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Monitoring phospholipids for assessment of matrix effects in a liquid chromatography–tandem mass spectrometry method for hydrocodone and pseudoephedrine in human plasma

Omnia A. Ismaiel [a,1], Matthew S. Halquist [a,2], Magda Y. Elmamly [b], Abdalla Shalaby [b], 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] *Zagazig University, Faculty of Pharmacy, Department of Analytical Chemistry, Egypt*

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Abstract

Matrix effects resulting in ion suppression or enhancement have been shown to be a source of variability and inaccuracy in bioanalytical mass spectrometry. Glycerophosphocholines may cause significant matrix ionization effects during quantitative LC/MS/MS analysis and are known to fragment to form characteristic ions (*m/z* 184) in electrospray mass spectrometry. This ion was used to monitor ion suppression effects in the determination of hydrocodone and pseudoephedrine in human plasma as a means to track and avoid these effects. The *m/z* 184 ion fragment was detected in both plasma extracts and solutions of phosphatidylcholine. Post-column infusion studies showed that the ion suppression for both drugs and internal standards correlated with the elution of phospholipids. HPLC conditions were adjusted to chromatographically resolve the peaks of interest from the phospholipids. Upon repeated injection, the elution time of the phospholipids decreased while elution of the analyte peaks remained unchanged. This resulted in co-elution and significantly affected peak shape and internal standard response for the analytes. It was decided to use the phospholipid fragment to monitor this matrix effect in validation samples. The resulting method demonstrated intra-day and inter-day precision within 4.5 and 5.6% for hydrocodone and pseudoephedrine, respectively, and accuracy within 8.9 and 8.7% for hydrocodone, and pseudoephedrine, respectively. There was no statistically significant difference in the internal standard response for the determination with and without monitoring the phospholipid fragment ion. We found that monitoring the phospholipid fragment was useful in method development to avoid the matrix effects, and in routine analysis to provide a practical way to ensure the avoidance of matrix effects in each individual sample.

Keywords: Matrix effects; LC/MS/MS; HPLC; Validation

1. Introduction

High-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) is the method of choice for quantitative bioanalysis of small molecules due to its sensitivity and selectivity. However, there are several endogenous matrix components that may co-elute with the target analytes, which are often invisible to the MS detector at analytical method masses but that may significantly affect the efficiency and reproducibility of the ionization process [1]. These result in signal enhancement or suppression, which is termed a matrix effect. The exact mechanism of matrix effects is unknown but it is likely due to competition reactions between an analyte and the co-eluting matrix component [2–4]. Because of the effects on ionization efficiency, precision, accuracy, and sensitivity may be affected; matrix effects should be studied and evaluated during method development. Rogatsky and Stein [5]

* Corresponding author. Tel.: +1 804 8283819; fax: +1 804 8288359.
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.T. Karnes).
[1] Tel.: +1 804 7260093, fax: +1 804 8281273.
[2] Tel.: +1 804 827 2078.

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stated that there are only 20% of quantitative analysis studies from plasma samples that investigated matrix effects and less than 5% investigated matrix effects in detail. Post-column infusion and post-extraction addition methods are the most common method for matrix effect evaluation [4]. There are few studies that have evaluated and minimized matrix effects by optimization of the sample extraction procedures, assessment of differential suppression from multiple sources of matrix, and the use of isotopically labeled internal standards [3,4,6,7]. Labeled internal standards may not be adequate for matrix effect compensation, especially if the analyte and internal standard elute near a large and sloping matrix suppression peak. A slight change in the retention time may yield different levels of matrix effects on analyte and internal standard peaks, which could affect the accuracy and precision of quantification [1].

Phospholipids are the main constituents of cell membranes and the main class of compounds that cause significant matrix effects [8,9]. Van Horn and Bennett [8] developed an approach to reduce these effects by removing phospholipids from the biological matrix by use of a specific lanthanide sorbent column. Phospholipids are composed of ester or amide derivatives of glycerol or sphingosine with fatty acids and phosphoric acid. The phosphate moiety is esterified with choline, serine or ethanolamine. Glycerophosphocholines (e.g. phosphatidylcholine) are considered the major phospholipids in plasma and cause significant matrix ionization effects during LC/MS/MS analysis [9]. Mono- and di-substituted glycerophosphocholines and also other phospholipids such as sphingomyelins, which are present at lower concentrations in plasma have been shown to fragment to form trimethylammonium-ethyl phosphate ions (*m/z* 184) in LC/MS/MS [9]. Little et al. [9] developed an approach, which was referred to as in-source multiple reaction monitoring (IS-MRM) for detection of glycerophosphocholines during LC/MS/MS analysis using only one channel in an MRM LC/MS/MS experiment. Complete elution of glycerophosphocholines occurred after each injection of biological extract. They adjusted the instrumental parameters as required for monitoring trimethylammonium-ethyl phosphate ions (*m/z* 184) without further fragmentation and studied various factors affecting elution of glycerophosphocholines.

Hydrocodone (4,5α-epoxy-3-methoxy-17-methylmorphinan -6-one) is a semi-synthetic opioid analgesic, more potent antitussive than codeine and more addictive. It is usually used for relief of moderate pain [10–12]. High-performance liquid chromatographic (HPLC) methods [12–14] and gas chromatographic (GC) methods [15–17] have been reported for determination of hydrocodone and its metabolites in plasma, blood, urine, human hair extracts, human liver microsomes and postmortem fluids. Pseudoephedrine (benzenemethanol, α-[1-(methylamino) ethyl]-, [S-(R*, R*)]) is an active stereoisomer of ephedrine, used for relief of nasal congestion in cases of rhinitis and effective in acute asthma treatment [10,11]. Several high-performance liquid chromatographic (HPLC) methods [18–22], gas chromatographic (GC) methods [23,24] and capillary electrophoresis [25,26] have been described for determination of pseudoephedrine in plasma and pharmaceutical preparations.

A simple and sensitive method for the determination of hydrocodone and pseudoephedrine in human plasma is reported that utilizes the *m/z* 184 mass fragment of the glycerophosphocholines to evaluate matrix effects. The analytes were isolated using methyl tertiary butyl ether as the extraction solvent. Hydrocodone-d_3 and pseudoephedrine-d_3 were used as isotopically labeled internal standards for hydrocodone and pseudoephedrine, respectively. Use of the phospholipids as a marker for the matrix components that cause suppression or enhancement effects was done to provide a means of tracking and avoiding these effects in method development. We also studied and compared the elution of glycerophosphocholines from different plasma sources, and investigated the effect of several biological extract injections on the elution time of glycerophosphocholines to determine the potential effect of repeated injections on analytical performance. Monitoring phospholipids may provide a means to ensure the avoidance of matrix effects in each individual sample and may provide a more practical tool for avoiding matrix effects than commonly used post-extraction addition and post-column infusion. Validation data was collected with and without monitoring the phospholipids mass transition *m/z* 184 > 184 to investigate the effect of increasing the number of mass transitions monitored on the intensity and reproducibility of each peak of interest.

2. Experimental

2.1. Reagents

Hydrocodone and pseudoephedrine were purchased from Alltech (State College, PA, USA), Hydrocodone-d_3 was obtained from Isotech Inc. (Miamisburg, OH, USA), and Pseudoephedrine-d_3 was purchased from Cerriliant (Round Rock, TX, USA). Phosphatidylcholine was 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 Liquid chromatograph, Shimadzu, System Controller, SCL-10A Vp, Pumps, LC 10AD Vp, Solvent Degasser, DGU14A, and autosampler, SIL-10AD Vp (Columbia, MD, USA). The Mass Spectrometer was Micromass Quattro API Micro, Waters Corp. with a Data acquisition, Masslynx version 4.0 and 4.1 installed on IBM think center com-

puter, Waters Corp. (Milford, MA, USA), which was operated in the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode.

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

Two separate 100 μg/mL stock solutions of each analyte were prepared in methanol and stored at approximately −20 °C in silylated glassware. The solutions were prepared by diluting 500 μL of a 1.00 mg/mL standard solution of each analyte to 5.0 mL with methanol. After checking responses form these two solutions, the two solutions were combined into one stock solution to prepare calibration standards and quality control (QC) samples. The calibration standards were prepared by adding appropriate amounts of the stock solution into pooled blank plasma. The nominal concentrations were 0.2, 0.45, 0.75, 5.0, 25.0, 50.0 and 100 ng/mL for hydrocodone and 50.0, 100.0, 250.0, 500.0, 750.0, 900.0 and 1000 ng/mL for pseudoephedrine. QC samples at concentrations of 0.5, 4.0 and 80.0 ng/mL for hydrocodone and 150, 400 and 800 ng/mL for pseudoephedrine were prepared. Dilution QC samples at 300 and 3000 ng/mL and LOQ QC samples at 0.2 and 50.0 ng/mL of hydrocodone and pseudoephedrine, respectively, were also prepared. Standards and controls were sub-aliquoted into 13 mm × 100 mm polypropylene tubes and stored at approximately −20 °C.

2.4. Sample preparation

Human plasma samples were thawed at room temperature and vortex mixed. A 500 μL aliquot of each sample was placed into a silanized 13 mm × 100 mm screw cap culture tube, 25 μL of freshly prepared working internal standard (combined solution of 3.00 μg/mL of pseudoephedrine-d_3 and 500 ng/mL hydrocodone-d_3), was added and mixed briefly, 50 μL of 0.1N sodium hydroxide, was added and 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, then the organic layer was transferred to a silanized 16 mm × 100 mm screw cap conical tube and evaporated in the TurboVap® under a nitrogen stream at approximately 40 °C to dryness, the residue was reconstituted with 50 μL of reconstitution solution, [20:80] 2.0 mM ammonium format in methanol:acetonitrile v/v, vortexed, and transferred to sample vials with silanized inserts. A 10 μL of the resulting solution was injected into LC/MS/MS.

2.5. Monitoring phospholipids

Optimized parameters for monitoring *m/z* 184 as a common ion fragment for glycerophosphocholines without further fragmentation were used [9]. These ions were detected in both plasma extracts and solutions of the phosphatidylcholine isolated from chicken eggs.

2.6. Post-column infusion

Post-column infusion experiments were conducted in which a 100 ng/mL solution of hydrocodone, pseudoephedrine, hydrocodone-d_3, and pseudoephedrine-d_3 in the reconstitution solution was constantly infused (10 μL/min) into the MS. A 500 μL aliquot of human plasma was extracted as described under Section 2.4, and the matrix extract injected pre-column. For the isolated phospholipids solutions, a 250 μg/mL phosphatidylcholine solution was prepared in the reconstitution solution and was injected pre-column.

2.7. Isocratic method for the analysis of hydrocodone and pseudoephedrine

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), Frit SS Nat 2 μm 0.094 × 0.65 × 0.2485 × 10 pk, A-100X, Upchurch Scientific, Inc. (Oak Harbor, WA, USA). Mobile phase A consisted of 2 mM ammonium formate in methanol, and mobile phase B was acetonitrile. An isocratic method using [20:80] 2 mM ammonium formate in methanol:acetonitrile v/v mobile phase with 0.35 mL/min flow rate was performed. The autosampler utilized a rinse solution comprised of 95:5 methanol:2% formic acid, with a run time of 10–12 min, rinse volume (500 μL) pre- and post-injection and injection loop volume (20 μL). The MS/MS System parameters were: capillary (3.00 kvolts), extractor (2.00 volts), source temperature (120 °C), desolvation temperature (400 °C), desolvation gas flow (300 L/h), collision gas (Argon), collision energy, mass transitions and MRM parameters as shown in Table 1.

Table 1
Multiple reaction monitoring (MRM) parameters for hydrocodone, pseudoephedrine, hydrocodone-d_3, pseudoephedrine-d_3 and glycerophosphocholines

Compound name	Nominal parent (*m/z*)	Nominal daughter (*m/z*)	Dwell (s)	Cone (volts)	Collision energy (eV)
Hydrocodone	300.0	199.0	0.250	40.0	30.0
Pseudoephedrine	166.0	148.0	0.150	20.0	12.0
Hydrocodone-d_3	303.0	199.0	0.150	40.0	30.0
Pseudoephedrine-d_3	169.0	151.0	0.150	20.0	12.0
Phospholipids	184.0	184.0	0.05	90.0	7.0

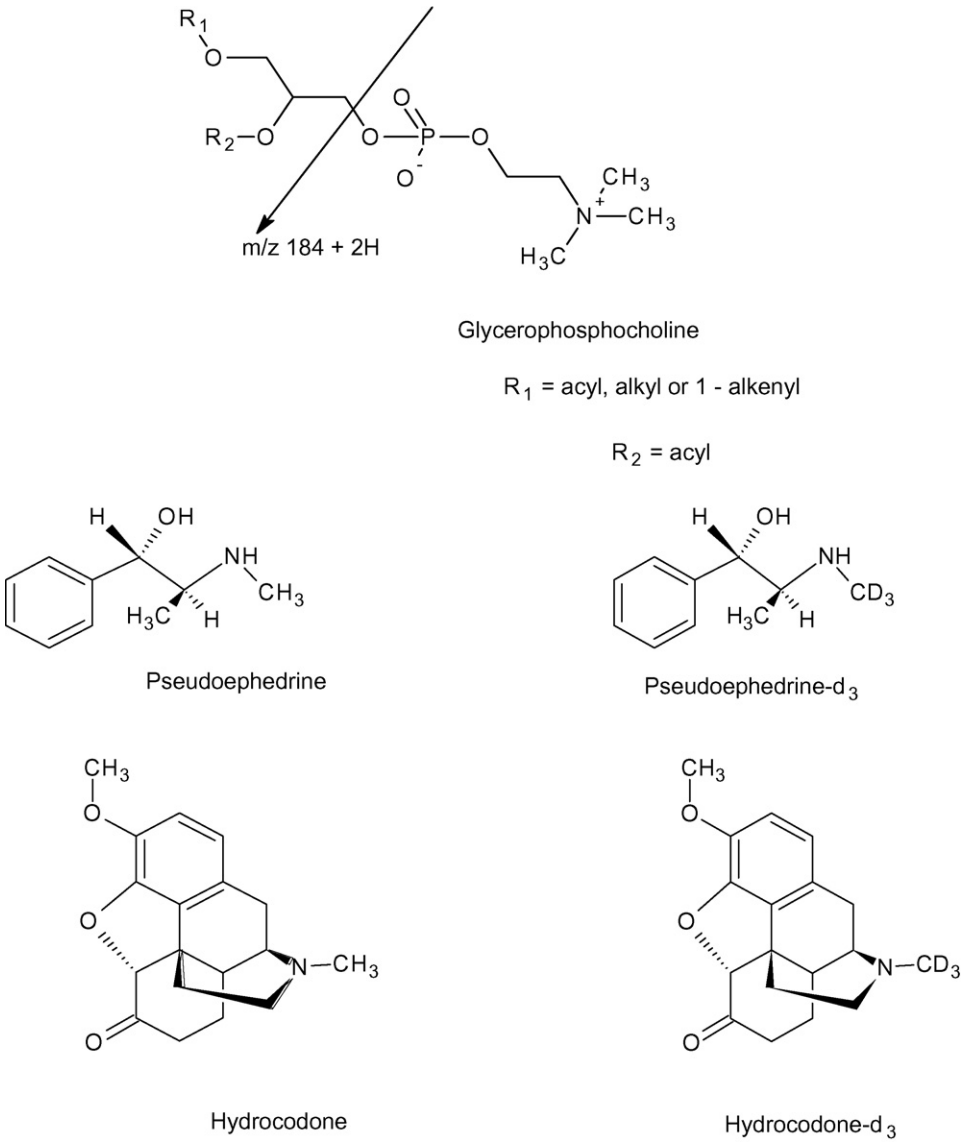

Fig. 1. Chemical structures of glycerophosphocholines, pseudoephedrine, pseudoephedrine-d_3, hydrocodone and hydrocodone-d_3 [9,28].

2.8. Validation

2.8.1. 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 hydrocodone, pseudoephedrine, hydrocodone-d_3 and pseudoephedrine-d_3, vortex mixed transferred to sample vials with silanized inserts and injected (10 μL) onto the LC/MS/MS. Solutions containing an equivalent amount of hydrocodone, pseudoephedrine, hydrocodone-d_3 and pseudoephedrine-d_3 in the reconstitution solution ($n = 3$) were injected as described under Section 2.4.

2.8.2. Determination of hydrocodone and pseudoephedrine while monitoring phospholipids

Validation runs were analyzed with and without monitoring the phospholipids mass transition m/z 184 > 184. Human plasma samples were aliquoted and extracted as described under Section 2.4. The relative abundance for the internal standard was compared for the two analyses using a t-test [27].

2.8.3. Monitoring phospholipids in different plasma sources

Fifteen sources of human plasma were extracted as described under Section 2.4, the phospholipid peaks were monitored as described under Section 2.7.

2.8.4. Effect of repeated injections on the elution of phospholipids

Quality control samples of hydrocodone and pseudoephedrine were extracted as described under Section 2.4. The phospholipids peaks were monitored as described under Section 2.7 for approximately 350 injections using two Betasil Diol-100, 50 mm × 2.1 mm, 5 μm particle size columns to investigate the effect of repeated injections on the elution of phospholipids.

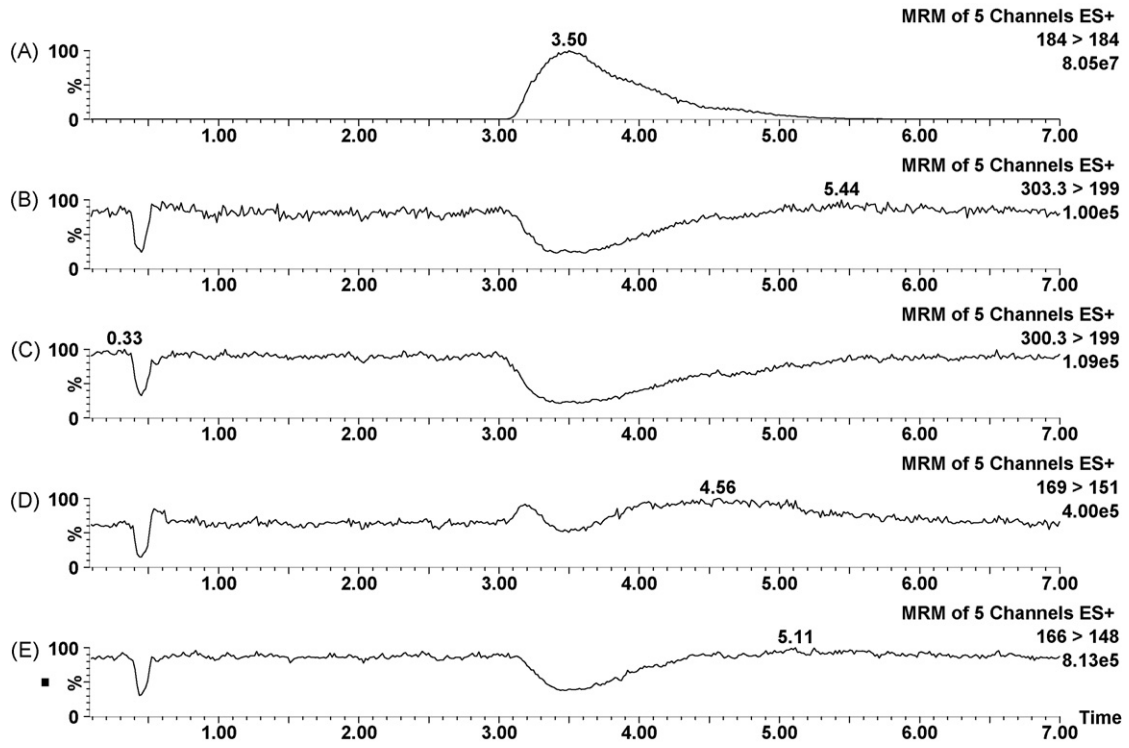

Fig. 2. (A) LC/MS/MS chromatograms of 250 μg/mL phosphatidylcholine solution, *m/z* 184 → 184 and matrix ionization effects with post-column infusion (100 ng/mL, 10 μL/min) of (B) hydrocodone-d_3, *m/z* 303 → 199; (C) hydrocodone, *m/z* 300 → 199; (D) pseudoephedrine-d_3, *m/z* 169 → 155; (E) pseudoephedrine, *m/z* 166 → 148.

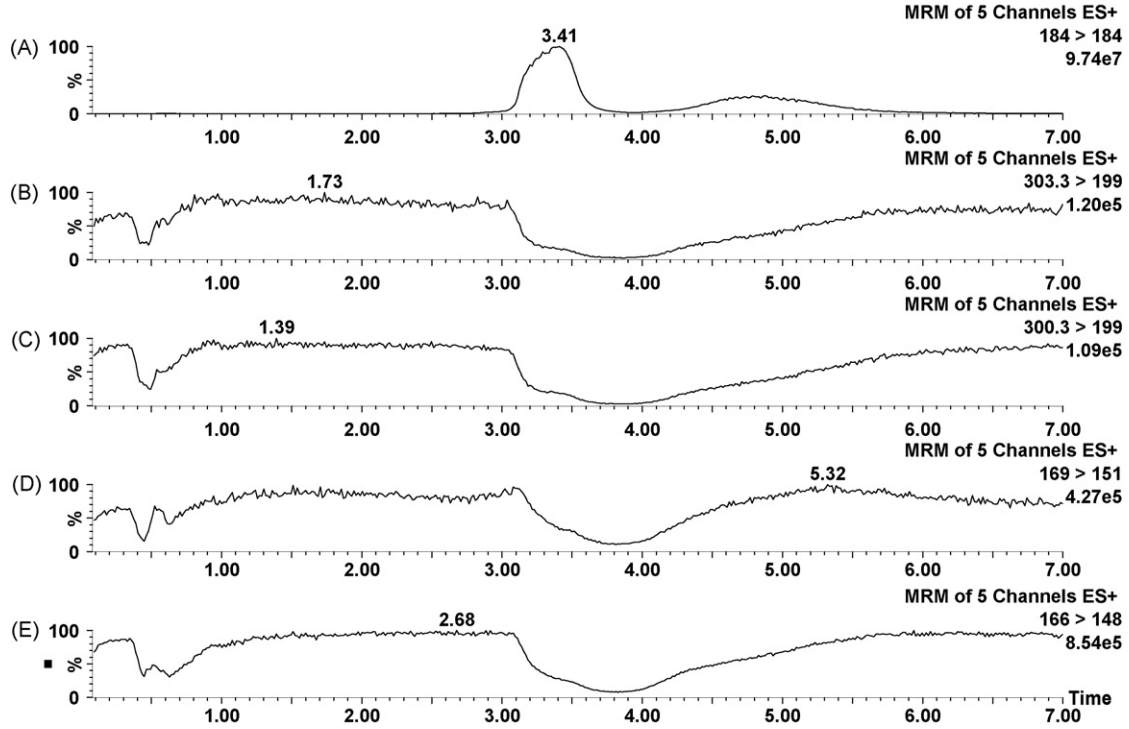

Fig. 3. (A) LC/MS/MS analysis of extracted plasma, *m/z* 184 → 184 and matrix ionization effects with post-column infusion (100 ng/mL, 10 μL/min) of (B) hydrocodone-d_3, *m/z* 303 → 199; (C) hydrocodone, *m/z* 300 → 199; (D) pseudoephedrine-d_3, *m/z* 169 → 155; (E) pseudoephedrine, *m/z* 166 → 148.

3. Results and discussion

3.1. Monitoring phospholipids

A wide variety of glycerophosphocholines can be monitored using one mass transition at *m/z* 184. Mono- and di-substituted glycerophosphocholines and also other phospholipids, such as sphingomyelins, which are present at lower concentrations in plasma, have been shown to fragment to form trimethylammonium-ethyl phosphate ions (*m/z* 184) in LC/MS/MS [9]. The trimethylammonium-ethyl phosphate ion fragment (*m/z* 184) was detected in both plasma extract after liquid–liquid extraction and in standard solutions of phosphatidylcholine using a cone voltage at 90 V, collision energy at 7 V, with the dwell time at 0.05 s and an interscan delay time of 0.05 s (Fig. 1).

3.2. Post-column infusion

Post-column infusion experiments were carried out to determine ionization effects on the analytes from phospholipids during LC/MS/MS analysis with both blank plasma extracts and a standard solution of phosphatidylcholine. The ion suppression observed at the beginning of the chromatograms (0.5–0.8 min) may be due to the presence of salts and highly polar non-retained compounds [9]. By comparing the post-column infusion using phosphatidylcholine solutions (Fig. 2) with the extracted matrix (Fig. 3), it can be concluded that the 250 μg/mL phosphatidylcholine solution yielded a suppression window at essentially the same time in the chromatogram. The response of the phosphatidylcholine solution was approximately the same as that of the endogenous phospholipid related peaks in the matrix, according to peak height intensities. The post-column infusion studies showed that, the ion suppression noted for the drugs and internal standards correlated in time with the elution of the phospholipids (Figs. 2 and 3).

3.3. Method development for the analysis of hydrocodone and pseudoephedrine

Five extraction solvents (ether, MTBE, ethyl acetate:*n*-hexane (1:1), (1:9) and (9:1) were evaluated for hydrocodone and pseudoephedrine extraction ($n = 2$) for of extraction recovery and matrix effects (Fig. 4). Methyl tertiary butyl ether (MTBE) provided the highest recoveries for both compounds using hydrocodone-d_3 and pseudoephedrine-d_3 as labeled internal standards for hydrocodone and pseudoephedrine, respectively. Using the phospholipids as an indicator of matrix effects, HPLC conditions were adjusted to resolve the peaks of interest from the phospholipids (Fig. 5). An isocratic mobile phase using [20:80] 2.0 mM ammonium formate in methanol:acetonitrile v/v at a 0.35 mL/min flow rate was found to yield optimal retention and good peak shape for all compounds. A mobile phase containing methanol provided 50% of the hydrocodone and 30% of the pseudoephedrine response as compared to acetonitrile. Both analytes also demonstrated short retention times and poor peak shape for hydrocodone when methanol was used in the mobile phase. These results support monitoring phospholipids as a marker for matrix components during method development since this was helpful to maintain resolution of the peaks of interest from the phospholipids.

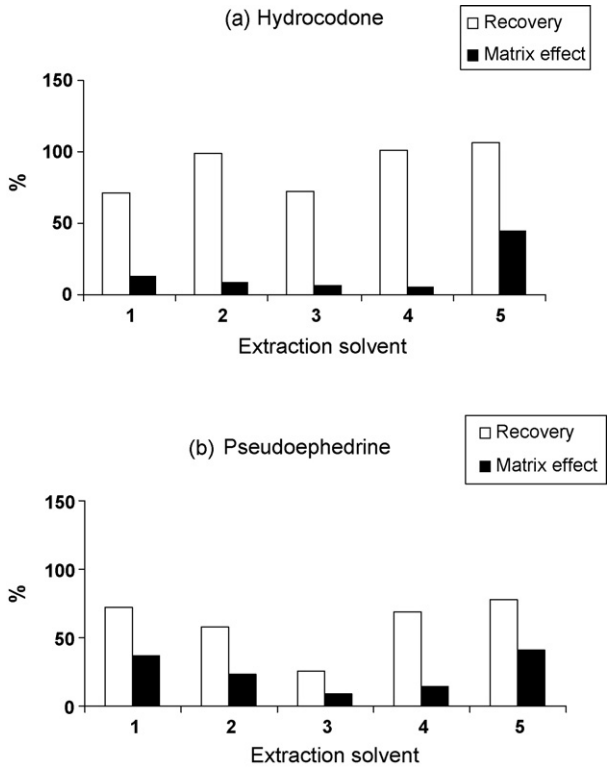

Fig. 4. Absolute recovery and matrix effect of (a) 50 ng/mL hydrocodone and (b) 500 ng/mL pseudoephedrine samples after liquid–liquid extraction using 3 mL of (1) ether, (2) ethyl acetate:*n*-hexane (1:1) v/v, (3) ethyl acetate:*n*-hexane (1:9) v/v, (4) ethyl acetate:*n*-hexane (9:1) v/v and (5) MTBE.

3.4. Post-extraction addition experiment

A post-extraction addition experiment was conducted to determine the degree of matrix effects that occurred during LC/MS/MS analysis. By comparing responses of sample extracts with the same concentrations of the analytes of interest added post-extraction from neat solution, the degree of matrix effects were evaluated. Matrix effects were reduced from 0.55 to 0.92 for hydrocodone and from 0.59 to 0.90 for pseudoephedrine by adjusting the HPLC conditions to resolve the peaks of interest from the phospholipids.

3.5. Determination of hydrocodone and pseudoephedrine with monitoring phospholipids

Increasing the number of mass transitions can potentially decrease the signal intensity and the signal to noise ratio for each peak of interest. A validation run was analyzed with and without monitoring the phospholipids to investigate this. The calibration parameters, slope, intercept and correlation coeffi-

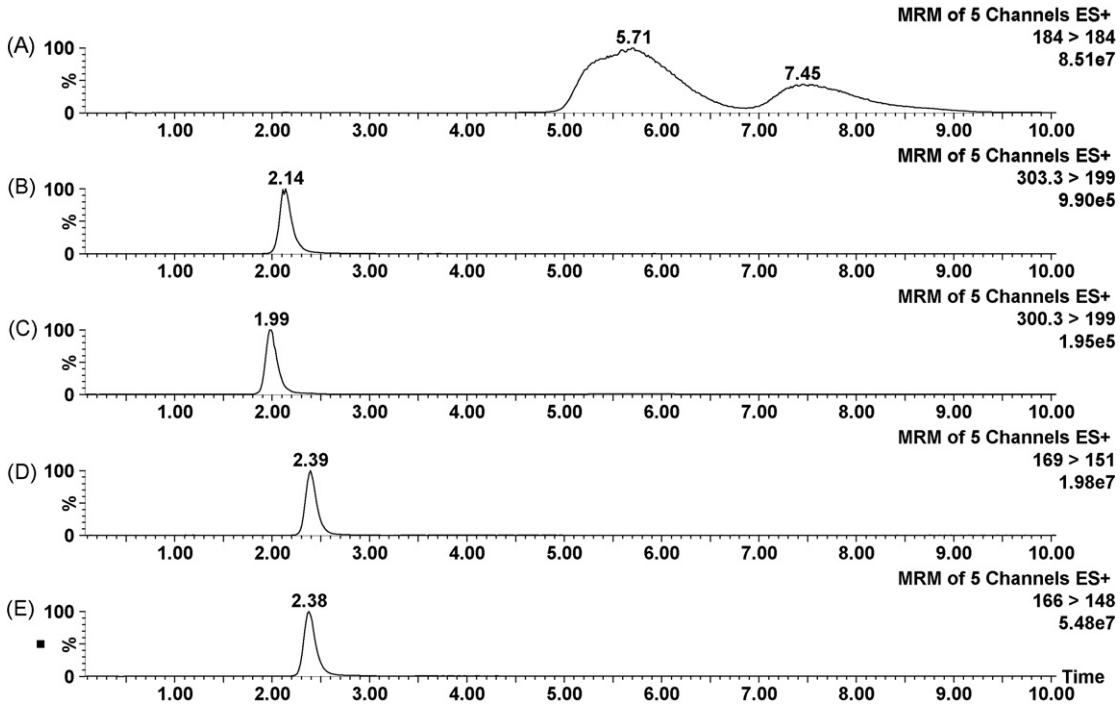

Fig. 5. Chromatograms of the resolved peaks, using an isocratic mobile phase of [20:80] 2.0 mM ammonium formate in methanol:acetonitrile v/v at a 0.35 mL/min flow rate. (A) Glycerophosphocholines, *m/z* 184 → 184, (B) 25 ng/mL hydrocodone-d_3, *m/z* 303 → 199, (C) 4 ng/mL hydrocodone, *m/z* 300 → 199, (D) 150 ng/mL pseudoephedrine-d_3, *m/z* 169 → 155 and (E) 400 ng/mL pseudoephedrine, *m/z* 166 → 148.

cient, for hydrocodone were 0.0416, 0.00025 and 0.996 with monitoring the 184 mass transition and were 0.0375, −0.000017 and 0.990 without monitoring the phospholipids mass transition. The calibration parameters, slope, intercept and correlation coefficient, for pseudoephedrine were 0.0696, 0.0665 and 0.986 with monitoring the phospholipids mass transition *m/z* 184 > 184 and were 0.0633, 0.0689 and 0.993 without monitoring the phospholipids mass transition *m/z* 184 > 184. The higher slope without monitoring the *m/z* 184 was unexpected and could have been due to day to day signal fluctuations of the mass spectrometer. In order to evaluate this, the relative abundance for the internal standards ($n = 25$) was compared for the two analytes using a *t*-test [27], The *t*-test results show that the mean response without monitoring *m/z* 184 is higher than that with monitoring *m/z* 184, but the difference is statistically non-significant. The additional mass transition for phospholipids therefore had no statistically significant impact on the sensitivity of the other mass transitions.

In order to investigate the effect of the additional mass transition on measurement at the LLOQ for both analytes, LLOQ samples ($n = 3$) were analyzed in the previous run with and without monitoring phospholipids. The intra-assay precision was 4.26% and 7.84% for hydrocodone and pseudoephedrine respectively, and the intra-assay accuracy was 5.0 and 5.36% for hydrocodone and pseudoephedrine, respectively. The results showed that there was no significant impact of the additional mass transition on the measurement at the LLOQ for both analytes.

3.6. Monitoring phospholipids in different blank plasma sources

The *m/z* 184 > 184 mass transition was monitored in fifteen different sources of human plasma, phospholipid signals were detected in all human plasma sources using the isocratic method, the retention times of the phospholipid peaks ranged from 3.7 to 4.2 min, the relative abundance of phospholipids ranged from 4.41×10^7 to 6.25×10^7 peak height intensity and the peak width ranged from 1.6 to 3.6 min. The intensity of the peak height and the peak width of the *m/z* 184 > 184 mass transition varied from one plasma source to another and may have been due to different levels and different types of phospholipids in each source. This observation further supports the need to monitor phospholipids during quantitative bioanalytical mass spectrometry.

3.7. Effect of repeated injections on the elution of phospholipids

The effect of multiple biological extract injections on the elution time of glycerophosphocholines was investigated using two Betasil Diol-100, 50 mm × 2.1 mm, 5 μm particle size columns (I and II) to determine the potential effect on analytical performance and matrix effects. It was found upon repeated injection that peaks associated with the phospholipids eluted faster while the elution of the analyte peaks remained unchanged. Phospholipids may have accumulated on the column upon repeated

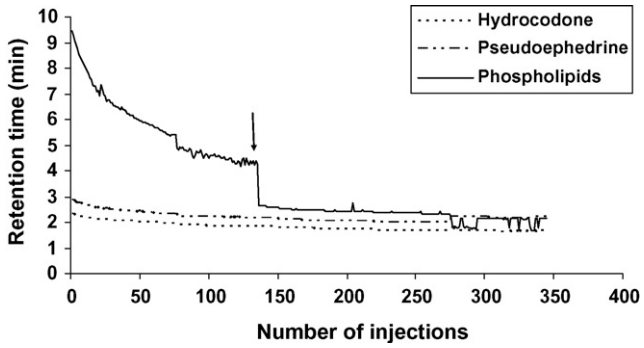

Fig. 6. The effect of repeated injection on the elution of the phospholipids using column (I). The arrow indicates a point at which the run was stopped and the column was left in the mobile phase overnight before starting the next batch of injections.

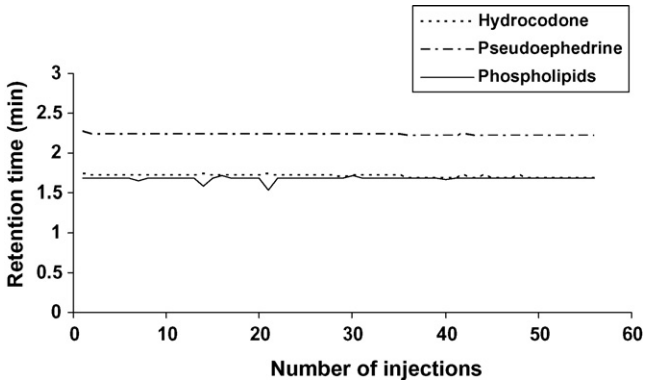

Fig. 7. Effect of repeated injection on the elution of the phospholipids using column II (used for approximately 300 injections before starting monitoring retention time of phospholipids).

injection and the column may have been saturated with phospholipids causing the phospholipids to elute faster, which indicates that phospholipids and the analytes may not competitively bind to the same sites on the stationary phase. Various organic solvents (acetonitrile, methanol, methylene chloride, isopropanol, chloroform, *n*-hexane) were used for column washing although these did not remove the accumulated phospholipids. As shown in Fig. 6 after 350 injections using column I, the phospholipids peaks co-eluted with the pseudoephedrine peak and very closely to the hydrocodone peak. Using column II as shown in Fig. 7 the phospholipid peaks co-eluted with hydrocodone after approximately 350 injections. The chromatograms in Figs. 5 and 8 show the resolved and the co-eluting peaks, respectively. Fig. 8 shows the effect of co-elution on the peak shape of the analytes. These results show inconsistent retention of the phospholipids.

The signal to noise S/N ratio for hydrocodone was 101.3 and 36.8 for resolved and co-eluting peaks, respectively, and for pseudoephedrine, it was 342.33 and 304.25 for resolved and co-eluting peaks, respectively. The relative abundance for the internal standards was compared for the resolved and the co-eluting peaks ($n = 12$) using a *t*-test [27]. The *t*-test results show that the difference between the mean response in case of resolved and co-eluting peaks is statistically significant. Elution of phospholipids at different retention times result in inaccurate results due to the presence of an inconsistent degree of ion suppression on the analyte, This suggests that monitoring phospholipids in routine samples may be beneficial to ensure that co-elution resulting in ion suppression effects does not occur.

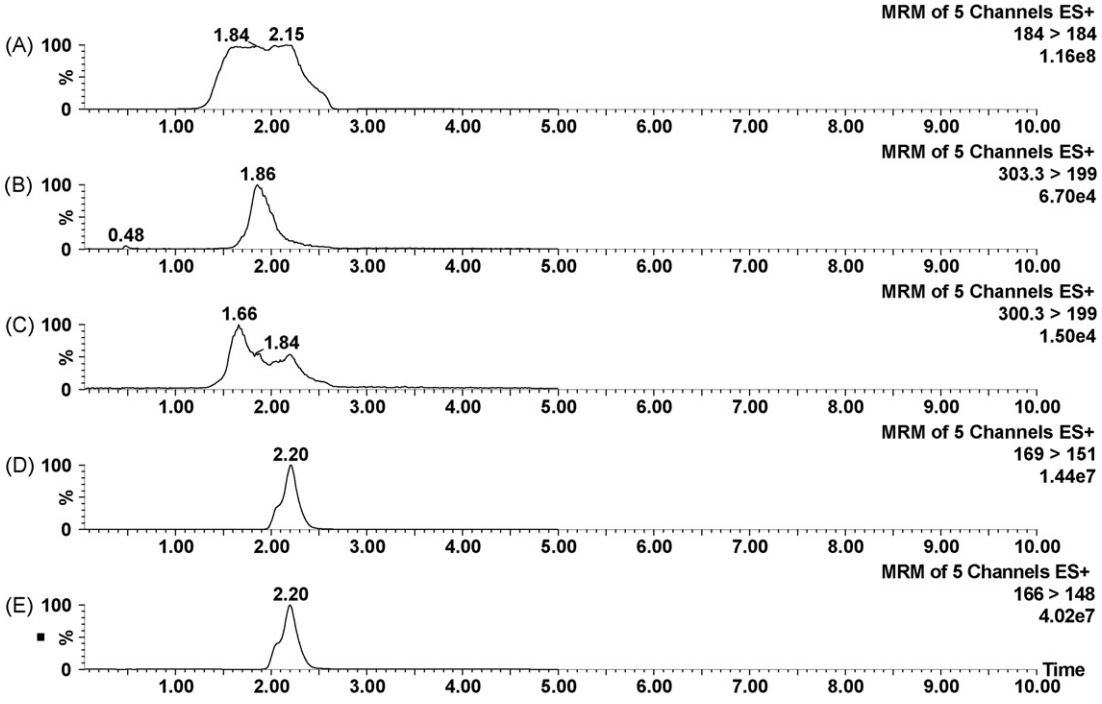

Fig. 8. Chromatograms of unresolved peaks, using an isocratic mobile phase of [20:80] 2.0 mM ammonium formate in methanol:acetonitrile v/v at a 0.35 mL/min flow rate. (A) Glycerophosphocholines, *m/z* 184 → 184, (B) 25 ng/mL hydrocodone-d_3, *m/z* 303 → 199, (C) 4 ng/mL hydrocodone, *m/z* 300 → 199, (D) 150 ng/mL pseudoephedrine-d_3, *m/z* 169 → 155 and (E) 400 ng/mL pseudoephedrine, *m/z* 166 → 148.

Table 2
Precision and accuracy calculated from quality control (QC) samples

QC sample (ng/mL)	Inter-day ($n=15$)				Intra-day ($n=6$)			
	Mean (ng/mL)	S.D.	%R.S.D.	%DFN	Mean (ng/mL)	S.D.	%R.S.D.	%DFN
Hydrocodone								
0.5	0.5	0.02	4.42	−0.57	0.5	0.02	3.46	−0.33
4.0	3.99	0.12	3.09	−0.20	3.97	0.06	1.58	−0.71
80.0	84.09	3.63	4.32	5.11	87.11	0.72	0.83	8.89
Pseudoephedrine								
150.0	162.91	8.98	5.51	8.61	156.66	7.28	4.65	4.44
400.0	420.86	12.67	3.01	5.21	417.62	7.27	1.74	4.40
800.0	797.15	43.77	5.49	−0.36	777.89	15.84	2.04	−2.76

3.8. Validation

The resulting method using the *m/z* 184 fragment to monitor co-elution was validated. The total run time was based on the elution of phospholipids. Before starting each run, the analyte retention times and responses were checked and 10 extracted blank plasma samples were injected. The baseline resolution of analytes and phospholipids was visually checked before and after each run to ensure the absence of co-elution in each sample. The results were accepted only if the phospholipid peaks were completely resolved from the analyte and the internal standard peaks. The estimated column lifetime ($n=2$) was approximately two hundreds injections without co-elution of phospholipids with the analyte or internal standard peaks. Loss of resolution is an indicator for changing the column.

The lower limit of quantitation (LLOQ) was 0.2 ng/mL for hydrocodone and 50 ng/mL for pseudoephedrine, linearity was obtained over the concentration range of 0.2–100 ng/mL for hydrocodone and 50–1000 ng/mL for pseudoephedrine using linear regression weighted by inverse concentration squared. The means of the calibration parameters, slope ± standard deviation (S.D.), intercept ± standard deviation (S.D.) and correlation coefficient, were 0.0427 ± 0.0049, -0.000038 ± 0.0003 and 0.9964 for hydrocodone and 0.00766 ± 0.0007, 0.102 ± 0.0760 and 0.9927 for pseudoephedrine, respectively. The percent relative standard deviation (%R.S.D.) of back calculated standards was less than 8.4 and 6.3% for all standards of hydrocodone and pseudoephedrine, respectively. The percent difference from nominal concentration (%DFN) was less than 2.0 and 7.3% for all standards of hydrocodone and pseudoephedrine, respectively. Six blank plasma samples from different individuals were analyzed 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 limit of quantification prepared in pooled plasma) at the retention times for hydrocodone, pseudoephedrine and their internal standards.

Inter- and intra-assay precision and accuracy were calculated from quality controls samples at three concentration levels over four validation days as summarized in Table 2. The overall inter-assay precision (measured as percent relative standard deviation, %R.S.D.) was less than 4.5 and 5.6% for hydrocodone and pseudoephedrine, respectively, and the intra-assay precision was less than 3.5 and 4.7% for hydrocodone and pseudoephedrine, respectively. The inter-assay accuracy (measured as percent difference from nominal, %DFN) was less than 5.2 and 8.7% for hydrocodone and pseudoephedrine, respectively, and the intra-assay accuracy was less than 8.9 and 4.5% for hydrocodone and pseudoephedrine, respectively.

The inter-assay precision at the lower limit of quantitation (LLOQ) was 3.8 and 8.2% for hydrocodone and pseudoephedrine, respectively. The intra-assay precision of the (LLOQ) was 2.05 and 6.39% for hydrocodone and pseudoephedrine, respectively. The inter-assay accuracy of the (LLOQ) was −2.33 and 3.32% for hydrocodone and pseudoephedrine, respectively. The intra-assay accuracy of the (LLOQ) was −0.83 and 6.18% for hydrocodone and pseudoephedrine, respectively.

The extraction recoveries of hydrocodone at 0.2, 5.00 and 100 ng/mL were 67.0, 75.3 and 79.5%, respectively, and the extraction recoveries of pseudoephedrine at 50.0, 500.0 and 1000 ng/mL were 61.0, 61.8 and 62.1%, respectively.

Freeze/Thaw stability was evaluated over four Freeze/Thaw cycles from −20 °C to room temperature, using quality control (QC) samples ($n=3$) with concentrations of 0.500, 4.00, and 80.0 ng/mL for hydrocodone and 150.0, 400.0, and 800.0 ng/mL for pseudoephedrine. Samples for cycle one were frozen for at least 24 h then each consecutive cycle was frozen for at least 12 h before thawing at room temperature, bench stability was investigated by removing (QC) samples ($n=3$) from −20 °C storage, thawing to room temperature and incubating for 4 h before starting analysis. Post-preparative stability was determined by storing the samples after preparation in the autosampler at approximately 4 °C for 24 h. Samples were stable for 75 days in long-term storage stability experiments at −20 °C; stability was tested by comparison with freshly prepared QC samples.

4. Conclusion

A sensitive and selective LC/MS/MS method for determination of hydrocodone and pseudoephedrine in human plasma monitoring phospholipids was developed and validated. The total run time is determined according to the elution of phospholipids and was 10–12 min in this work. The ion suppression noted for both drugs and internal standards correlates in time with the elution of phospholipids that were monitored using

the m/z 184 > 184 mass transition. We found that monitoring phospholipids was useful in method development to avoid the matrix ionization effects and that the additional mass transition for phospholipids did not significantly affect quantitative measurements when monitored in sample analysis. It was found upon repeated injection that peaks associated with the phospholipids eluted faster while the elution of the analyte peaks remained unchanged. This resulted in the eventual co-elution of the phospholipid and analyte peaks. It was found that there was a statistically significant difference in the internal standard responses for the resolved and co-eluting peaks and co-elution significantly affected peak shape. This suggests that monitoring phospholipids elution may be beneficial to ensure that co-elution resulting in ion suppression does not occur upon repeated injection. Monitoring phospholipids in routine analysis has been shown to be a practical way to ensure avoidance of matrix effects is maintained in each individual sample.

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