



Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations

Omnia A. Ismaiel^{a,1}, Matthew S. Halquist^{b,2}, Magda Y. Elmamly^c, Abdalla Shalaby^c, H. Thomas Karnes^{a,*}

^a Virginia Commonwealth University School of Pharmacy Department of Pharmaceutics, PO Box 980533, 410 N 12th Street, Richmond, VA 23298-0533, United States

^b Virginia Commonwealth University School of Pharmacy Department of Pharmaceutics, PO Box 980533, 410 N 12th Street, Richmond, VA 23298, United States

^c Zagazig University Faculty of Pharmacy Department of Analytical Chemistry Egypt

ARTICLE INFO

Article history:

Received 18 October 2007

Accepted 26 August 2008

Available online 18 September 2008

Keywords:

Phospholipids

LC/MS/MS

Matrix effects

Ion suppression

ABSTRACT

Biological matrix effects are a source of significant errors in both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) LC/MS. Glycerophosphocholines (GPChos) and 2-lyso-glycerophosphocholines (2-lyso GPChos) are known to fragment to form ions at m/z 184 and m/z 104, respectively. Phospholipids were used as markers to evaluate matrix effects resulting in both ion suppression and enhancement using ESI and APCI modes in the determination of chlorpheniramine in human plasma. Results revealed that GPChos and 2-lyso GPChos demonstrated very low ionization efficiency in the APCI mode, post-column infusion experiments were performed to confirm that suppression and enhancement matrix ionization effects coincided with the elution profiles of the phospholipids. The mean matrix effect for chlorpheniramine using APCI was 75% less than the mean matrix effect in ESI, making APCI the ionization method of choice initially even though the absolute response was lower than in the ESI mode. The resulting APCI method showed acceptable results according to the FDA guidelines; however, a multiple source relative matrix effects study demonstrated variability. It was concluded that an absolute matrix effects study in one source of biological fluid may be not sufficient to ensure the validity of the method in various sources of matrix. In order to obviate the multiple matrix source variability, we employed an isotopically labeled internal standard for quantification of chlorpheniramine in the ESI mode. An additional validation was completed with the use of chlorpheniramine- d_6 as the internal standard. This method met all acceptance criteria according to the FDA guidelines, and the relative matrix effects study was successful.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most commonly used soft ionization sources in mass spectrometry. Both ESI and APCI are susceptible to errors in quantification caused by matrix ion suppression and enhancement effects that are due to co-elution of matrix components [1–5]. ESI has been shown to be more susceptible to matrix effect than APCI [1,2,6]. In the ESI mode, the presence of neutral

species in the gas phase with higher proton affinity than the analytes may neutralize the ionized analytes through gas phase proton transfer reactions. Salts with high ionization efficiency may also affect ion evaporation. The presence of high levels of non-volatile co-eluting components may also prevent droplets from reaching the required radius and surface charge for ion transfer into the gas phase [7].

In the APCI mode, the analytes are in the gas phase before ionization, which may explain the lower degree of matrix effects observed in APCI in comparison with ESI [7]. Bonfiglio et al. [8] stated that the degree of matrix effect in ESI is compound dependent; polar analytes are more affected by ion suppression than less polar analytes. On the other hand, APCI is often less sensitive than ESI [7] and may contribute to decomposition of thermolabile compounds [6].

Endogenous matrix components (e.g. phospholipids) may lead to many analytical problems such as retention time shifts, elevated baseline, and divergent curves [9]. For these reasons, matrix effects

* Corresponding author. Tel.: +1 804 828 3819; fax: +1 804 828 8359.

E-mail addresses: oismaiel@vcu.edu (O.A. Ismaiel), halquistms@vcu.edu (M.S. Halquist), Abdallashalaby@yahoo.com (A. Shalaby), tom.karnes@vcu.edu (H. Thomas Karnes).

¹ Tel.: +1 804 7260093; fax: +1 804 8281273.

² Tel.: +1 804-827-2078.

should be evaluated during method development and in some cases in routine sample analysis [10]. Use of a stable isotope labeled internal standard is the most common approach for coping with matrix effects. When significant matrix suppression exists any slight differences in retention times between the analyte and internal standard may lead to different matrix effects [3]. Labeled internal standards are expensive and may not be commercially available [11].

Phospholipids are present in abundant concentrations in biological matrices such as plasma, and are the major class of endogenous components that cause ion suppression or enhancement in LC/MS/MS analyses [12,13]. Glycerophosphocholines (GPCos) constitute up to 70% of total plasma phospholipids and lysophospholipids constitute up to 10% [13]. GPCos are known to fragment to form ions at (m/z 184) in mass spectrometry, whereas 2-lyso glycerophosphocholines (2-lyso GPCos) fragment to form ions primarily at m/z 104 [Fig. 1] [12].

Chlorpheniramine (2-[*p*-chloro- α -(2-(dimethylamino)ethyl)benzyl] pyridine) is an H_1 -receptor antagonist anti-histamine, used for the common cold and allergic rhinitis, it has less sedation effects than diphenhydramine [14,15]. High-performance liquid chromatography coupled with mass spectrometry (LC/MS) [16], tandem mass spectrometry (LC/MS/MS) [17] or UV detection [18,19], and capillary electrophoresis methods [20] have been reported for determination of chlorpheniramine in human plasma and pharmaceutical preparations. Phospholipids were used in this work as markers for endogenous matrix components and for comparison between APCI and ESI in the determination of chlorpheniramine in human plasma. Initial results indicated the APCI mode to be superior to the ESI mode due to lower matrix effects. A multiple source relative matrix effects study performed during the validation of the APCI method indicated unacceptable variability; therefore, the determination of chlorpheniramine was also validated using a labeled internal standard (chlorpheniramine- d_6) which overcame this variability. Chemical structures of GPCos, 2-lyso GPCos, chlorpheniramine maleate and diphenhydramine hydrochloride are shown in Fig. 1 [12,21].

2. Experimental

2.1. Reagents

Chlorpheniramine maleate and diphenhydramine hydrochloride were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Chlorpheniramine- d_6 was purchased from Toronto Research Chemicals (Toronto, Canada). Phosphatidylcholine and lysophosphatidylcholine (1-behenoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) were purchased from Avanti polar Lipid Inc. (Alabaster, AL, USA). Blank human plasma with K_2 -EDTA as an anti-coagulant was obtained from BioChemed Services (Winchester, VA, USA). Acetonitrile, methanol, and methyl tertiary butyl ether (MTBE) suitable for HPLC were obtained from Burdick and Jackson (Muskegon, MI, USA), formic acid was purchased from Sigma Chemical Company (St. Louis, MO, USA), ammonium formate was purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and sodium hydroxide was obtained from GFS chemicals Inc. (McKinley, Columbus, OH, USA).

2.2. Apparatus

The HPLC system consisted of a Shimadzu, System Controller, SCL-10A Vp, Pumps, LC 10AD Vp, Solvent Degasser, DGU14A, and autosampler, HTC PAL (Zwingen, Switzerland). The Mass Spectrometer was Micromass Quattro API Micro, Waters Corp. with a Data acquisition, Masslynx version 4.1 installed on IBM think center computer, Waters Corp (Milford, MA, USA), which was operated in either the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode or atmospheric pressure chemical ionization (APCI) positive multiple reaction monitoring (MRM) mode.

2.3. Preparation of standards and quality control (QC) samples

Two separate 100 $\mu\text{g/mL}$ stock solutions of chlorpheniramine were prepared in methanol and stored at approximately -20°C .

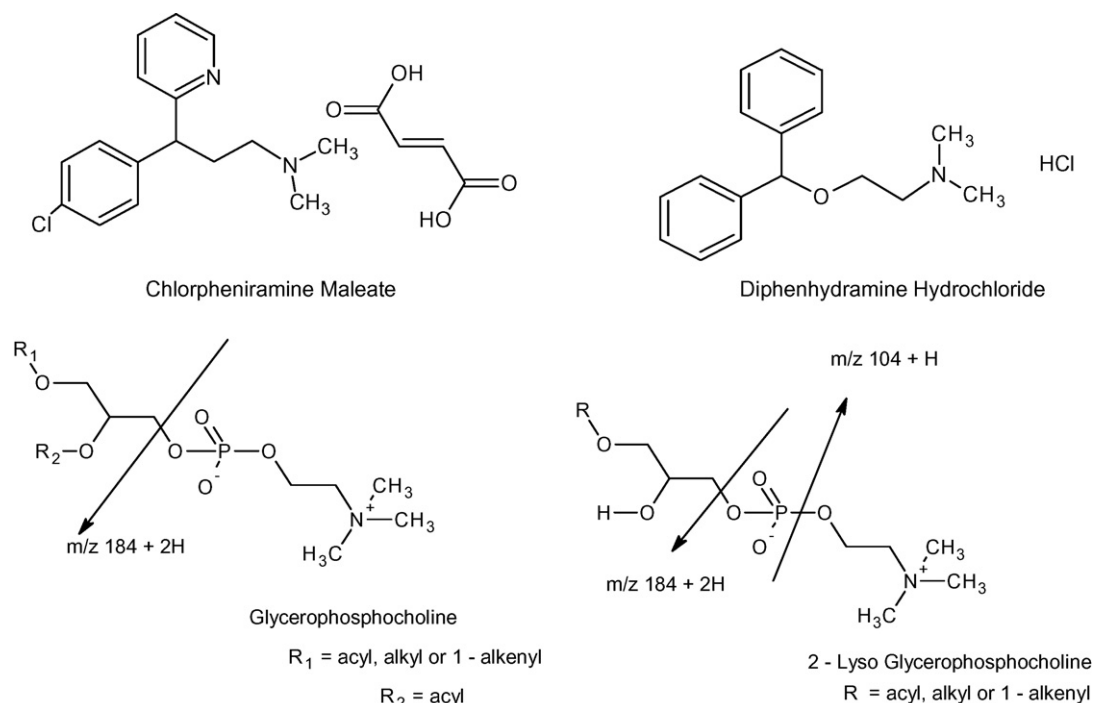


Fig. 1. Chemical structures of chlorpheniramine maleate, diphenhydramine hydrochloride, glycerophosphocholine and 2-lyso glycerophosphocholine [21].

The solution was prepared by weighing approximately 1.00 mg of chlorpheniramine and quantitatively transferring it to a glass container. The solution was diluted to approximately 10 mL. The calibration standards were prepared by adding the appropriate amounts of the stock solution into pooled blank plasma. Nominal concentrations were 0.2, 0.45, 0.75, 5.0, 25.0, 50.0 and 100.0 ng/mL of chlorpheniramine, QC samples at concentrations of 0.5, 4.0 and 80.0 ng/mL of chlorpheniramine were prepared and lower limit of quantification (LLOQ) QC samples at 0.2 ng/mL of chlorpheniramine were also prepared. Standards and controls were sub-aliquoted into 13 mm × 100 mm polypropylene tubes and stored at approximately –20 °C.

2.4. Initial method sample preparation

Human plasma samples were thawed at room temperature and vortex mixed. A 500 µL aliquot of each sample was placed into a 13 mm × 100 mm screw cap culture tube, 50 µL of freshly prepared working internal standard (25.0 µg/mL of diphenhydramine hydrochloride) was added and the tubes were mixed briefly. 50 µL of 0.1N sodium hydroxide was added and the tubes were again mixed briefly. The mixed samples were extracted by addition of 3.0 mL of methyl tertiary butyl ether (MTBE) followed by rotation for approximately 5 min. After centrifugation at approximately 3000 rpm for 10 min, the samples were placed in the freeze bath at –40 °C, the organic layer was transferred to a 16 mm × 100 mm screw cap conical tube, and evaporated to dryness in the TurboVap® under a dry nitrogen stream at approximately 40 °C. For ESI, the residue was reconstituted with 50 µL of reconstitution solution [20:80] 2.0 mM ammonium formate in methanol:acetonitrile (v/v), vortex mixed, and transferred to 96-well plates with silanized inserts. After centrifugation at approximately 2500 rpm for 3 min, a 10 µL portion of the resulting solution was injected. For APCI, the dried extract was reconstituted with 100 µL of reconstitution solution [70:30:1] acetonitrile:water:formic acid (v/v/v), vortex mixed, and transferred to the 96-well plates with silanized inserts. After centrifugation at approximately 2500 rpm for 3 min, a 25 µL portion of the resulting solution was injected.

2.5. Modified method sample preparation

The newly validated method for the determination of chlorpheniramine using chlorpheniramine-*d*₆ as an internal standard used a similar extraction procedure. However, in changing to the ESI mode, the additional sensitivity allowed for a reduction in the sample aliquot size to 200 µL. A freshly prepared internal standard was added (400 ng/mL of Chlorpheniramine-*d*₆ in replace of 25.0 µg/mL of diphenhydramine hydrochloride). A reduction in the volume of extraction solvent (1 mL) was also employed to compensate for the smaller sample size.

2.6. Monitoring of GPChos and 2-lyso GPChos

Optimized parameters for monitoring *m/z* 184 and *m/z* 104 as common ion fragments for GPChos and 2-lyso GPChos, respectively

without further fragmentation were used [12]. The *m/z* 184 and *m/z* 104 ions were detected in both plasma extracts and solutions of the phosphatidylcholine isolated from chicken eggs and synthetic lysophosphatidylcholine under ESI and APCI modes using a cone voltage of 90 V, collision energy 7 V, the dwell time set at 0.05 s, and an interscan delay time of 0.05 s.

2.7. Post-column infusion

Post-column infusion experiments were conducted in which a 100 ng/mL (for ESI) or a 250 ng/mL (for APCI) solution of chlorpheniramine and diphenhydramine in their respective reconstitution solutions were constantly infused (10 µL/min) into the MS. A 500 µL human plasma sample was aliquoted and extracted as described under Section 2.4 and the matrix extract injected pre-column. For the isolated GPChos and synthetic 2-lyso GPChos solutions, a 10 µg/mL phosphatidylcholine solution and a 10 µg/mL lysophosphatidylcholine (for ESI) or a 500 µg/mL phosphatidylcholine solution and a 25 µg/mL lysophosphatidylcholine solution (for APCI) were prepared in the reconstitution solutions and were injected pre-column.

2.8. Isocratic method for the analysis of chlorpheniramine using the ESI mode

The samples were analyzed using a Betasil Diol-100, 50 mm × 2.1 mm, 5 µm particle size analytical column, Thermo Electron Corp. (Bellefonte, PA, USA) and Frit SS Nat 2 µm 0.094 × 0.65 × 0.2485, A-100X, Upchurch Scientific, Inc. (Oak Harbor, WA, USA). An isocratic method using a mobile phase consisting of 20% 2 mM ammonium formate in methanol and 80% acetonitrile (v/v) with a 0.35 mL/min flow rate was performed. The autosampler utilized a rinse solution comprised of 95% methanol and 5% formic acid (2%) (v/v) with a run time of 5 min. Five cycles of post-syringe and injection port washing were used. MS/MS system parameters were as shown in Table 1. For the dueterated internal standard method, all chromatographic conditions were kept consistent with the exception of the addition of a Phenomenex (Torrance, CA) Silica guard column, 4.0 mm × 2.0 mm, for extended life of the analytical column.

2.9. Isocratic method for the analysis of chlorpheniramine using APCI mode

The samples were analyzed using a ZORBAX-SB C₁₈, 4.6 mm × 50 mm, 3.5 µm particle size analytical column, Agilent Technologies (Santa Clara, CA, USA) and Frit SS Nat 2 µm 0.094 × 0.65 × 0.2485, A-100X, Upchurch Scientific, Inc. (Oak Harbor, WA, USA). An isocratic method using [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase with a 0.65 mL/min flow rate was employed. The autosampler utilized a rinse solution comprised of 95:5 methanol:2% formic acid (v/v) with a run time of 5 min and five cycles of post (syringe and injection port) washing. The MS/MS system parameters were as shown in Table 1.

Table 1

Multiple reaction monitoring (MRM) parameters for chlorpheniramine, chlorpheniramine-*d*₆, diphenhydramine, glycerophosphocholines and 2-lyso glycerophosphocholines.

Compound name	Nominal parent (<i>m/z</i>)	Nominal daughter (<i>m/z</i>)	Dwell (s)	Cone (V)	Collision energy (eV)
Chlorpheniramine	275.0	230.0	0.30	25.0	25.0
Chlorpheniramine- <i>d</i> ₆	281.0	230.0	0.30	25.0	25.0
Diphenhydramine	256.0	167.0	0.30	25.0	25.0
Glycerophosphocholines	184.0	184.0	0.05	90.0	7.0
2-Lyso glycerophosphocholines	104.0	104.0	0.05	90.0	7.0

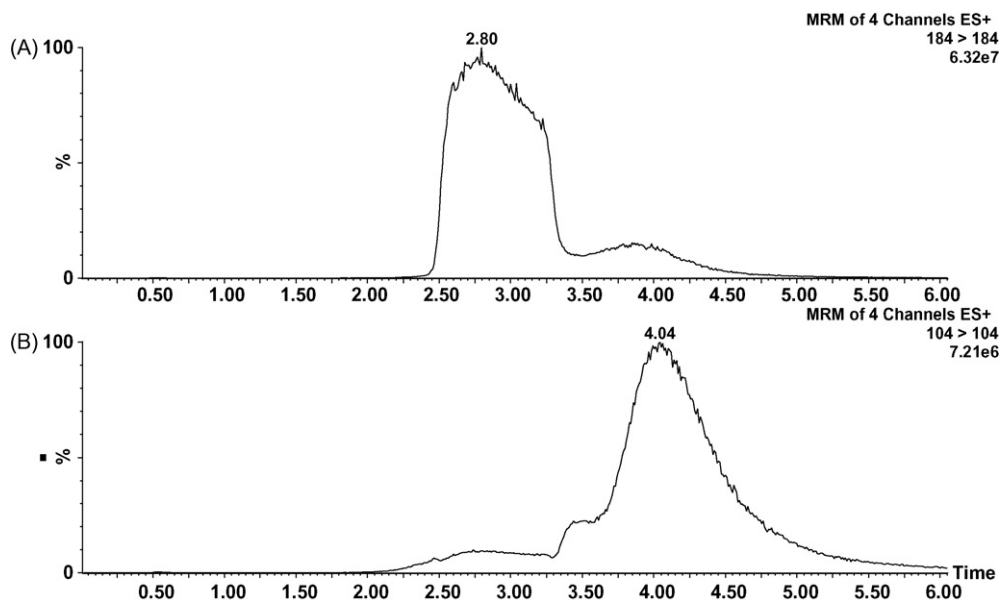


Fig. 2. LC/MS/MS-ESI analysis of extracted plasma using a Betasil diol column with [20:80] 2 mM ammonium formate in methanol:acetonitrile (v/v) mobile phase: (A) glycerophosphocholines, m/z 184 \rightarrow 184 and (B) 2-lyso glycerophosphocholines, m/z 104 \rightarrow 104.

2.10. Post-extraction addition

Post-extraction addition experiments were conducted in which human blank plasma ($n=3$) was aliquoted and extracted as described under Section 2.4, reconstituted with a standard solution of chlorpheniramine and diphenhydramine, mixed, then transferred to a 96-well plate with silanized inserts and injected (10 μ L for ESI and 25 μ L for APCI). Solutions containing an equivalent amount of chlorpheniramine and diphenhydramine in the reconstitution solutions ($n=3$) were injected as described under Section 2.4.

3. Results and discussion

3.1. Monitoring GPChos and 2-lyso GPChos

Di-substituted GPChos and sphingomyelins are known to fragment to form trimethylammonium-ethyl phosphate ions (m/z 184) in LC/MS/MS. Mono-substituted GPChos (2-lyso GPChos) can fragment to form ions at both m/z 184 and m/z 104. [12]. m/z 184 and m/z 104 were detected in plasma extracts after liquid–liquid extraction using methyl tertiary butyl ether (MTBE) and in standard solutions of phosphatidylcholine and lysophosphatidylcholine, respectively.

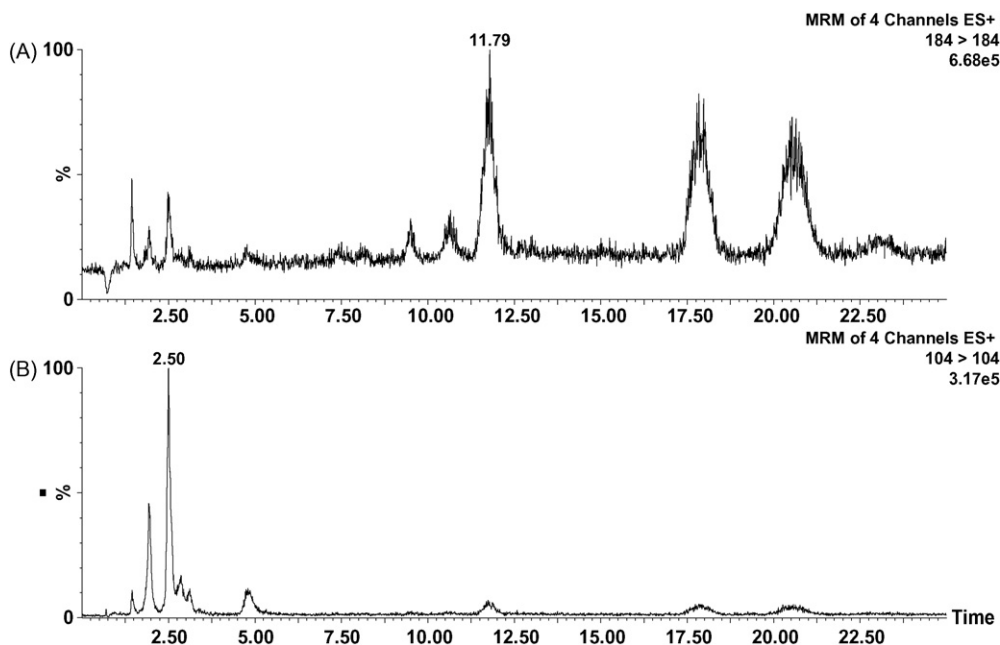


Fig. 3. LC/MS/MS-ESI analysis of extracted plasma using a ZORBAX-SB C_{18} column with [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase: (A) glycerophosphocholines, m/z 184 \rightarrow 184 and (B) 2-lyso glycerophosphocholines, m/z 104 \rightarrow 104.

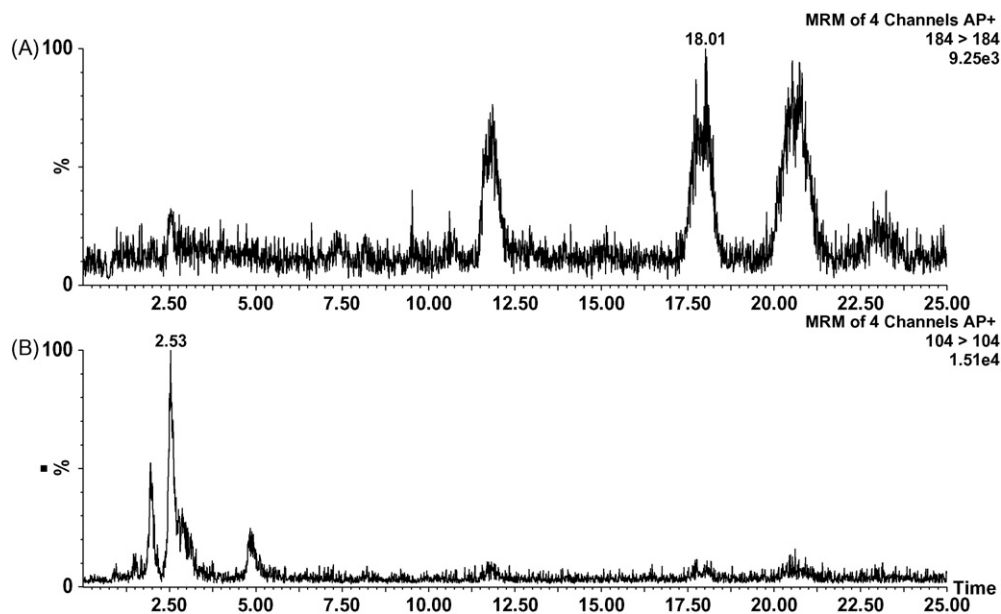


Fig. 4. LC/MS/MS-APCI analysis of extracted plasma using a ZORBAX-SB C_{18} column with [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase: (A) glycerophosphocholines, m/z 184 \rightarrow 184 and (B) 2-lyso glycerophosphocholines, m/z 104 \rightarrow 104.

3.1.1. Electrospray ionization (ESI) using a Betasil diol column

GPChos and 2-lyso GPChos were monitored in the positive (ESI) multiple reaction-monitoring (MRM) mode. Normal phase HPLC separates phospholipids by class according to the polarity of the head group [22]. GPChos eluted from the column as only one peak, 2-lyso GPChos eluted after the GPChos also as one peak (Fig. 2). The retention times of chlorpheniramine and diphenhydramine were 2.7 and 2.1 min, respectively. GPChos and 2-lyso GPChos eluted later, however the retention times of the phospholipids decreased upon repeated injection. We found in a previous study using similar chromatographic conditions for LC/MS/MS analysis of hydrocodone and pseudoephedrine that upon repeated injection, the elution rate

of phospholipids increased causing co-elution with the analytes [10].

3.1.2. Electrospray ionization (ESI) mode using a ZORBAX-SB C_{18} column

GPChos and 2-lyso GPChos were monitored in the positive (ESI) multiple reaction monitoring (MRM) mode using a ZORBAX-SB C_{18} analytical column, the reversed phase C_{18} column demonstrated the ability to separate different GPChos and 2-lyso GPChos into several peaks. Reversed phase HPLC separates phospholipids based primarily on their lipophilicity, the fatty-acyl chains rather than the polar head groups determine their elution [22].

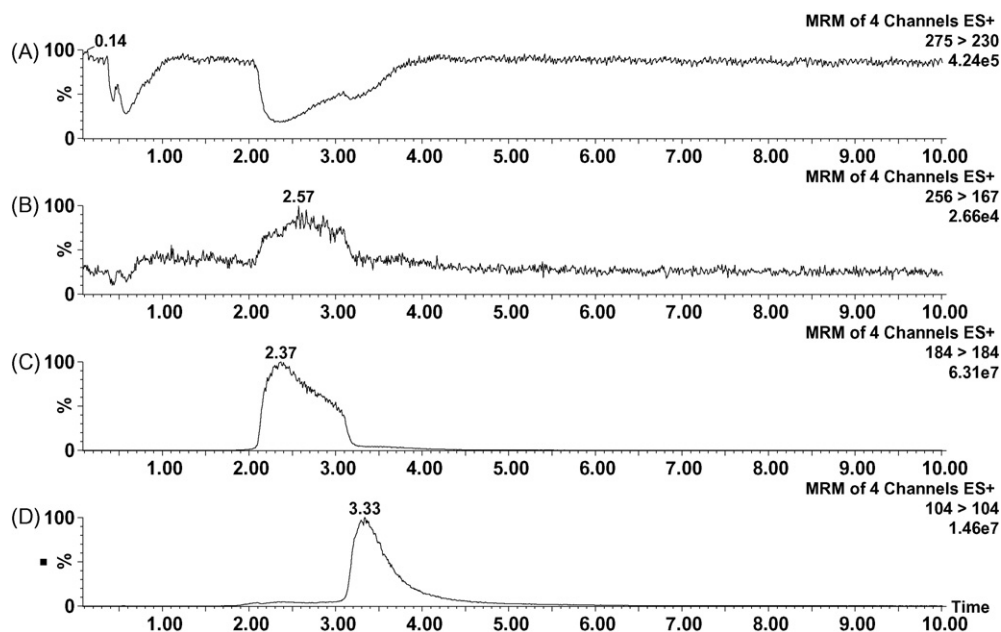


Fig. 5. Matrix ionization effects as shown with a post-column infusion experiment (100 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167 and LC/MS/MS-ESI analysis of extracted plasma, (C) glycerophosphocholines, m/z 184 \rightarrow 184, (D) 2-lyso glycerophosphocholines, m/z 104 \rightarrow 104, using a Betasil diol column with [20:80] 2 mM ammonium formate in methanol:acetonitrile (v/v) mobile phase.

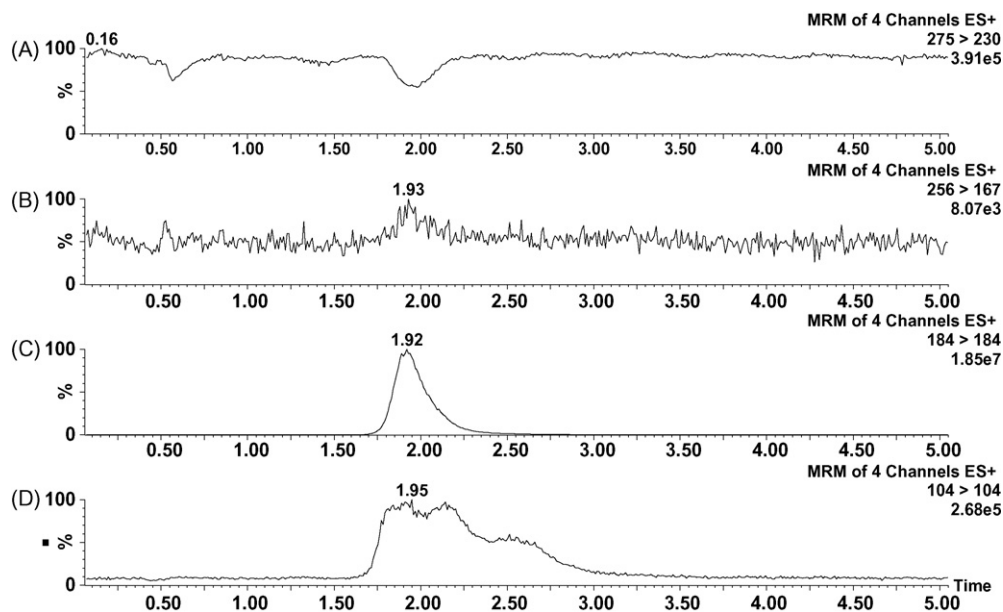


Fig. 6. Matrix ionization effects as shown with a post-column infusion experiment (100 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167, with LC/MS/MS-ESI analysis of 10 μ g/mL of phosphatidylcholine using a Betasil diol column with [20:80] 2 mM ammonium formate in methanol:acetonitrile (v/v) mobile phase, (C) m/z 184 \rightarrow 184, and (D) m/z 104 \rightarrow 104.

2-lyso GPChos peaks eluted earlier at 1.5–5 min (Fig. 3). Blank plasma ($n=3$) were extracted and injected as described under Section 2.4. The peak height intensity responses of phospholipids were compared. The peak height intensity of the highest peak of the 2-lyso GPChos using a mobile phase consisting of [70:30:1] acetonitrile:water:formic acid (v/v/v) was less than 5% of the peak height intensity using a Betasil diol column with a mobile phase consisting of 20% 2 mM ammonium formate in methanol and 80% acetonitrile (v/v). These results were observed using the same extraction procedure. Several GPChos peaks eluted at retention times from 1.5 to 22 min (Fig. 3), the peak height intensity of the highest peak of GPChos was less than 1% of the

peak height intensity of GPChos under the previously described conditions).

Changing the stationary phase and modifying the chromatographic conditions can significantly reduce the responses of the phospholipids. However, the relative peak height intensity responses of GPChos and 2-lyso GPChos were still relatively abundant and late elution of phospholipids could possibly cause inconsistent results in subsequent injections. The reduction in phospholipid response is most likely due to low ionization efficiency in the mobile phase and also to the ability of the reversed phase C_{18} column to separate different GPChos and 2-lyso GPChos into several separate peaks.

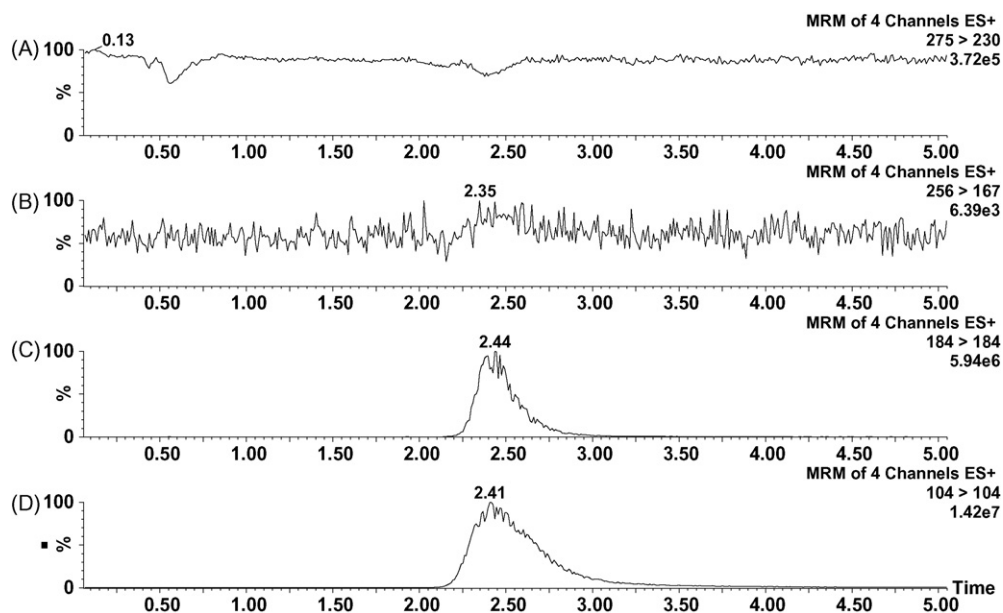


Fig. 7. Matrix ionization effects as shown with a post-column infusion experiment (100 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167, with LC/MS/MS-ESI analysis of 10 μ g/mL of lysophosphatidylcholine using a Betasil diol column with [20:80] 2 mM ammonium formate in methanol:acetonitrile (v/v) mobile phase, (C) m/z 184 \rightarrow 184, and (D) m/z 104 \rightarrow 104.

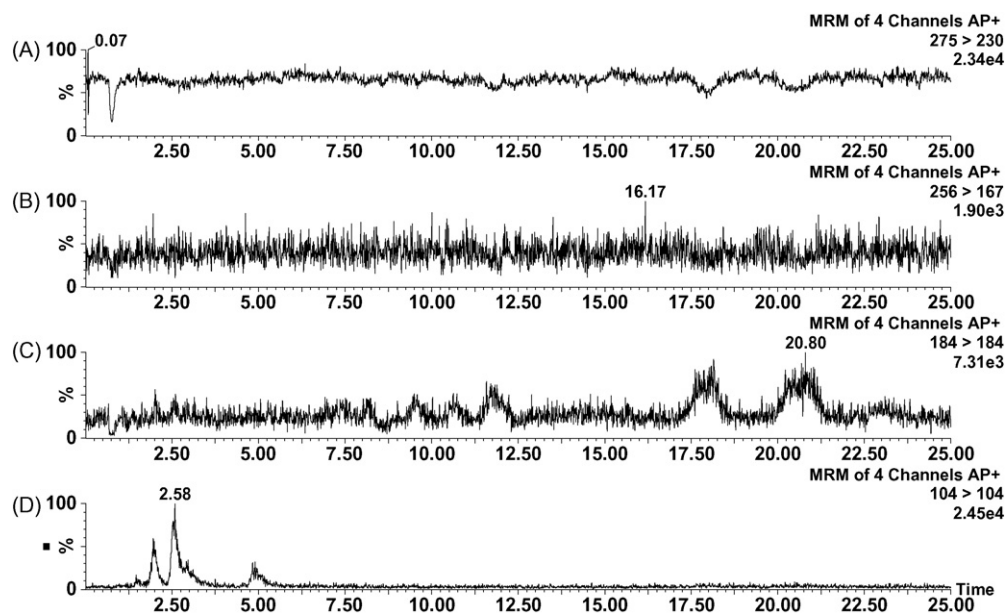


Fig. 8. Matrix ionization effects as shown with a post-column infusion experiment (250 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167, and LC/MS/MS-APCI analysis of extracted plasma using a ZORBAX-SB C_{18} column with [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase, (C) glycerophosphocholines, m/z 184 \rightarrow 184, and (D) 2-lyso glycerophosphocholines m/z 104 \rightarrow 104.

3.1.3. Atmospheric pressure chemical ionization (APCI) mode using a ZORBAX-SB C_{18} column

GPChos and 2-lyso GPChos were monitored in the atmospheric pressure positive chemical ionization (APCI) multiple reaction-monitoring (MRM) mode. The response of GPChos and 2-lyso GPChos under APCI conditions became very low (Fig. 4). The peak height intensity responses of the GPChos was less than 14% of the response when the ESI mode was used under the same chromatographic conditions and using the same extraction procedure. The peak height intensity of the 2-lyso GPChos was less than 37% of the mean response using the ESI mode under the same chromatographic conditions and the same extraction procedure. The ionization efficiency of phospholipids using APCI was much lower than the ionization efficiency using ESI especially for GPChos. The overall response for endogenous phospholipids using APCI under the modified chromatographic conditions was less than 2% of the ESI response under the initial chromatographic conditions according to the peak height intensities.

3.2. Post-column infusion

Post-column infusion experiments were performed to confirm that suppression and enhancement ionization effects coincided with the elution profiles of the phospholipids using blank plasma extracts to show suppression in plasma samples and a standard solution of phosphatidylcholine and lysophosphatidylcholine to show suppression from the phospholipids.

3.2.1. ESI mode using a Betasil diol column

Ion suppression commonly observed at the beginning of the chromatographic run may be due to the presence of salts and non-retained polar compounds [12]. This could explain the suppression observed at 0.5 min shown in (Fig. 5). Post-column infusion experiments in the ESI mode showed a 100% ion enhancement for diphenhydramine, whereas chlorpheniramine demonstrated 70% ion suppression (Fig. 5).

Chlorpheniramine is a strong basic compound with an aliphatic amine ($\text{CH}_3\text{-N-CH}_3$) group, which acts as proton acceptor site and a

pyridine nucleus (with a lone pair of electrons) that acts as an active site for charge transfer. There are two possibilities for the interaction between chlorpheniramine and phospholipid ion fragments, the aliphatic amine group may accept a proton from the hydroxyl group of the phospholipids and the pyridine ring may donate two electrons to the positively charged tri-methyl ammonium site of the phospholipid. Diphenhydramine may react with the phospholipid ion fragments only through accepting a proton from the hydroxyl group. This may explain why chlorpheniramine is suppressed while diphenhydramine is enhanced. Comparison of the post-column infusion experiments using 10 μ g/mL phosphatidylcholine solutions (Fig. 6) and 10 μ g/mL lysophosphatidylcholine solutions (Fig. 7) with the extracted matrix, show that the suppression and enhancement windows were correlated in time with the elution times of phosphatidylcholine and lysophosphatidylcholine. 10 μ g/mL of phosphatidylcholine yielded approximately 30% of the extracted fraction of endogenous GPChos response according to the overall peak height intensities and approximately caused 30% ion suppression for chlorpheniramine and 30% ion enhancement for diphenhydramine. 10 μ g/mL lysophosphatidylcholine yielded approximately the same response as the extracted fraction of the endogenous 2-lyso GPChos and caused approximately 10% ion suppression for chlorpheniramine and 10% ion enhancement for diphenhydramine. It can be concluded from these results that, GPChos contribute to matrix effects more so than 2-lyso GPChos. Both the phosphatidylcholine and lysophosphatidylcholine fragmented to form m/z 184 and m/z 104 ions but to a different extent. Phosphatidylcholine yielded mainly m/z 184 ions and lysophosphatidylcholine yielded mainly m/z 104 ions according to the overall peak height intensity ratios of the m/z 184 and m/z 104 peaks. The m/z 184 ion fragment for both GPChos and 2-lyso GPChos is due to the loss of the phosphocholine head group [$\text{H}_2\text{PO}_4\text{-(CH}_2\text{)}_2\text{-N-(CH}_3\text{)}_3\text{]}^+$ [12,23] whereas m/z 104 was due to loss of the choline head group [$\text{HO-CH-CH}_2\text{-N}^+\text{-(CH}_3\text{)}_3$] [24,25].

3.2.2. APCI mode using a ZORBAX-SB C_{18} column

A similar suppression window due to the presence of salts and non-retained polar compounds was also observed at the begin-

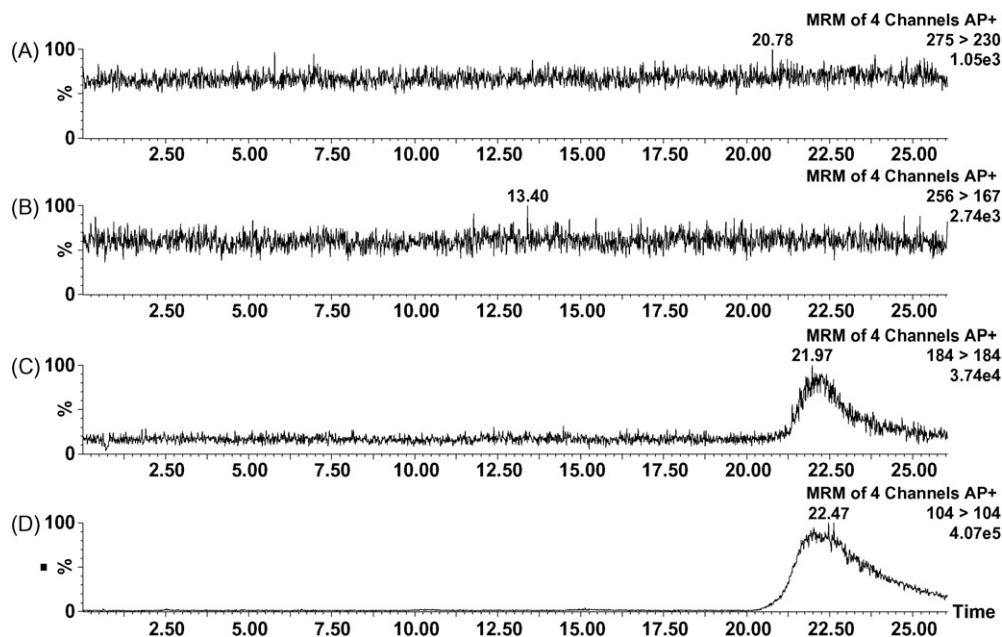


Fig. 9. Matrix ionization effects as shown with a post-column infusion experiment (250 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167, with LC/MS/MS-APCI analysis of 25 μ g/mL of lysophosphatidylcholine using a ZORBAX-SB C_{18} column with [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase, (C) m/z 184 \rightarrow 184, and (D) m/z 104 \rightarrow 104.

ning of chromatograms in the APCI mode. Post-column infusion studies revealed that the other matrix ionization effects coincided with the elution profiles of phospholipids (Fig. 8). However, these effects were less than 20% which may have been due to the very low ionization efficiency of the GPChos and 2-lyso GPChos in the APCI mode as indicated by their poor response. The response of the 25 μ g/mL solution of synthetic lysophosphatidylcholine yielded approximately the same response as the extracted fraction of the endogenous 2-lyso GPChos related peaks in the blank plasma matrix. Lysophosphatidylcholine eluted at 22 min due to its long

side chain and yielded no suppression or enhancement at this elution time (Fig. 9). Due to the very low response of the GPChos in the APCI mode, a 500 μ g/mL phosphatidylcholine solution was used. The standard solution of phosphatidylcholine yielded both m/z 184 and m/z 104 ions at 2.6 and 2.5 min, respectively and demonstrated non-significant matrix ionization effect as shown in Fig. 10.

Post-column infusion results showed that poor ionization efficiency of phospholipids in the APCI mode significantly affect the matrix ionization effects.

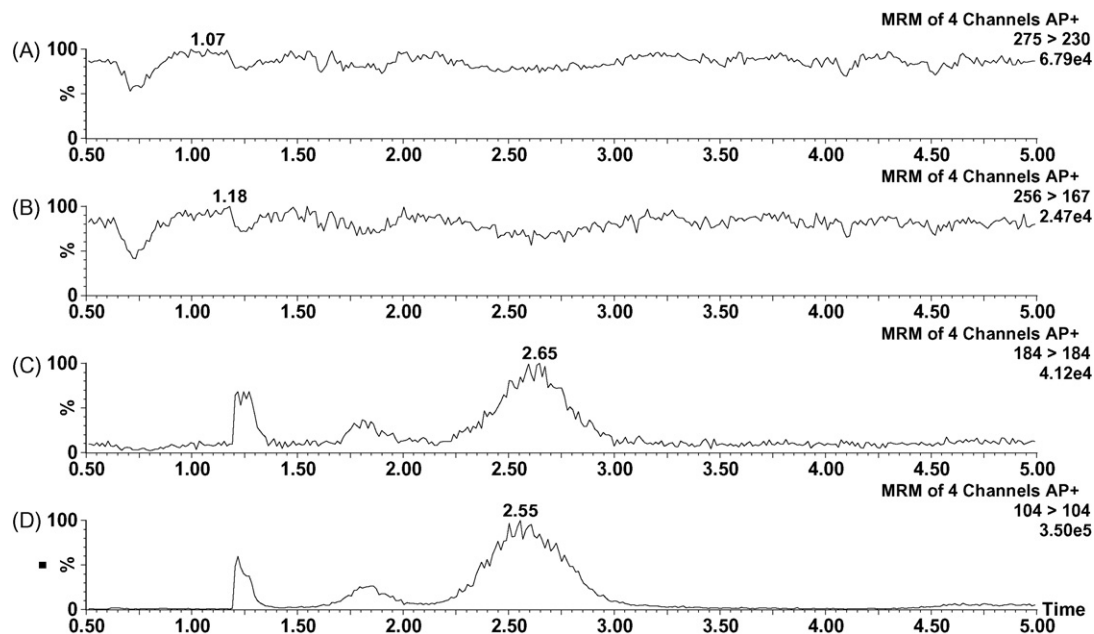


Fig. 10. Matrix ionization effects as shown with a post-column infusion experiment (250 ng/mL, 10 μ L/min) of (A) chlorpheniramine, m/z 275 \rightarrow 230, (B) diphenhydramine, m/z 256 \rightarrow 167, with LC/MS/MS-APCI analysis of 500 μ g/mL of phosphatidylcholine using a ZORBAX-SB C_{18} column with [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase, (C) m/z 184 \rightarrow 184, and (D) m/z 104 \rightarrow 104.

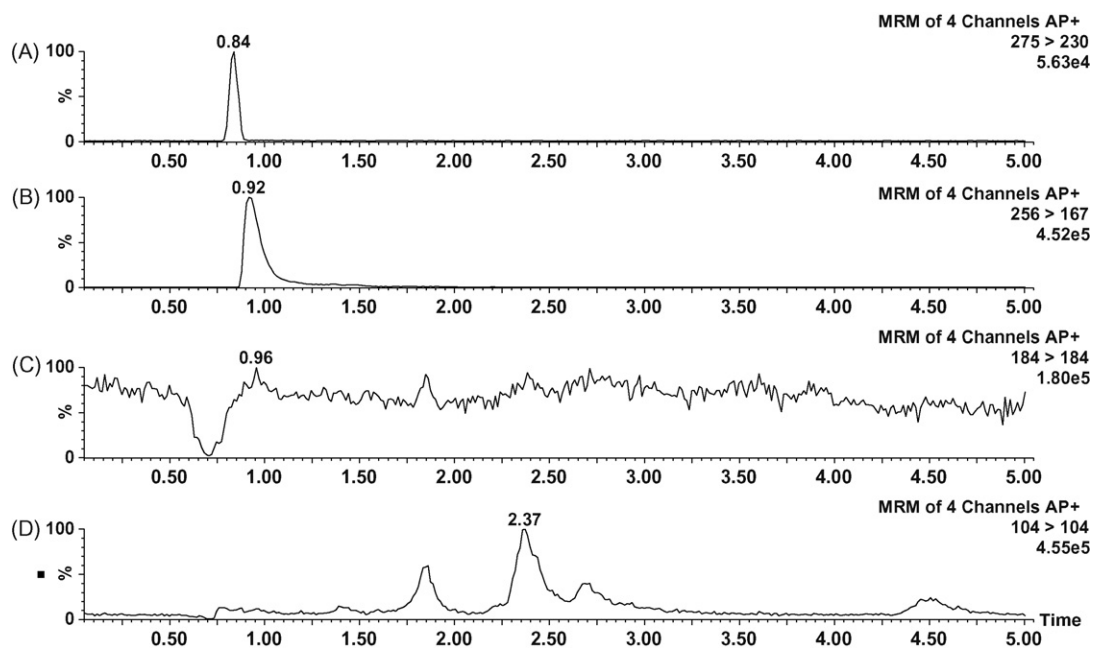


Fig. 11. Chromatogram of (A) 5 ng/mL chlorpheniramine, m/z 275 \rightarrow 230, (B) 2.5 μ g/mL diphenhydramine, m/z 256 \rightarrow 167, (C) glycerophosphocholines, m/z 184 \rightarrow 184, and (D) 2-lyso glycerophosphocholines, m/z 104 \rightarrow 104.

3.3. Method development for quantitative analysis of chlorpheniramine using the APCI mode

A mobile phase consisting of [70:30:1] methanol:water:formic acid (v/v/v) was described before for LC/MS/MS analysis of chlorpheniramine in human plasma using a C_{18} column [26]. However, it was found that acetonitrile was a stronger solvent and provided more consistent elution than methanol for phospholipids. Other technical problems were observed with using methanol such as high back pressure and emulsification during of the extract reconstitution with mobile phase. [70:30:1] acetonitrile:water:formic acid (v/v/v) mobile phase was found to be the most appropriate mobile phase. The analyte peaks were chromatographically resolved from the phospholipids peaks (Fig. 11). Relatively low sensitivity for diphenhydramine in the APCI mode was observed so the concentration of diphenhydramine was increased to obtain a suitable response and good internal standard tracking.

3.4. Post-extraction addition experiment

Post-extraction addition experiments were conducted to determine the degree of matrix effects that occurred during LC/MS/MS analysis of chlorpheniramine under both ESI and APCI modes using the adjusted chromatographic conditions in each case as described under Sections 2.7 and 2.8. The average area response of sample extracts with analytes added post-extraction with the same concentrations of the analytes prepared in the reconstitution solutions were compared. Matrix effects were reduced from 65% in the ESI mode to only 15% in the APCI mode. Using the phospholipid elution profile as a marker for matrix components was helpful in minimizing the matrix ionization effects by adjusting the HPLC conditions and selecting the appropriate stationary phase, mobile phase and ionization source.

3.5. Validation

3.5.1. ESI mode

The initial LC/MS/MS for determination of chlorpheniramine in human plasma using the ESI mode and the diphenhydramine inter-

nal standard showed inaccurate and imprecise results mainly due to a high degree of matrix effects and poor internal standard tracking. The inter-assay precision of quality control QC samples at concentrations of (0.5, 4.0 and 80.0 ng/mL) was between 16.6% and 25.2% and the inter-assay accuracy was between -2.9% and 37.8% . The intra-assay precision of quality control QC samples was between 5.9% and 19.9% and the intra-assay accuracy was between -3.9% and 69.9%.

3.5.2. APCI mode

The LC/MS/MS method for determination of chlorpheniramine in human plasma with monitoring GPChos and 2-lyso GPChos

Table 2

Precision and accuracy calculated from quality control (QC) samples for chlorpheniramine using APCI mode and the modified ESI method.

Quality control samples (ng/mL)			
	0.5	4.0	80.0
APCI method			
Inter-run precision and accuracy			
Mean	0.54	4.31	83.30
S.D.	0.05	0.3	8.3
%R.S.D.	8.5	7.0	10.0
%DFN	8.0	7.6	4.1
Intra-run precision and accuracy			
Mean	0.56	4.17	81.50
S.D.	0.05	0.2	6.9
%R.S.D.	9.5	5.5	8.5
%DFN	11.7	4.3	1.9
Modified ESI method			
Inter-run precision and accuracy			
Mean	0.51	4.23	81.86
S.D.	0.03	0.2	5.9
%R.S.D.	6.2	5.4	7.2
%DFN	2.9	5.7	2.3
Intra-run precision and accuracy			
Mean	0.51	4.34	87.40
S.D.	0.02	0.07	0.7
%R.S.D.	4.0	1.5	0.8
%DFN	2.5	8.4	9.3

Table 3

Relative matrix effects experimental results of 10 different lots of plasma fortified with chlorpheniramine using the APCI method and the modified ESI method.

Individual sources	Relative matrix effects study of chlorpheniramine (0.5 ng/mL)									
	1	2	3	4	5	6	7	8	9	10
APCI data										
Mean	0.51	0.54	0.61	0.70	0.49	0.43	0.52	0.44	0.48	0.56
S.D.	0.04	0.04	0.06	0.04	0.01	0.01	0.33	0.04	0.09	0.07
%R.S.D.	8.0	7.7	9.1	6.0	1.2	1.3	63.5	8.1	17.6	11.5
%DFN	1.3	8.7	21.3	39.3	−1.3	−13.3	4.7	−13.0	−3.3	12.7
ESI data										
Mean	0.43	0.51	0.46	0.47	0.44	0.45	0.46	0.48	0.48	0.52
S.D.	0.00	0.06	0.02	0.02	0.04	0.03	0.02	0.02	0.01	0.02
%R.S.D.	0.7	11.6	4.5	4.9	8.9	5.6	5.1	4.2	2.5	4.2
%DFN	−14.0	1.7	−7.5	−5.2	−12.3	−9.7	−8.2	−3.8	−3.2	3.5

as markers for matrix effects was validated. Ten extracted blank plasma samples were injected before each run for column conditioning. The total run time was 5 min. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with accuracy (%DFN) within $\pm 20\%$ and a precision (%R.S.D.) within 20%, the LLOQ in this study was 0.2 ng/mL. A quadratic regression weighted by inverse concentration was used to generate the calibration curves over the concentration range of 0.2–100 ng/mL. The percent relative standard deviation (%R.S.D.) of back-calculated standards was between 4.4% and 13.2% for all standards of chlorpheniramine. The percent difference from nominal concentration (%DFN) was between −6.0% and 4.6% for all standards of chlorpheniramine. Selectivity was studied by analyzing six blank plasma samples from different individuals for interference at the retention times of the analytes. All matrix samples were free from interferences (less than 20% of the mean response at the lower limit of quantification prepared in pooled plasma) at the retention times of chlorpheniramine and diphenhydramine.

Inter- and intra-assay precision and accuracy were calculated from quality control samples at three concentration levels over three validation days as summarized in Table 2. The overall inter- and intra-assay precision (measured as percent relative standard deviation, %R.S.D.) was $\leq 10.0\%$ and 9.5% , respectively. The inter- and intra-assay accuracy (measured as percent difference from nominal, %DFN) was $\leq 8.0\%$ and 11.7% , respectively.

The inter- and intra-assay precision at the lower limit of quantification (LLOQ) was 13.5% and 12.1%, respectively. The inter- and intra-assay accuracy of the (LLOQ) was 0.4% and −0.8%, respectively. The extraction recoveries for chlorpheniramine at 0.2, 5.0 and 100.0 ng/mL were 92.4%, 92.1% and 86.6% (%R.S.D. 3.61), respectively. Freeze/thaw stability was investigated over four freeze and thaw cycles from -20°C to room temperature, quality control (QC) samples ($n=3$) at concentrations of 0.500, 4.00, and 80.0 ng/mL of chlorpheniramine were frozen for at least 24 h for cycle one then each consecutive cycle was frozen for at least 12 h prior to thawing at room temperature. Bench stability was studied by removing (QC) samples ($n=3$) from -20°C storage, thawing to room temperature and allowing them to sit on the bench 4 h before starting analysis. Post-preparative stability was evaluated by storing the samples after preparation in the autosampler at approximately 4°C for 72 h. All stability studies showed results to within 15% of the reference. Long-term stability was tested by comparing frozen QC samples with freshly prepared QC samples. Samples were stable for 204 days at -20°C .

The validation demonstrated acceptable results according to FDA guidelines [27]. The post-extraction addition experiment showed 15% absolute matrix effects in the plasma source selected. Inter-subject variability is a potential problem during LC/MS/MS analysis due to variable matrix effects that may exist from different sources and it is rarely addressed [5]. To investigate the

effect of variable matrix effects from multiple individuals, quality control samples ($n=3$) at a concentration of 0.5 ng/mL of chlorpheniramine were spiked into 10 different human blank plasma sources, extracted and analyzed as described under Sections 2.4 and 2.9, respectively. Seven out of 10 subjects showed acceptable accuracy and precision within 15%, three subjects were outside of the acceptable range (Table 3). This may have been related to a different amount of phospholipids from different sources or may be due to matrix effects associated with the extraction process. These results would indicate that caution is necessary when analysis of diverse sample lots is necessary and that acceptable results in one source of plasma may be insufficient to ensure the validity of the method in multiple sources.

3.5.3. ESI mode with deuterated internal standard

Because of the problem indicated by the multiple source matrix effect experiment using the APCI method, further investigation was carried out to investigate the source of the problem and to modify the method to overcome the problem. Post-column infusion studies were performed for the same 10 lots of plasma used in the APCI multiple source matrix effect study. There was no indication from this investigation that the phospholipids monitored caused the matrix effect through suppression or enhancement and it was speculated that the unacceptable results could be due to poor internal standard tracking associated with the extraction procedure when multiple sources of matrix are used. A modified method using a deuterated internal standard for chlorpheniramine that was unavailable from a commercial source previously was then employed. The use of a deuterated internal standard permitted re-examination of the ESI mode which demonstrated sensitivity greater than that of the APCI mode. The gain in sensitivity during these experiments also allowed us to reduce the sample size. The validation results were acceptable as were the APCI mode results using diphenhydramine as an internal standard (see Table 2). The multiple source matrix effect experiment was also repeated using the same 10 lots of plasma as used during the previous APCI mode study. Quality control samples ($n=3$) at concentration of 0.5 ng/mL of chlorpheniramine were spiked into 10 different human blank plasma sources, extracted and analyzed as described under Sections 2.4 and 2.8, respectively. All 10 showed acceptable accuracy and precision (Table 3). This shows the importance of extending matrix effect evaluations to include multiple sources of matrix. Using a quantitative assessment in addition to evaluating post-column infusion, and post-extraction addition.

4. Conclusion

The ion suppression noted for chlorpheniramine and the ion enhancement noted for diphenhydramine coincided with the elution profiles of phospholipids that were monitored using the m/z

184 and m/z 104 ions. Ion enhancement correlating to the phospholipid peaks was observed previously in the negative ion ESI mode [28]. This is the first report of correlation of ion enhancement to phospholipids in the positive mode. We found that monitoring phospholipids was useful in method development to avoid matrix effects by selection of the appropriate stationary phase, mobile phase and ionization source. It was also found that GPChos and 2-lyso GPChos demonstrated very low ionization efficiency in the APCI mode. The apparent decrease in the ionization efficiency of phospholipids in the APCI mode would be considered an advantage analytically and may explain why APCI is traditionally less susceptible to matrix effects than ESI. Quantitative studies carried out in multiple sources of plasma matrix indicated intersource variability with an otherwise valid method. This variability was apparently not due to ion suppression or enhancement as indicated by post-column infusion studies. The importance of an isotopically labeled internal standard was shown when the variability was corrected by use of a deuterated chlorpheniramine. The results indicate the appropriateness of extending matrix effects experiments, especially when a labeled internal standard is unavailable. Multiple source matrix effect evaluations should be investigated quantitatively to ensure the validity of a method when different plasma sources are used.

References

- [1] S. Souverain, S. Rudaz, J.-L. Vouthey, *J. Chromatogr. A* 1058 (2004) 61.
- [2] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, *J. Am. Soc. Mass Spectrom.* 14 (2003) 1290.
- [3] S. Wang, M. Cyronak, E. Yang, *J. Pharm. Biomed. Anal.* 43 (2007) 701.
- [4] J.C. Van De Steene, K.A. Mortier, W.E. Lambert, *J. Chromatogr. A* 1123 (2006) 71.
- [5] P.J. Taylor, *Clin. Biochem.* 38 (2005) 328.
- [6] J. Schuhmacher, D. Zimmer, F. Tesche, V. Pickard, *Rapid Commun. Mass Spectrom.* 17 (2003) 1950.
- [7] C. Chen, Z.P. Zhang, H.T. Karnes, *J. Pharm. Biomed. Anal.* 35 (2004) 1149.
- [8] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, *Rapid Commun. Mass Spectrom.* 13 (1999) 1175.
- [9] P. Bennett, H. Liang, *Proceedings of the American Society for Mass Spectrometry (ASMS) Conference*, Nashville, Tennessee, 2004, p. 1. Available at http://www.tandemlabs.com/capabilities_publications.html.
- [10] O.A. Ismaiel, M.S. Halquist, M.Y. Elmamly, A. Shalaby, H.T. Karnes, *J. Chromatogr. B* 859 (2007) 84–93.
- [11] A. Kloepper, J.B. Quintana, T. Reemtsma, *J. Chromatogr. B* 1067 (2005) 153.
- [12] J.L. Little, M.F. Wempe, C.M. Buchanan, *J. Chromatogr. B* 833 (2006) 219.
- [13] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, *J. Chromatogr. B* 852 (2007) 22.
- [14] The U.S. P 24 revision, NF 19, United States Pharmacopeial convention, Rockville, MD, 392, 2000.
- [15] A. Goth, *Medical Pharmacology Principals and Concepts*, 10th edition, C.V. Mosby Company, St. Louis, 1981.
- [16] T. Takagaki, M. Matsuda, Y. Mizuki, Y. Terauchi, *J. Chromatogr. B* 776 (2002) 169.
- [17] C. Celma, J.A. Allue, J. Prunonosa, C. Peraire, R. Obach, *J. Chromatogr. A* 870 (2000) 77.
- [18] A. Marín, E. García, A. García, C. Barbas, *J. Pharm. Biomed. Anal.* 29 (2002) 701.
- [19] A. García, F.J. Rupérez, A. Marín, A. de la Maza, C. Barbas, *J. Chromatogr. B* 785 (2003) 237.
- [20] Y. Dong, X. Chen, Y. Chen, X. Chen, Z. Hu, *J. Pharm. Biomed. Anal.* 39 (2005) 285.
- [21] ACD/ChemSketch Freeware, version 10.00, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2006. www.acdlabs.com.
- [22] M. Pulfer, R.C. Murphy, *Mass Spectrom. Rev.* 22 (2003) 332.
- [23] A. Takatera, A. Takeuchi, K. Saiki, T. Morisawa, N. Yokoyama, M. Matsuo, *J. Chromatogr. B* 838 (2006) 31.
- [24] X. Han, R.W. Gross, *J. Am. Chem. Soc.* 118 (1996) 451.
- [25] F.-F. Hsu, J. Turk, A.K. Thukkani, M.C. Messner, K.R. Wildsmith, D.A. Ford, *J. Mass Spectrom.* 38 (2003) 742.
- [26] X. Chen, Y. Zhang, D. Zhong, *Biomed. Chromatogr.* 18 (2004) 248.
- [27] US Department of Health and Human Services, Food and Drug Administration, 2001. <http://www.fda.gov/cder/guidance/4252fn1.htm>.
- [28] T. Zhang, M. Meng, K.V. Horne, P.K. Bennett, *Proceedings of the American Association of Pharmaceutical Scientists (AAPS) conference*, Nashville, TN, 2005, p. 9. Available at http://www.tandemlabs.com/capabilities_publications.html.