preparation and Alkaline Hydrolysis of PC Liposome

Phosphatidylcholine liposome suspension was formed by the evaporation–hydration method. Exactly 0.224 g of PC was solubilized in 50 mL chloroform and transferred to a 250-mL round bottom flask. A thin lipid film was formed by evaporating the chloroform under nitrogen stream and then exposing the film to vacuum in a Rotary Evaporator (LABCONCO, Kansas City, MO) for 4 h to remove traces of chloroform from the film. The lipid film was then hydrated with 50 mL of 0.9% NaCl solution at 40°C. The concentration of PC in the liposome was found to be 4.48 mg/mL. The multilamellar vesicle suspension was then sonicated for 30 min to produce unilamellar liposomes. The pH of the liposome suspension was then adjusted to pH 9 or 10 using

0.1 N NaOH. The suspensions were autoclaved for 15 min at 121°C. Samples were stored at −20°C prior to analysis.

Preparation of the Standard and Sample Solutions

About 5 mg/mL standard stock solutions of PC, LPC, and linoleic acid were prepared in chloroform–methanol (50:50%, *v*/*v*), stored at −20°C, and protected from light. Working standard solutions in the range 82–816 μg/mL for PC, 30–250 μg/mL for LPC, and 55–290 μg/mL for linoleic acid were prepared by serial dilution from the respective stock solutions. Mixture of standards was prepared by mixing equal concentrations of PC, LPC, and four fatty acids: linoleic acid, oleic acid, stearic acid, and palmitic acid. FFA sample was prepared by mixing equal concentrations of linoleic acid, oleic acid, stearic acid, and palmitic acid. Sample solutions were prepared by diluting 0.5 mL of the liposome in 10 mL of the chloroform–water (50:50%, *v*/*v*) solvent system.

HPLC Analysis

The composition of the solvents and running program for the HPLC-ELSD method is summarized in Table I. Resolution of PC, LPC, and FFA was achieved within a total run time of 30 min, including 5 min for column re-equilibration using a flow rate of 1.0 mL/min. The column temperature was 25°C. The optimized ELSD conditions include: temperature of the drift tube 40°C, gain 6, and pressure of 3.2 bar. Ultra high purity nitrogen gas (Scott-Gross Co. Inc.) was used as nebulizing gas. The volume of the standards injected was 20 μL. The identification of each compound was performed by comparing the retention time with that of the corresponding peak in the standard. Calibration and validation of the system demonstrated that the HPLC method is capable of detecting PC, LPC, and FFA as linoleic acid at the level of about 0.1 μg. For the HPLC analysis of PC, LPC, and FFA, 50 μL of the sample solution was injected.

Validation of the HPLC-ELSD Method

The HPLC-ELSD analytical method was validated in terms of precision (repeatability and reproducibility), correlation coefficient of calibration curve, and limits of detection and quantification. A linear relationship between detector response and concentration was observed for PC and LPC. A logarithmic relationship for FFA was observed. In all cases, triplicate measurement of the standard solutions and the correlation coefficients were determined. Repeatability of the method was evaluated by repeated measurements of the standard solutions ($n = 6$). Reproducibility of the method was evaluated by estimation of the percent relative standard deviation (%RSD) of the peak area of the standards solutions. The %RSD obtained on three different days within a week determined the interday variability. The limits of detection (LOD) and limit of quantification (LOQ) values were estimated as: $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$, where σ is the standard deviation of the response (peak area) of the lowest standard concentration used and S is the sensitivity which is the slope of the calibration curve for the linear models (PC and LPC). For the log–log model (FFA), S was calculated from the lowest three concentrations used in the calibration curve.

Table I. Solvent Program Gradient

Time (min)	Mobile phases		
	A[a] (%)	B[b] (%)	C[c] (%)
0	100	0	0
5	0	100	0
10	0	0	100
16	0	0	100
21	0	100	0
25	100	0	0
30	100	0	0

[a] Chloroform
[b] Chloroform–methanol (70:30%, *v*/*v*)
[c] Chloroform–methanol–water–ammonium hydroxide (45:45:9.5:0.5%, *v*/*v*/*v*/*v*)

RESULTS AND DISCUSSION

HPLC-ELSD Method for the Determination of PC, LPC, and FFA

The HPLC method enabled baseline resolution of the L-α-PC and its degradation products: LPC and FFA in a single run. Chromatograms of the standards and typical baseline resolved spectra of the mixture of PC, LPC, and FFA are shown in Fig. 2. The peaks are symmetric and well resolved and their retention times show excellent reproducibility. In the case of FFA (Fig. 2c), individual fatty acids are not resolved, although the peak is symmetric and well resolved from PC and LPC. Several investigators have addressed the topic of HPLC analysis of fatty acids (26–28). Routine determinations of free fatty acids in pharmaceutical products often require the measurement of the total amount, not individual fatty acids (12). As shown in Fig. 2, a single symmetrical peak was obtained for the mixture of fatty acids, which indicates that the FFA could be determined using a representative standard. In this work, linoleic acid was used as representative standards as it is the major fatty acid component in PC and LPC (Table II). In order to validate this hypothesis, the response factors of linoleic acid and that of stearic acid were compared. After adjustment was made for the purity of the standards, the response factor (peak area/amount injected) of linoleic acid and stearic acid at concentration of 80 μg/mL (1.6 μg, amount injected) was found to be 77,334 and 76,346 mVs/μg, respectively, which is 98.7% similar.

Response Model

Calibration standards were diluted with chloroform–methanol (50:50%, *v*/*v*) to appropriate concentration, and triplicate injections were made from each solution. The PC was investigated between 1.64 and 16.32 μg mass injected. LPC and FFA were evaluated at lower mass injected (from 0.6 to 5.0 μg and from 1.1 to 5.8 μg, respectively) so as to enable the method to follow the degradation of PC at early stages of the reaction. To quantify the relationship between the amount injected and the ELSD response (peak area),

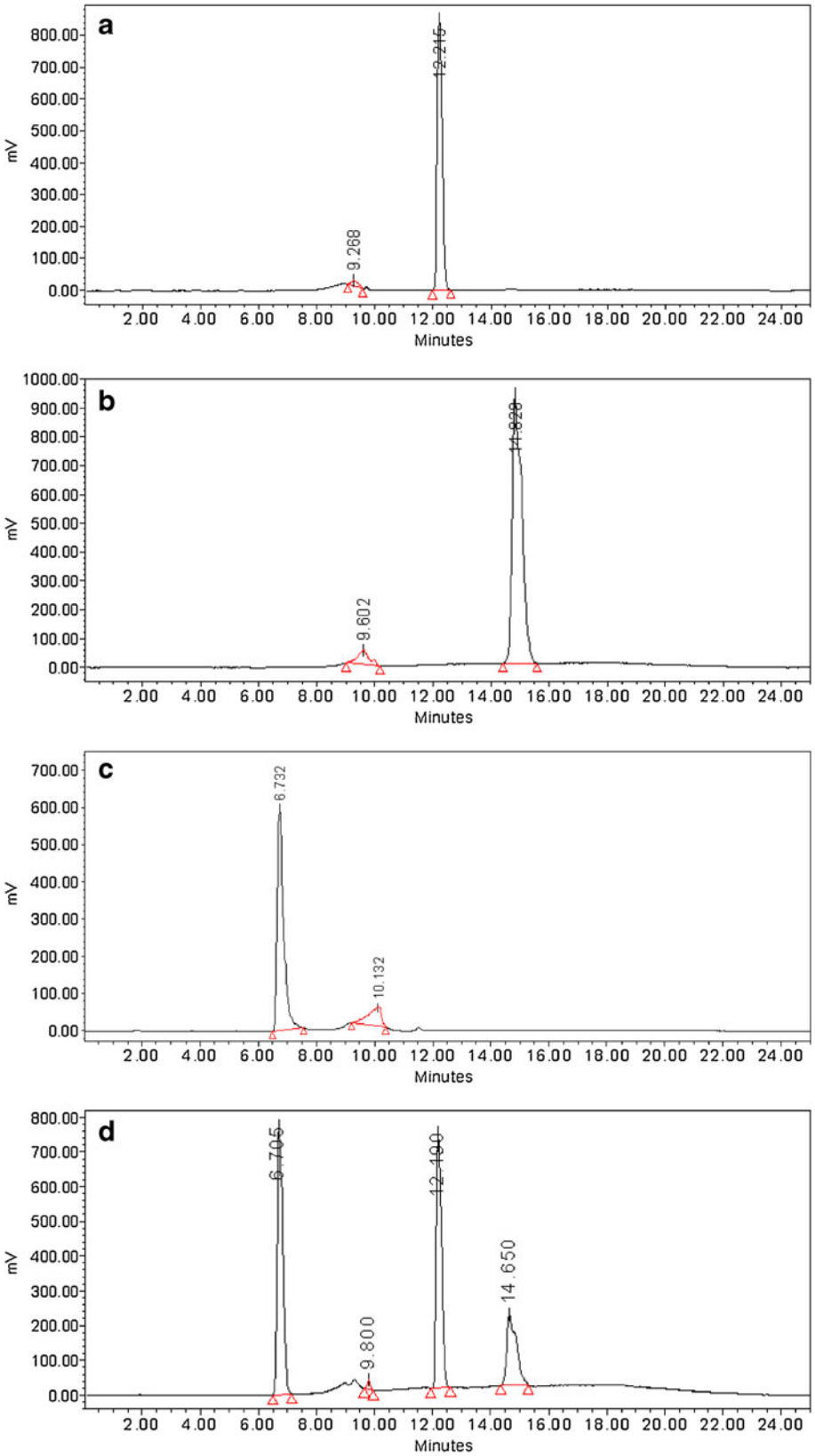

Fig. 2. Chromatographic separation of the standards on Allsphere Silica column with ELSD detection: **a** PC standard; **b** LPC standard; **c** equimolar mixture of linoleic acid, oleic acid, stearic acid, and palmitic acid; **d** mixture of standards (elution times: FFA mixture, 6.7 min; PC, 12.2 min; LPC, 14.7 min)

Table II. Fatty Acid Composition (%) of soy L-α-PC and Soy L-α-LPC

Fatty acid	Fatty acid composition (%)[a]	
	PC	LPC
Palmitic acid (16:0)	14.9	26.1
Stearic acid (18:0)	3.7	7.3
Oleic acid (18:1)	11.4	8.5
Linoleic acid (18:2)	63.0	50.1
Linolenic acid (18:3)	5.7	5.1
Unknown	1.2	2.9

[a] Data obtained from Avanti Polar Lipids, Inc.

Table IV. Retention Time Repeatability of Standards and Results of Sensitivity Study

Compound	Retention time (min)	%RSD[a]	S^b (mV/μg)	LOD[c] (μg)	LOQ[d] (μg)
PC	12.261	0.338	753,177	0.18	0.56
LPC	14.918	0.411	101,800	0.04	0.11
Linoleic acid	6.726	0.220	113,331	0.03	0.09

[a] Repeatability of the retention time
[b] Sensitivity
[c] Limits of detection
[d] Limits of quantification

linear and log–log models were evaluated (Table III). Several authors have established that the ELSD response is linear for a broad range of concentrations (29–31). Nevertheless, other authors have reported that the response of the ELSD detector more closely follows a log–log model (17,32,33).

Table III lists the results of fitting the ELSD response data of PC, LPC, and FFA to the linear and log–log models. PC and LPC showed a good linear relationship with correlation coefficients of 0.9991 and 0.9966, respectively, while the logarithmic relationship resulted in slightly lower correlation coefficients. Kang *et al.* (34) reported that the response of ELSD to PC was nonlinear in the mass range of 10–25 μg. Our findings showed that a linear relationship provides a better correlation coefficient in the range of 1.64–16.32 μg. On the other hand, linoleic acid showed a good logarithmic relationship with correlation coefficients of 0.9967. Moreover, when applying the linear model to linoleic acid, a large negative *y*-intercept value was observed.

Precision, Sensitivity, Limits of Detection, and Quantification

The repeatability of the method was evaluated on the basis of six independent runs with the same standard concentration. For PC at low injected mass (1.64 μg), the repeatability was found to be 4.2% RSD, and at higher injected amount, 0.75% RSD can be reached. A repeatability of <3% RSD and <4% RSD was obtained for LPC and linoleic acid, respectively.

Retention time repeatability and sensitivity data for PC, LPC, and FFA are summarized in Table IV. The IUPAC has defined the sensitivity of a linear detector to be the slope of the calibration curve (35). For the fatty acid, where the calibration curve is nonlinear, the sensitivity varies with the analyte amount. As sensitivity is more critical at lower concentrations, the lowest three concentrations of the calibration curve were used to calculate sensitivity. The LOD and LOQ values for PC were estimated to be 0.18 and 0.56 μg, respectively, whereas the LOD and LOQ for LPC were found to be 0.04 and 0.11 μg, respectively.

Proof of Concept: Application of the Assay to the Alkaline Hydrolysis of PC Liposomes

We illustrate the utility and robustness of the assay by applying it to the study of the alkaline hydrolysis of PC in a liposome suspension. Our goal is to validate that the assay is capable of simultaneously quantifying PC, LPC, and FFA in a model system. The success of the assay is to be based on the mass balance of PC before exposure to hydrolytic conditions with the masses of PC, LPC, and FFA after exposure.

Figure 3 depicts typical HPLC-ELSD chromatograms of the PC liposome suspension at a pH of 4.8 (Fig. 3a) and after stressed hydrolysis of PC at elevated pH and high temperature of steam sterilization (Fig. 3b). As shown in the figures, the peak areas of FFA and LPC increased after pH adjustment and thermal treatment of PC that resulted in hydrolytic degradation.

The alkaline hydrolysis of phosphatidylcholine initially yields lysophosphatidylcholine and a single fatty acid, as shown in Fig. 1. The LPC may further hydrolyze, producing a second fatty acid and glycerophosphorylcholine. These reactions are expressed in Eqs. 1 and 2.

$$\text{PC} \longrightarrow \text{LPC} + \text{FFA} \quad (1)$$

$$\text{LPC} \longrightarrow \text{FFA} + \text{Glycerophosphorylcholine} \quad (2)$$

Table III. Results of Fitting of Response Models for PC, LPC, and FFA Data

Compound	Range[a] (μg)	Linear model[b] ($y = ax + b$)			Logarithmic model[b] $\log y = a \log x + \log b$		
		a	b	r^2	a	$\log b$	r^2
PC	1.64–16.32	753,177	237,145	0.9991	1.067	5.793	0.9974
LPC	0.60–5.00	101,800	3,829	0.9966	1.029	4.9875	0.9948
Linoleic acid	1.10–5.80	159,789	−139,185	0.9893	1.518	4.766	0.9967

[a] Six individual calibration concentrations employed in each range
[b] y = response, peak area; x = mass injected

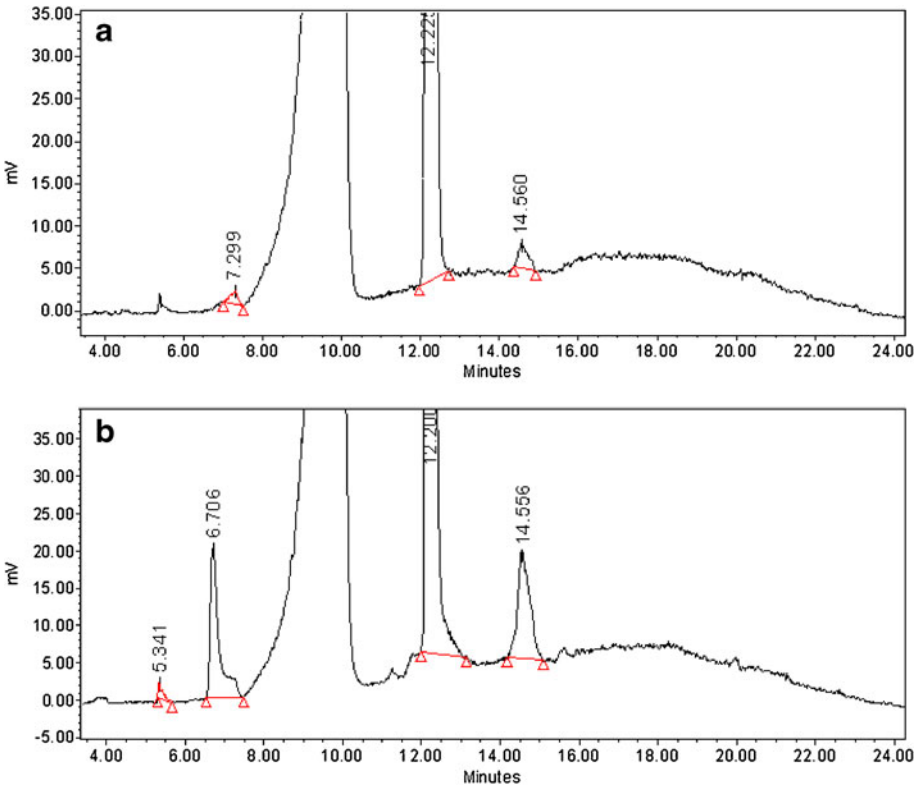

Fig. 3. Typical HPLC-ELSD chromatography of the PC liposome: **a** pH 4.8 and before sterilization; **b** pH was adjusted to 10 with 0.1 N NaOH and steam-sterilized (121°C for 15 min; elution times: FFA, 6.7 min; PC, 12.2 min; LPC, 14.6 min). High magnification was used to illustrate the peaks at 6.7 and 14.6 min (note the *y*-axis). The peak at 8.5–10 min was observed in the standards (Fig. 2) as well as in blank run

Based on Eqs. 1 and 2, the amount of LPC and FFA assayed can be defined by Eqs. 3 and 4, respectively.

$$\mathrm{LPC}_{assayed} = \mathrm{LPC}_{formed\ from\ PC} - \mathrm{LPC}_{hydrolyzed\ to\ FFA} \quad (3)$$

$$\mathrm{FFA}_{assayed} = \mathrm{FFA}_{formed\ from\ PC} + \mathrm{FFA}_{formed\ from\ LPC} \quad (4)$$

From stoichiometry, the number of moles of LPC hydrolyzed to FFA is equal to the number of moles of FFA formed from LPC (χ) in both Eqs. 3 and 4. Using the values of χ into Eq. 1, the amount of PC hydrolyzed could be calculated according to Eqs. 5a and 5b.

$$\mathrm{PC}_{hydrolyzed} = \mathrm{LPC}_{assayed} + \chi \quad (5a)$$

$$= \mathrm{FFA}_{assayed} - \chi \quad (5b)$$

Focusing on Eq. 5a, the mass balance of PC could be calculated from the assay results for PC, LPC, and FFA (via χ) according to Eq. 6.

$$\text{Mass balance}\ (\%) = [(\mathrm{PC}_{assayed} + \mathrm{LPC}_{assayed} + \chi)/\mathrm{PC}_{assayed\_pH4.8}] \times 100 \quad (6)$$

Table V. Alkaline Hydrolysis of PC Liposome Suspensions

	Assay results[a]					
pH	PC (mol, RSD)	LPC (mol, RSD)	FFA (mol, RSD)	Calculated χ[b]	Mass balance[c] (%, RSD)	Propagation error[d]
4.8[e]	1.48×10^{-8} (1.35)	1.17×10^{-10}	–	–	–	
9.0	9.29×10^{-9} (3.25)	4.19×10^{-9} (9.24)	6.03×10^{-9} (4.16)	9.11×10^{-10}	97.00 (±7.91)	5.40×10^{-19}
10.0	6.57×10^{-9} (0.89)	5.94×10^{-9} (2.98)	1.02×10^{-8} (4.67)	2.13×10^{-9}	98.65 (± 9.20)	3.65×10^{-19}

[a] Moles per volume of injection (50 μL); mean of three determinations; (SD)
[b] Amount of LPC hydrolyzed to FFA, calculated from Eqs. 5a and 5b
[c] Mass balance was determined based on Eq. 6 and RSD was calculated from the square root of the propagation error
[d] Calculated according to Eq. 7 (N = at least 3)
[e] PC liposome sample not autoclaved

As determination of the mass balance was based on the measurement of three variables, propagation of error of the total assay ($S_{\text{Total Assay}}^2$) was calculated using Eq. 7.

$$S_{Total\ Assay}^2 = S_{PC}^2 + S_{LPC}^2 + 2\text{Cov}_{(LPC,\ PC)} + S_{FFA}^2 + 2\text{Cov}_{(FFA,\ LPC)} \quad (7)$$

S_{PC}^2, S_{LPC}^2, and S_{FFA}^2 are the experimental variances for PC, LPC, and FFA, respectively. The covariance terms ($\text{Cov}_{(\text{LPC, PC})}$ and $\text{Cov}_{(\text{FFA, LPC})}$) were added in order to account for the correlation between the values of PC and LPC as well as LPC and FFA via χ (36).

Table V shows the result for the hydrolysis of PC liposome at pH 9 and 10. Mass balance of 97.0±7.9% and 98.7±9.2% was observed at pH 9 and 10, respectively. Based on the PC liposome model, a mass balance of PC, LPC, and FFA of 100% (within error) was observed before and after alkaline hydrolysis of the liposome suspension. This result provides a strong support that the proposed method for simultaneous analyses of PC and its degradation products is valid for a model liposome system. Additional work will be necessary to adapt the proposed method to other lipid-based systems.

CONCLUSION

A suitable HPLC method using ELSD has been developed for a direct and simultaneous determination of phosphatidylcholine and its degradation products, lysophosphatidylcholine and free fatty acids, in a single run. The method is sensitive and quantitative analyses showed good repeatability. It has been shown to be accurate for all major lipid products in a model liposome suspension subjected to high pH and sterilizing temperatures. The simplicity of the assay, the absence of baseline drift, and the short analysis time make the method suitable for application to various phospholipid-based liposome products.

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