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Quantitation and characterization of phospholipids in pharmaceutical formulations by liquid chromatography–mass spectrometry

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Abstract

A simple and fast method for phospholipid analysis was developed using high-performance liquid chromatography–mass spectrometry with an atmospheric pressure ionization interface. Separation of the phospholipid molecular species was achieved using a linear gradient of a mixture of chloroform–10 m*M* ammonium acetate–methanol (30:5:65) on a silica column. Optimization of the mass spectrometer conditions has allowed the method to separate and detect the phospholipids mainly as protonated molecular species. In comparison to existing LC–MS methods, improvement in the total analysis time and sensitivity were achieved. Separation of all major phospholipid molecular classes was achieved in less than 6 min. Marked improvement was observed in the linearity of the response of the phospholipids studied providing a linear response over three orders of magnitude. Data supporting the validation of this method for the characterization of major phospholipids molecular species are also presented.

Keywords: Pharmaceutical analysis; Phospholipids; Lipids

1. Introduction

Phospholipids are major constituents of membranes in plants, animals and microorganisms and are important constituents of the lipid bilayer of cell membrane. Natural phospholipids can be divided into two main groups, glycerophospholipids and sphingophospholipids; glycerophospholipids are derived from glycerol while sphingophospholipids are derived from sphingosine. However, most of the phospholipids used in the preparation of phospholipid-based pharmaceutical formulations and nutritional supplements are glycerophospholipids. The structures of glycerophospholipids consist of three parts: a glycerol backbone, a polar head group and two fatty acid chains esterified at the *sn*-1 and *sn*-2 positions.

Phospholipids have been given increased attention in pharmaceutical drug delivery systems. Formulation of drugs through the use of liposomes, i.e., phospholipid bilayer vesicles is an example of one of the approaches utilized. Although liposomes are more suited for water-soluble drugs they can also be used in very limited cases to deliver lipophilic drugs [1,2]. Several phospholipid-based formulations, including Intralipid (a lipid emulsion nutritional supplement), Diprivan (a lipid emulsion pharmaceutical formulation of propofol), and Daunoxome and Ambisome (liposomal formulations of daunorubicin and amphotericin B), are currently marketed. The

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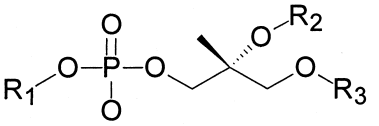
phospholipids primarily used are pure synthetic or lipids derived from egg or soybean, and typically contain phospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE) with minor amounts of lysophospholipids. Among the new technologies is RTP Pharma's IDD (Insoluble drug delivery) drug delivery systems [3–5]. This technology is used mainly for formulating water-insoluble drugs. It involves the formation of micron or submicron size particles of a solid or oily drug through the use of phospholipid surface modifying and stabilizing agents. Accordingly, methods for accurate identification of phospholipid classes and molecular species will become increasingly important.

The various quantitative methods for the analysis of molecular species of phospholipids have evolved over the last 20 years, becoming increasingly sophisticated and diverse. The method of choice is influenced by different factors such as the amount of material available for analysis, the existing equipment and available expertise within the laboratory. A variety of methods using thin-layer chromatography (TLC) [6–9] or high-performance liquid chromatography (HPLC) [10–23] are now available for quantitative analysis of phospholipids with a variety of detection systems. HPLC separation using both normal [10,11,13,15] and reversed-phase [16,18,19] chromatography have been proposed for the separation of phospholipids. Bonanno et al. [17] have used an on-line combination of silica/reversed-phase HPLC system to determine the phospholipids content from pulmonary surfactant. Detection of phospholipids has been performed by different spectrophotometric techniques such as UV. However with this technique, serious constraints are imposed on the mobile phase selection since underivatized phospholipids absorb near 200 nm with a low extinction coefficient [10,11,16,20]. A novel derivatization approach was proposed to increase the UV sensitivity of phospholipid analysis by Rastegar et al. [19] using naproxen chloride. Although interesting, this approach is labor intensive and is not suitable for routine and high throughput analysis. Alternatively, phospholipids can be analyzed by HPLC with evaporative light-scattering detection (ELSD) [13–15,21–23]. Although this technique is compatible with gradient elution and permits quantitation of phospholipids, the poor selectivity of this detector implies that the identification of the different phospholipids present must be made by retention behavior in comparison to known standards.

The possibility of coupling HPLC on-line with mass spectrometry (MS) has opened new possibilities in the separation and structural analysis of intact phospholipids. Several methods have been reported using different techniques including thermo-



General structure

R_1:	$CH_2-CH_2-NH_3^+$	Egg Phosphatidylethanolamine (PE)
R_2:	Oleic acid chain	
R_3:	Palmitic acid chain	
R_1:	$CH_2-CH_2-N^+(CH_3)_3$	Egg Phosphatidylcholine (PC)
R_2:	Oleic acid chain	
R_3:	Palmitic acid chain	
R_1:	$CH_2-CH_2-N^+(CH_3)_3$	Egg Lyso-phosphatidylcholine (Lyso-PC)
R_2:	H	
R_3:	Palmitic acid chain	

Fig. 1. Chemical structures of the phospholipids studied.

spray/plasmaspray [24,25], electrospray [26,27], particle beam [28] and atmospheric pressure chemical ionization (APCI) [26].

Although these methods allow good separation and identification of the major phospholipids, total analysis time is significant. For example, Careri et al. [29] reported a total analysis time of 12 min for the characterization of PE, phosphatidylinositol (PI) and PC. In order to quickly characterize the phospholipid content used in drug delivery systems, we have developed a rapid and sensitive method for the characterization of PE, PC and lyso-phosphatidylcholine (Lyso-PC). Fig. 1 gives the chemical structures of the phospholipids studied. The main objective of the method described in this communication is to enable quick assessment of the stability of the phospholipids during the formulation development stage. Furthermore, the method developed must be stability indicating in terms of the phospholipids content, detecting degradation of the phospholipids to lyso-phospholipids. The present details the development of an HPLC–MS method for the quantitation and characterization of phospholipids.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol (MeOH) and chloroform were purchased from J.T Baker (Phillipsburg, NJ, USA). HPLC-grade ammonium acetate was purchased from Sigma (Ontario, Canada). L-α-Phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE) and L-α-lyso-phosphatidylcholine (Lyso-PC) derived from egg yolk were purchased from Sigma. Glass distilled water (>18 MΩ), available in the laboratory was filtered prior to use through a 0.2-μm filter. The following reference phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA): 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE).

2.2. Chromatographic system and conditions

HPLC–MS analysis was carried out using an HP 1100 (Agilent Technologies, Canada) system composed of the following units: a solvent delivery module, an automatic sample injector (100-well capacity), a controller module, a column oven, and a model series 1100 MSD mass spectrometer. The analysis was performed on an Hypersil Silica column 10.0 cm×0.46 cm I.D., 5 μm particle size (Agilent Technologies), maintained at 25°C. The mobile phase consisted of a linear gradient as described in Table 1. The ammonium acetate was prepared using deionized water. The mobile phase was filtered through a 0.45-μm polypropylene filter and degassed under vacuum. The mobile phase was pumped at 1.0 ml/min.

2.3. Mass spectrometric conditions

MS was carried out on an HP 1100 mass spectrometer (Agilent Technologies) equipped with an atmospheric chemical ionization source used in the

Table 1
Linear gradient composition

	Time (min)	Flow-rate (ml/min)	Channel A (%)	Channel B (%)	Channel C (%)	Curve
Sample	0.0	1.0	30	5	65	Linear
	1.0	1.0	30	5	65	Linear
Analysis	1.1	1.0	30	15	55	Linear
	5.0	1.0	30	15	55	Linear
	5.1	1.0	30	5	65	Linear
Re-equilibration	6.0	1.0	30	5	65	Linear

positive ionization mode. Data handling was performed with Chemstation V. A.06.03 data handling system. Full scan spectrum were obtained by scanning masses between m/z 200 and 800. The vaporizer temperature was held at 450°C. Nitrogen was used both as drying gas and nebulizing gas at flowrates of 3.0 l/h and 20 p.s.i., respectively (1 p.s.i.= 6894.76 Pa). The temperature of the drying gas was set to 350°C. Quantitative analysis of different phospholipids were conducted in single-ion monitoring (SIM) mode scanning the quasi-molecular ions $[M+H]^+$ with a dwell time of 100 ms.

2.4. Sample preparation

Samples were prepared by diluting the different drug formulations (containing phospholipids, a drug and other excipients) to approximately solutions of 100 μg/ml with methanol.

3. Results and discussion

3.1. Atmospheric pressure ionization (API) mass spectra of phospholipids

Initially standard solutions of phospholipids were prepared in order to study their mass spectra profiles. Fig. 2A–D shows typical spectra obtained for various phospholipids (DPPE, DPPC, DMPE and DMPC) the major constituents of egg and soybean based phospholipids using the APCI technique in the positive ion mode. Analysis were performed using a fragmentor voltage of 80 V. Results obtained indicate the presence of the protonated $[M+H]^+$ and the diglyceride ions resulting from the loss of phosphate ester head group for both PE and PC. Positive ion APCI of the lipid classes studied here showed fragmentation patterns similar for both phosphatidylcholines and phosphatidylethanolamines.

The mass spectra obtained showed that for the PC and the PE phospholipids, the molecular ion and the diglyceride fragment were the major peaks observed. Under these conditions, the abundance of the molecular ions was favored over the diglyceride fragments. Results obtained for lyso-phosphatidylcholine obtained from egg yolk (Fig. 2E), showed that no molecular ion at m/z 496 was observed under these conditions. Ions obtained at 393.2 and 313.2 correspond, respectively, to ions resulting from the various losses at the phosphate ester head group position. The egg lyso-phosphatidylcholine used in these experiments is a mixture containing primarily palmitic or stearic acids in position 3 (Fig. 1). The mass spectrum (Fig. 2E) reflects the presence of both acids, since fragments corresponding to different mass losses were obtained with a mass difference of 28 mass units corresponding to the mass difference between the two fatty acids (421.3−393.2 and 341.2−313.2).

The abundance of these respective ions is affected by the fragmentor voltage of the instrument. The fragmentor voltage affects the intensity of the molecular ions and can induce formation of diglyceride fragments. In order to determine the origin of this effect, optimization of the fragmentor voltage used for the characterization of the different phospholipids was performed. Fig. 3 gives the intensity of molecular ions and diglyceride fragments for DMPE and DPPE as a function of the fragmentor voltage. Results obtained indicate that for voltages lower than 100 V, the molecular ion was the dominant ion. However for voltages higher than 120 V, the diglyceride fragment becomes the dominant peak in the mass spectrum obtained. Results obtained for other phospholipids studied were similar and showed that the production of the diglyceride fragments were less significant when fragmentor voltage lower than 100 V was applied. Fig. 4 represents mass spectrum obtained for DPPE for two different voltages, 100 V and 160 V. The spectrum obtained at 100 V (Fig. 4) shows that the protonated molecular species were near their maximum at this fragmentor voltage. However, the spectrum obtained with a much higher fragmentor voltage, 160 V, showed substantial fragmentation as indicated by the presence of dominant diglyceride ion at m/z 551.5.

3.2. LC–MS separation

Despite the many HPLC–MS methods previously reported for phospholipid analysis, few methods can resolve molecular species of phospholipids in a reasonable time. Typical analysis time reported is about 15 to 20 min [26,27]. The objective of this work was to develop a fast, reliable, stability indicat-

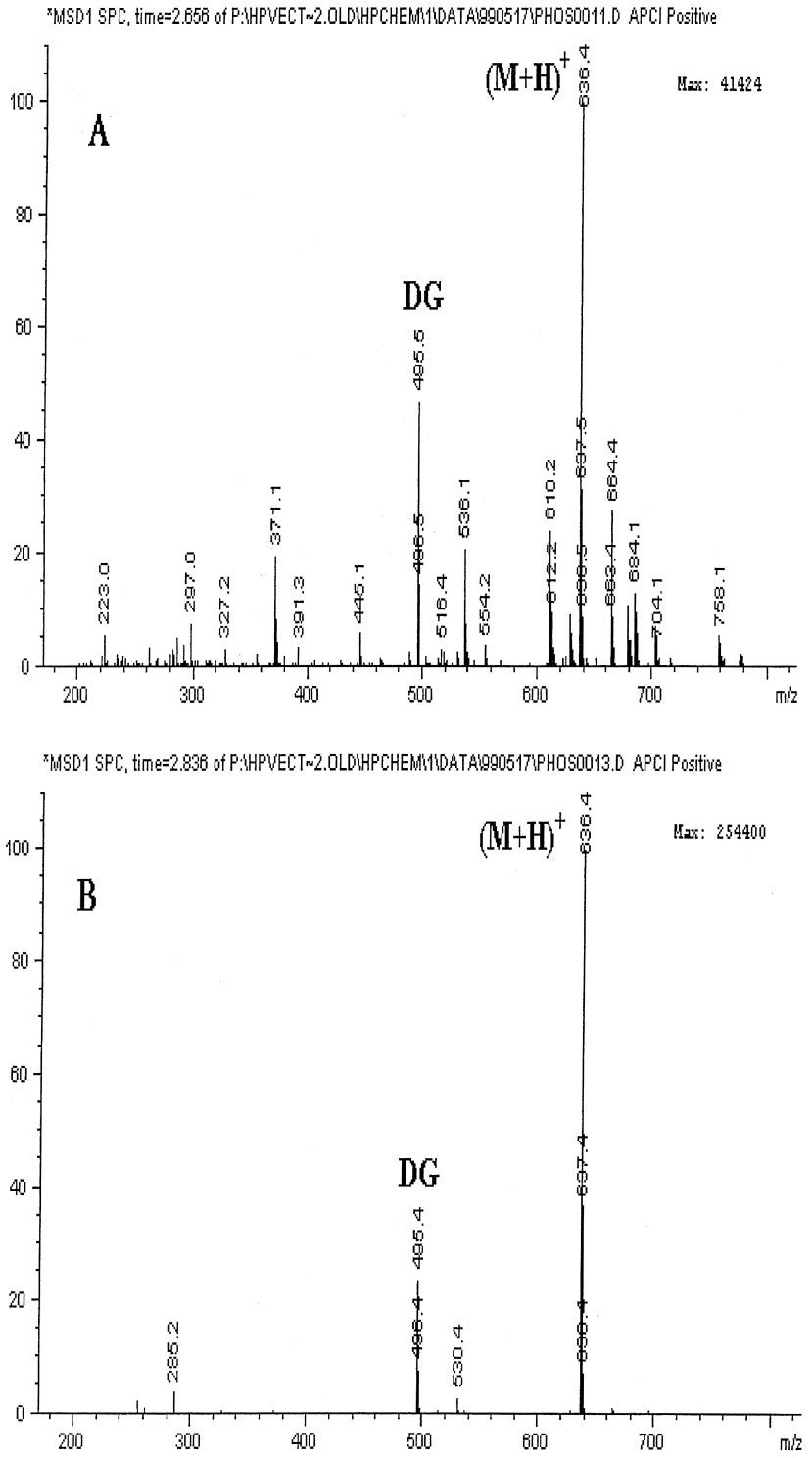


Fig. 2. Positive ion atmospheric pressure ionization mass spectra of (A) DMPC, (B) DMPE, (C) DPPC, (D) DPPE and (E) Lyso-PC. Fragmentor voltage, 80 V; scanning range from m/z 200–800. Molecular ion $[M+H]^+$ and diglyceride ion (DG).

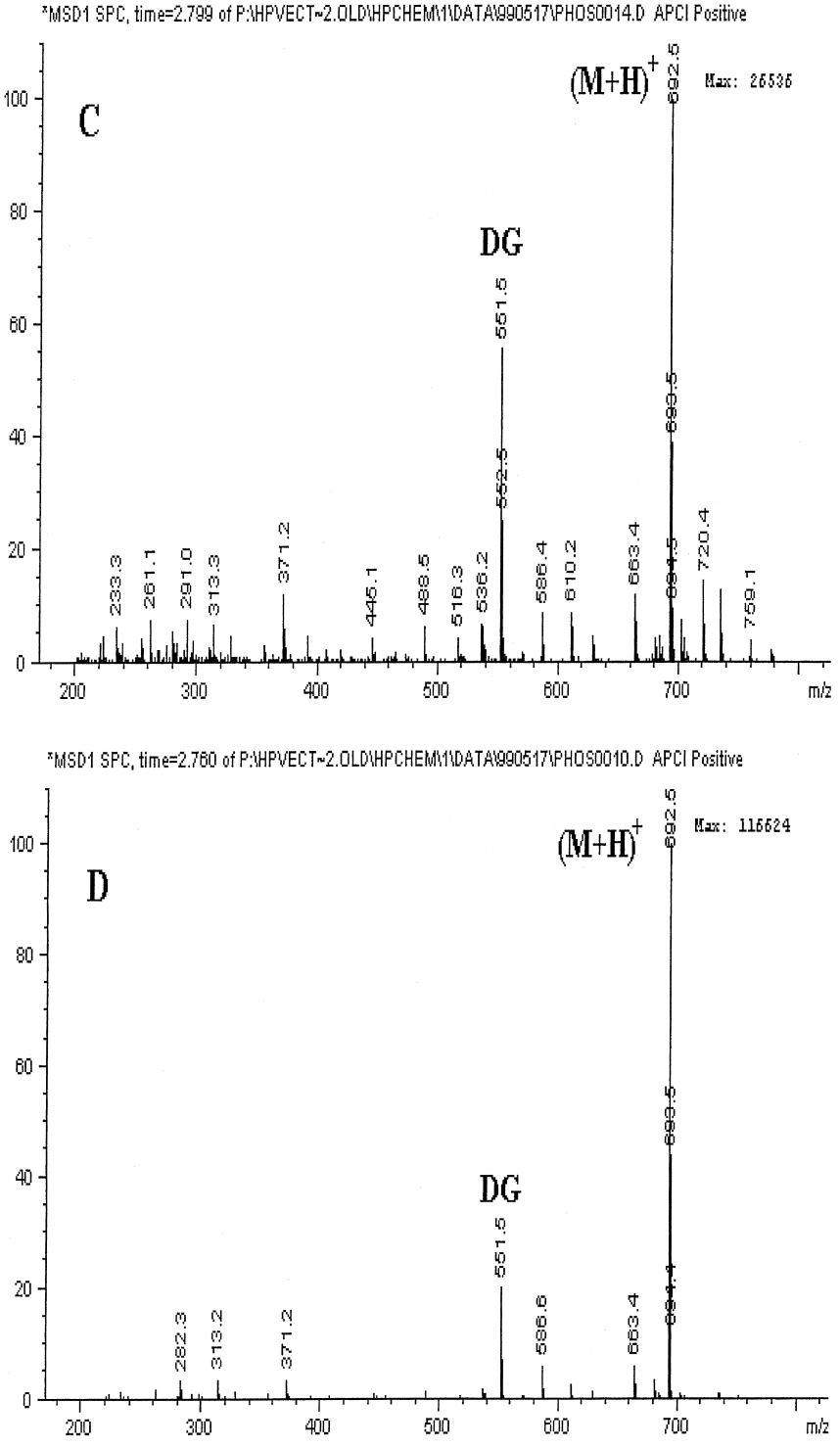


Fig. 2 (*continued*).

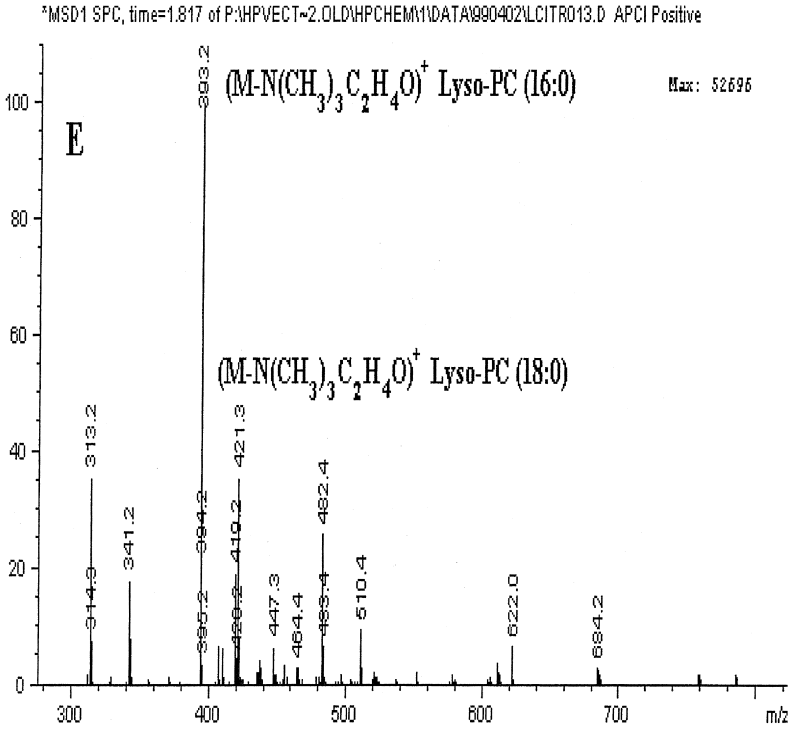


Fig. 2 (*continued*).

ing method to characterize and quantitate phospholipid content in phospholipid-based pharmaceutical formulations. In order to achieve these objectives a normal-phase LC separation assay with mass spectrometric detection was developed. The separation obtained for a standard mixture of PE, PC and Lyso-PC by the use of the above mentioned chromatographic system is illustrated in Fig. 5. This ion chromatogram was obtained using the SIM mode; the molecular ion and diglyceryl fragments were scanned. Under these conditions, retention times of PE, PC and Lyso-PC were, respectively, 1.6 min, 3.8 min and 4.5 min. Fig. 6 corresponds to the phospholipids profile obtained for an analysis of egg lecithin. The chromatogram obtained for that extracted material from natural source indicates the presence of both PE and PC.

3.3. Linearity

In order to verify the linearity of the method in the SIM mode, mixtures containing PE, PC and Lyso-PC in the range of 2 to 1600 μg/ml were prepared and injected in triplicate using the developed method. The $[M+H]^+$ ions and the diglyceride ions were monitored for each phospholipids studied. Nine standard solutions containing from 2.91 to 745 μg/ml for L-α-phosphatidylethanolamine, 3.05 to 1560 μg/ml for L-α-phosphatidylcholine and 17.3 to 553 μg/ml for L-α-lyso–phosphatidylcholine, were prepared and analyzed in triplicate. The corresponding peak areas were fitted to a straight line. Linearity was observed for the phospholipids studied over three-orders of magnitude. Correlation coefficients for the calibration curves were greater than or equal to 0.99 over the range studied.

Limits of quantitation (LOQs) were determined by preparing solutions containing various amount of phospholipids. These solutions were then injected in triplicate and the acceptance criteria was set to a relative standard deviation (RSD)<5%. Results obtained indicate that for L-α-phosphatidylethanolamine, the LOQ is 2.91 μg/ml (RSD=4.84%). For L-α-phosphatidylcholine, the LOQ is 3.05 μg/ml

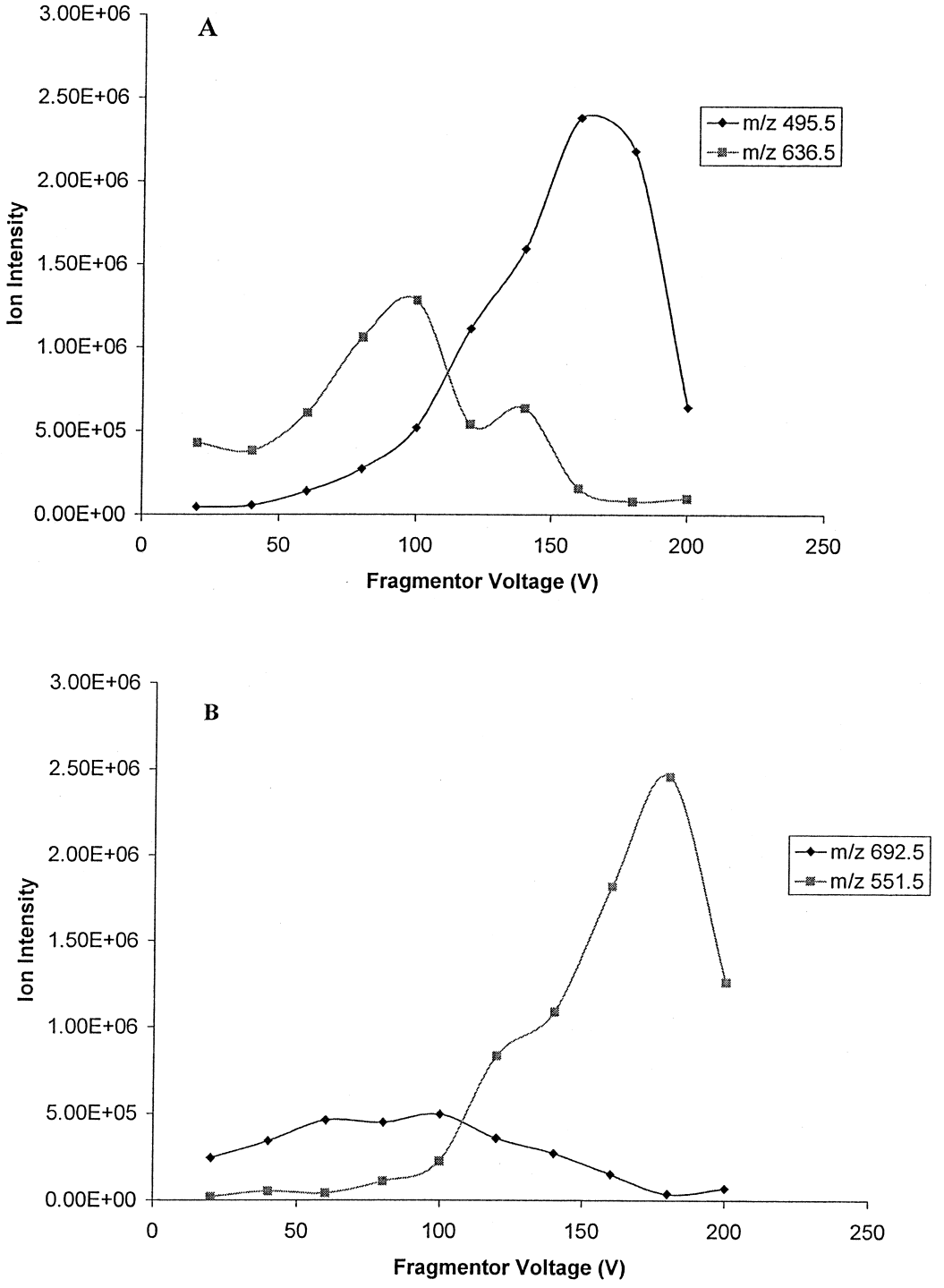


Fig. 3. Effect of fragmentor voltage on ion response of (A) DMPE m/z 636.5 (molecular ion) and m/z 495.5 (diglyceride ion) and (B) DPPE m/z 692.5 (molecular ion) and m/z 551.5 (diglyceride ion).

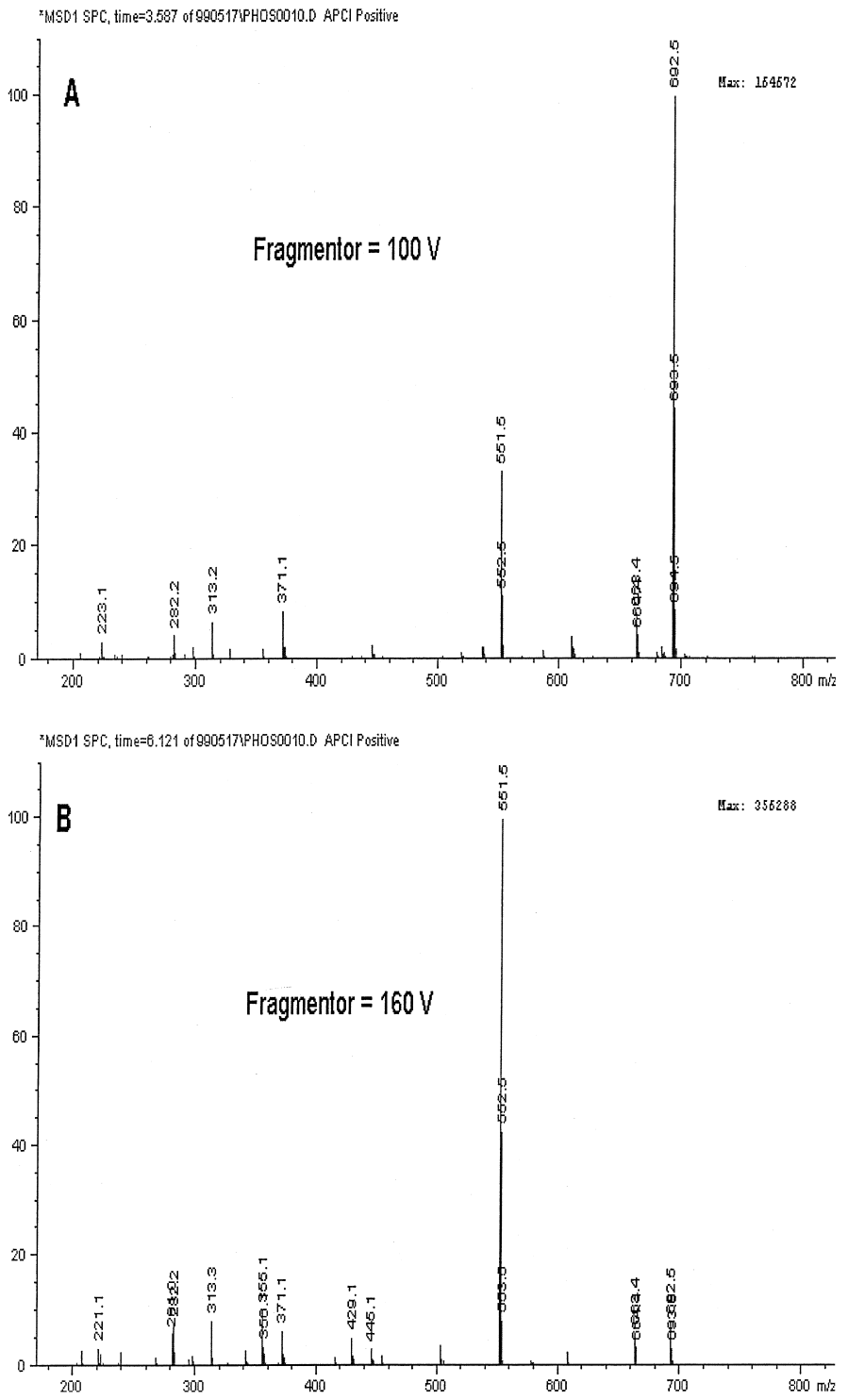


Fig. 4. Positive ion atmospheric pressure ionization mass spectra of DPPE obtained at two different fragmentor voltages (A) 100 V and (B) 160 V.

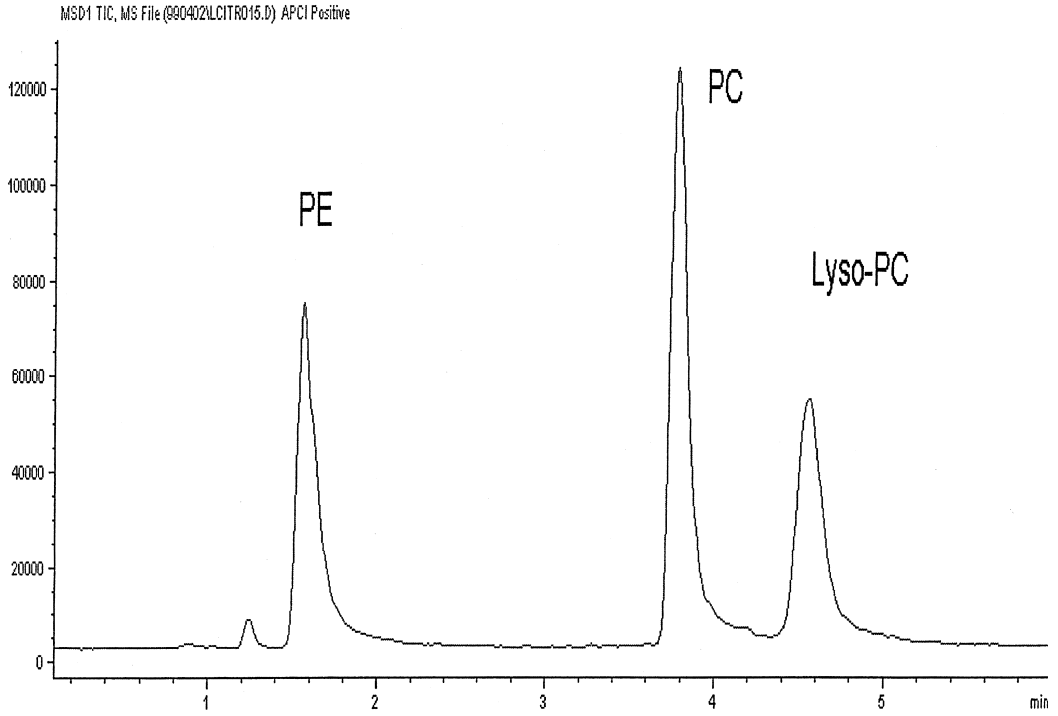


Fig. 5. Typical LC–MS separation obtained for a standard mixture in methanol of phospholipids containing 500 ng of PE, PC and Lyso-PC.

(RSD=4.35%) and for L-α-lyso-phosphatidylcholine the LOQ is 17.3 μg/ml (RSD=3.73%). The limit of detection, defined as a signal-to-noise ratio 3:1, of the phospholipids studied was approximately 250 ng/ml.

3.4. Precision and accuracy

As part of the validation process of the method developed, evaluation of the precision and accuracy was undertaken; samples were spiked at three levels (75%, 100% and 125%) of the working concentration (100 μg/ml) for each phospholipids studied. The three concentrations tested were 75, 100 and 125 μg/ml. Individual solutions of L-α-phosphatidylethanolamine, L-α-phosphatidylcholine and L-α-lyso-phosphatidylcholine were prepared and injected in triplicate.

The results obtained for the evaluation of the accuracy indicate that at 75% of the content, the recovery averaged 102±2% for L-α-phosphatidylethanolamine, 101±2% for L-α-phosphatidylcholine and 104±1% for L-α-lyso–phosphatidylcholine. For the 100% concentration, the results were 101±3% for L-α-phosphatidylethanolamine, 99±3% for L-α-phosphatidylcholine and 99±3% for L-α-lyso–phosphatidylcholine. At 125% of the content, the recovery averaged 100±2% for L-α-phosphatidylethanolamine, 99±2% for L-α-phosphatidylcholine and 100±2% for L-α-lyso–phosphatidylcholine.

For the evaluation of the precision of the method developed, samples were prepared at the 100% level of the working concentration (100 μg/ml) and injected in triplicate on three different days, giving the following results. The overall recovery was 100±2% for L-α-phosphatidylethanolamine, 100±2% for L-α-phosphatidylcholine and 100±1% for L-α-lyso–phosphatidylcholine.

3.5. Robustness

The robustness of the method was evaluated by verifying the sensitivity of the method to three minor independent changes in operational parameters.

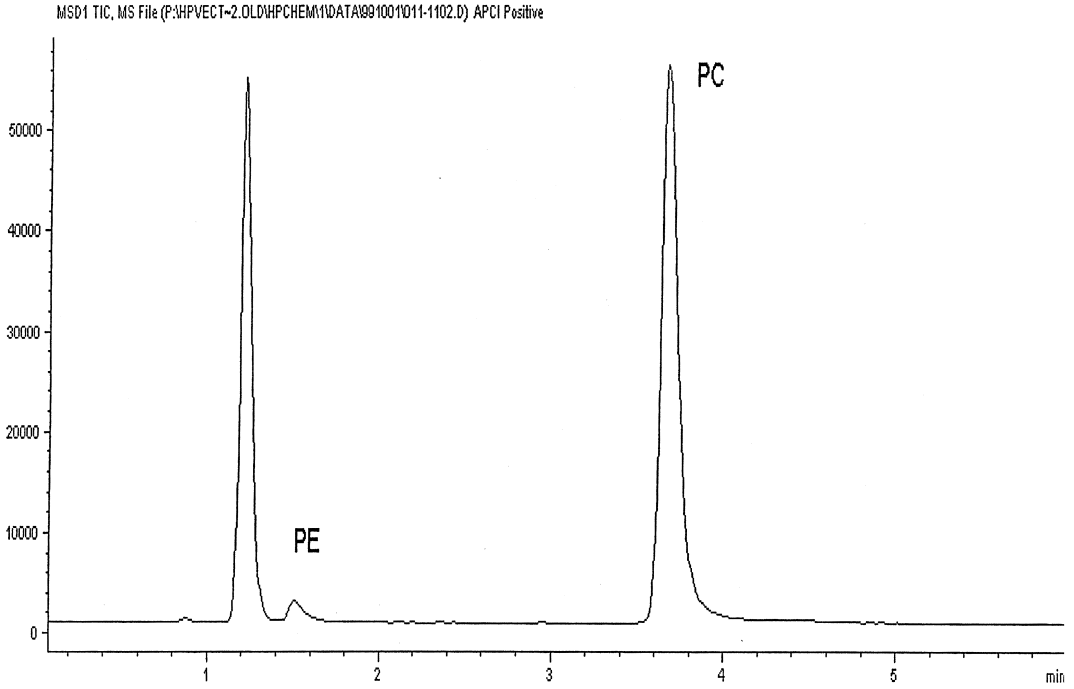


Fig. 6. LC–MS separation obtained for an injection of 1 ng of egg lecithin in methanol.

These parameters were column temperature, flow-rate and mobile phase composition. The evaluation of the robustness was done by examining the retention time and the resolution.

The results obtained indicate that minor changes in operational conditions do not significantly affect the results. Resolution between each of L-α-phosphatidylethanolamine, L-α-phosphatidylcholine and L-α-lyso-phosphatidylcholine was always greater than 1.5 (R_s>1.5).

3.6. Range

The range for which the HPLC assay for the determination of L-α-phosphatidylethanolamine, L-α-phosphatidylcholine and L-α-lyso-phosphatidylcholine has been shown to be linear, accurate and reproducible is between 2.91 and 745 μg/ml, 3.05 and 1560 μg/ml and 17.3 and 553 μg/ml, respectively. The method was shown to be accurate and precise over the range of 31.2 to 52.0 μg/ml for L-α-phosphatidylethanolamine, 63.6 to 106.0 μg/ml for L-α-phosphatidylcholine and 59.4 to 99.0 μg/ml for L-α-lyso–phosphatidylcholine.

3.7. Formulation testing

The method developed has been applied to various phospholipid-based formulations containing a variety of drugs and is routinely used in our laboratory to assess the stability of the different phospholipids used in both raw material and finished product. Fig. 7A corresponds to the phospholipid profiles obtained at initial stability time point for an egg phospholipid-based formulation. The results indicate that the formulation initially contained 0.34 mg/g of PE and 15.33 mg/g of PC and no Lyso-PC. In that particular case, the expected amounts of phospholipids were PE<1.0 mg/g, PC 15.5 mg/g and Lyso-PC<0.2 mg/g. Chromatogram obtained after storing the same sample at 40°C for 1 month is presented in Fig. 7B. The results obtained indicate that the PE (0.28 mg/g) content remained approximately the same; however a small decrease in the PC (14.17 mg/g) content was observed. This decrease was associated

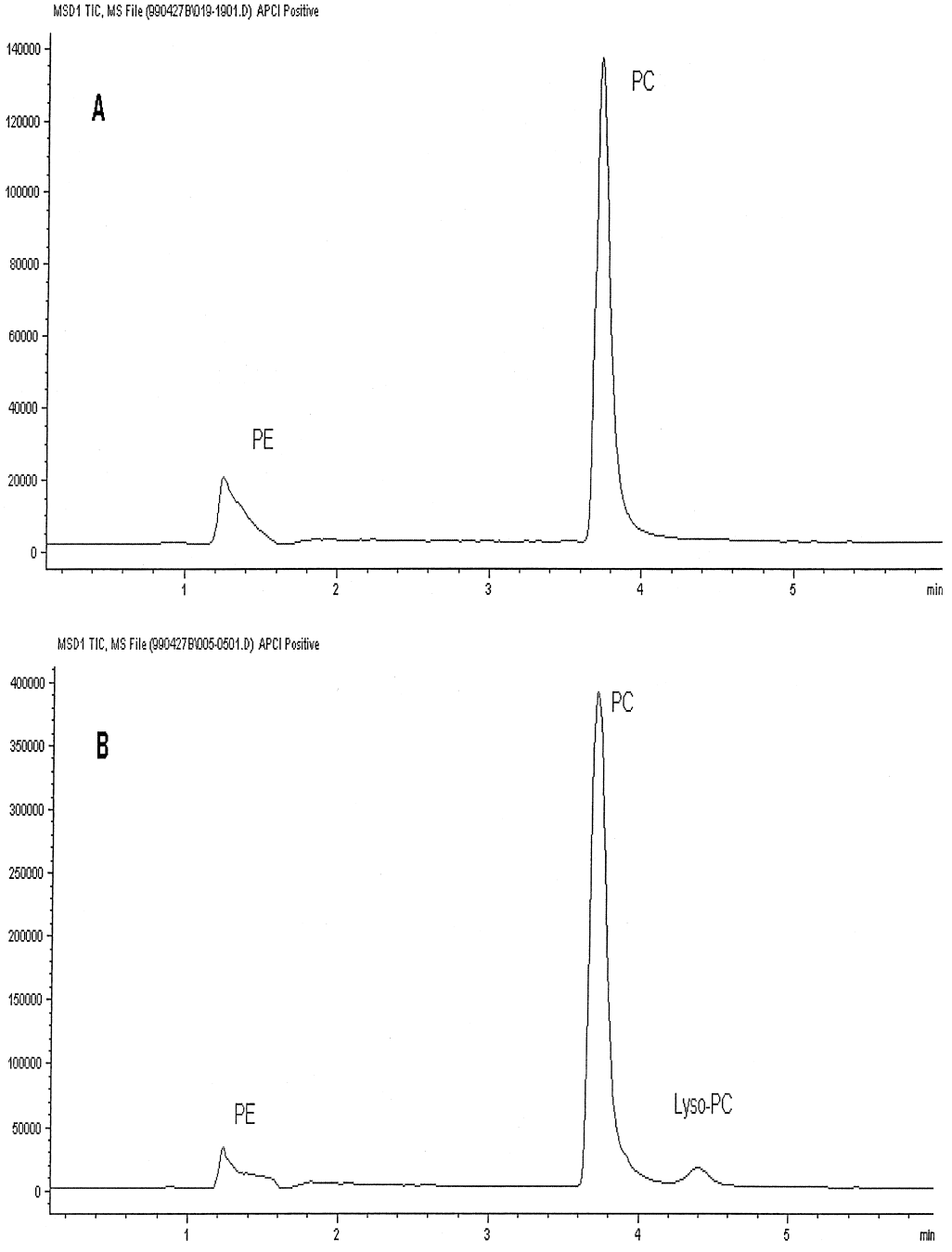


Fig. 7. Phospholipid profiles of an egg phospholipid-based formulation. (A) Initial time point, (B) 1 month at 40°C, 75% relative humidity.

with a concomitant increase in the Lyso-PC (1.08 mg/g) content.

4. Conclusion

A rapid LC–MS method for the determination of phospholipid molecular species of phosphatidylcholine, phosphatidylethanolamine and lyso-phosphatidylcholine was developed. The described method is useful for the characterization of phospholipids in pharmaceutical formulations and in natural egg lecithin. The LC–MS assay permits reproducible identification and quantitative analysis of all relevant phospholipids. The use of this method allows rapid and reliable assessment of the stability of the different phospholipid-based formulations developed and becomes an important tool in the development stage of phospholipid-based formulation. Data was presented to support the validation of this method. The assay was successfully used to assess the stability of potential phospholipid-based formulations exposed to various stress conditions.

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