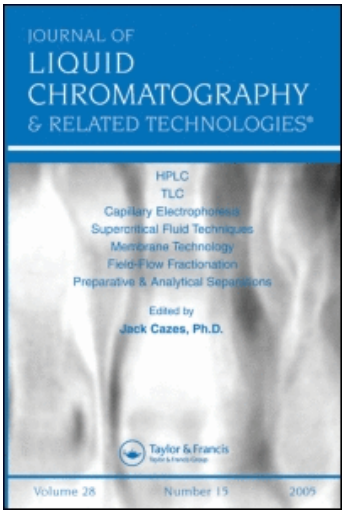

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Ismaiel, Omnia , Halquist, Matthew S. , El-Mammli, Magda Y. , Shalaby, Abdalla and Karnes, H. Thomas(2008) 'Development of a Liquid Chromatography-Negative ESI-Tandem Mass Spectrometry Method for Ibuprofen with Minimization of Matrix Effects Associated with Phospholipids', Journal of Liquid Chromatography & Related Technologies, 31: 20, 3194 — 3208
To link to this Article: DOI: 10.1080/10826070802480222
URL: http://dx.doi.org/10.1080/10826070802480222

Journal of Liquid Chromatography & Related Technologies®, 31: 3194–3208, 2008

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802480222

Development of a Liquid Chromatography-Negative ESI-Tandem Mass Spectrometry Method for Ibuprofen with Minimization of Matrix Effects Associated with Phospholipids

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Abstract: Phospholipids are known to cause matrix ionization effects during LC/MS/MS analysis of biological samples. Matrix effects involving endogenous phospholipids in the negative ESI mode have been investigated in this work for the determination of ibuprofen in human plasma. Glycerophosphocholines (GPChos) and 2-lysoglycerophosphocholines (2-lyso GPChos) were monitored as markers for endogenous matrix components. Various extraction solvents were evaluated to assess their abilities to remove phospholipids from plasma samples to minimize matrix effects. Post-column infusion experiments were applied to chromatographically resolve matrix effects that resulted from endogenous phospholipids. The resulting method was validated and linearity was obtained over a concentration range of 50 to 10,000 ng/mL.

Keywords: Ibuprofen, Matrix effects, Phospholipids, Post-column infusion

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INTRODUCTION

Phospholipids are considered the main class of endogenous components that cause matrix effects in LC/MS/MS.[1–3] Glycerophosphocholines GPChos (e.g., phosphatidylcholine) constitute up to 70% of total plasma phospholipids and result in significant matrix ionization effects in LC/MS/MS.[1,4] Phosphatidylcholine as a zwitterion has the ability to ionize in both positive and negative ESI modes and can cause matrix ionization effects in both modes.[5] Phospholipids are present at significant concentrations in biological samples, which is a problem in both positive and negative ESI modes.

Signal enhancement and signal suppression correlated to the phospholipid peaks were observed in the negative ESI mode and the positive ESI mode, respectively.[6] Many approaches have been developed to remove phospholipids from biological samples in order to minimize matrix ionization effects that resulted from phospholipids. The high oxophilicity of lanthanide metals that selectively bind to the phosphate group in the phospholipid molecules have been used to remove phospholipids from biological samples using a specific lanthanide column, the sorbents removed more than 90% of endogenous phospholipids from plasma and serum samples.[2] Protein precipitation (PP) methods using either methanol or acetonitrile were found to be inefficient for phospholipid removal.[4] Solid phase extraction (SPE) using either Waters Oasis® HLB polymeric sorbent or silica-based strong cation exchange sorbents have been shown to be more efficient for phospholipid removal.[4]

The capabilities of three ion exchange solid phase extraction plates to remove phospholipids from plasma samples have been evaluated using the peak height intensity of phosphatidylcholine as an indicator.[5] Phospholipids are efficiently ionized in the positive ESI mode and have been shown to be the main source of ion suppression in the positive ESI mode. Phospholipids can generate negatively charged ion fragments through demethylation $(M\text{-}15)^-$ and also generate $(M\text{-}H)^-$ ions.[6,7] The ionization of phospholipids in the positive ESI mode is more efficient than that in the negative mode. Phosphatidylcholine is efficiently ionized in the positive ESI mode due to the presence of the quaternary nitrogen in the head group. Phosphatidylcholine has been shown to fragment to form m/z 184 corresponding to the loss of the polar head group $(H_2PO_4CH_2CH_2N(CH_3)_3)^+$ and m/z 168 corresponding to the loss of the polar head group $(HPO_4CH_2CH_2N + (CH_3)_2)^-$ in the positive ESI mode and the negative ESI mode, respectively. It was found that, the production of fragment ions are dependent on the applied cone voltage, and at a high cone voltage, weak unfragmented parent ions and relatively abundant polar head group fragment ions were observed. However, at

low cone voltage more abundant unfragmented parent ions were observed and the polar head group fragment ions were absent.[7]

Ibuprofen ((±)-2-(*p*-isobutylphenyl) propionic acid) is a non-steroidal anti-inflammatory drug, it is effective in rheumatoid, arthritis, and osteoarthritis patients with a lower incidence of gastrointestinal toxicity than aspirin. Ibuprofen may act by inhibiting the synthesis of prostaglandins.[8,9] High performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) methods,[10–12] gas chromatography-tandem mass spectrometry (GC/MS/MS) methods,[13–14] and ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) methods[15] have been described for determination of ibuprofen in surface and wastewaters. Enantioselective determination of ibuprofen in plasma has been reported using liquid chromatography-electrospray mass spectrometry.[16]

Previous methods utilizing LC/MS/MS have not addressed the need to monitor matrix effects in biological samples. A simple and sensitive LC/MS/MS method for determination of ibuprofen in human plasma using phospholipids as markers to optimize the procedure for the avoidance of matrix effects in the negative ESI mode is described. Ibuprofen-d_3 was used as an isotopically labeled internal standard. Various liquid-liquid extraction solvents were compared in terms of extraction recovery of ibuprofen and the capacity for phospholipid removal. Phospholipid response was used as an indicator in order to minimize matrix ionization effects. Post-column infusion experiments were conducted to evaluate matrix ionization effects from endogenous phospholipids in negative ESI mode. Figure 1 shows the chemical structure of ibuprofen, ibuprofen-d_3, glycerophosphocholines, and 2 lyso-glycerophosphocholines.

EXPERIMENTAL

Reagents

Ibuprofen was purchased from Sigma Chemical Company (St. Louis, MO, USA). Ibuprofen-d_3 was purchased from C.D.N Isotopes (point-Claire, Quebec, 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, ethyl acetate, methyl tertiary butyl ether (MTBE), and n-hexane 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

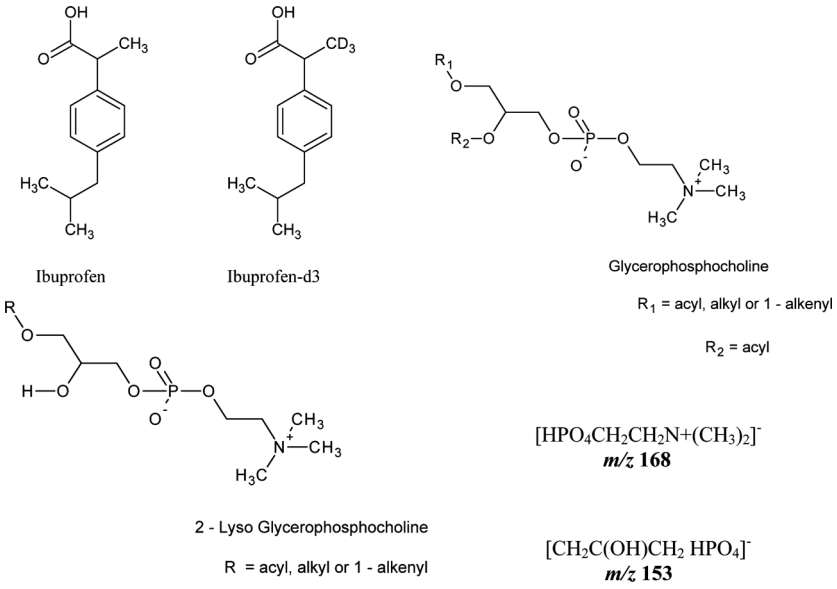

Figure 1. Chemical structures of ibuprofen, ibuprofen-d_3, Glycerophosphocholines and 2 lyso-glycerophosphocholines.

Chemical Company (Milwaukee, WI, USA), and hydrochloric acid was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

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 the electrospray ionization (ESI) negative multiple reaction monitoring (MRM) mode.

Preparation of Standards and Quality Control (QC) Samples

Two separate 1.0 mg/mL stock solutions of ibuprofen were prepared in methanol and stored at approximately −20°C. The solution was prepared by weighing approximately 5.0 mg of ibuprofen and quantitatively transferring it to a glass container. The solution was diluted to

approximately 5.0 mL but the exact volume was determined based on the amount of material weighed. After checking responses form these two solutions, one pooled stock solution was used to prepare calibration standards and QC samples. The calibration standards were prepared by adding the appropriate amounts of the stock solution into the pooled blank plasma. Nominal concentrations were 50.0, 100.0, 250.0, 500.0, 1000, 5000, 9000, and 10000 ng/mL of ibuprofen, QC samples at concentrations of 150.0, 900.0, and 8000 ng/mL were prepared along with lower limit of quantification (LLOQ) QC samples at 50.0 ng/mL of ibuprofen. Standards and controls were sub-aliquotted into 13×100 mm polypropylene tubes and stored at approximately $-20°C$.

Sample Preparation

Human plasma samples were thawed at room temperature and vortex mixed. A 250 μL aliquot of each sample was placed into a 13×100 mm screw cap culture tube, 25.0 μL of freshly prepared working internal standard (5.0 μg/mL of ibuprofen-d_3) was added and the tubes were mixed briefly. Hydrochloric acid (50 μL of 1.0 N) was added and the tubes were again mixed briefly. The mixed samples were extracted by addition of 1.0 mL of (10: 90) ethyl acetate: n-hexane v/v followed by rotation for approximately 3 minutes. After centrifugation at approximately 3000 rpm for 10 minutes, the samples were placed in a freeze bath at $-40°C$, and then the organic layer was transferred to a 16×100 mm screw cap conical tube and evaporated to dryness in the TurboVap® under a dry nitrogen stream at approximately 40°C. The residue was reconstituted with 100 μL of reconstitution solution and transferred to 96 well plates with silanized inserts. A 10 μL portion of the resulting solution was injected.

Monitoring of Phospholipids

High cone voltage and low collision energy conditions were described for monitoring the m/z 184 and the m/z 104 as common fragment ions for Glycerophosphocholines (GPChos) and 2-lyso glycerophosphocholines (2-lyso GPChos), respectively, without further fragmentation in the positive ESI mode.[1] Similar conditions were found here to be optimal for monitoring the m/z 168 and the m/z 153 fragment ions in the negative ESI mode. Both ions were detected in plasma extracts after liquid-liquid extraction, of phosphatidylcholine isolated from chicken eggs and synthetic lysophosphatidylcholine using the negative ESI mode and 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 was used.

Post-Column Infusion Experiment

Post-column infusion experiments were conducted in which a 250 ng/mL solution of ibuprofen and ibuprofen-d_3 in the reconstitution solution was constantly infused (10 μL/min) into the MS. A 250 μL human plasma sample was aliquotted and extracted as described under Sample Preparation and the matrix extract was injected precolumn. For the isolated GPChos and synthetic 2-lyso GPChos solutions, a 500 μg/mL phosphatidylcholine solution and a 500 μg/mL lysophosphatidylcholine solution were prepared in the reconstitution solution and were injected precolumn.

Isocratic Method for the Analysis of Ibuprofen using ESI Mode

The samples were analyzed using an Onyx Monolithic Si 100 × 4.6 mm analytical column, Phenomenex (Torrance, CA, USA) and HPLC guard cartridge 4.0 mm L × 2.0 mm ID Phenomenex (Torrance, CA, USA). An isocratic method using a mobile phase consisting of 20% 2 mM ammonium formate in methanol and 80% acetonitrile with a 0.5 mL/min flow rate was performed. The autosampler utilized a rinse solution comprised of 95:5 methanol:2% formic acid (v/v) with a run time of 7 min and five cycles of post (syringe and injection port) washing. MS/MS system parameters were as shown in Table 1.

RESULTS AND DISCUSSIONS

Monitoring Phospholipids

Zwitterionic phospholipids (e.g., Phosphatidylcholine and lysophosphatidylcholine) can be detected in both positive and negative ionization

Table 1. Multiple reaction monitoring (MRM) parameters for ibuprofen, ibuprofen-d_3, and phospholipids

Compound Name	Nominal parent (m/z)	Nominal daughter (m/z)	Dwell (sec)	Cone (volts)	Collision Energy (eV)
Ibuprofen	205.0	161.0	0.25	20.0	7.0
Ibuprofen-d_3	208.0	164.0	0.25	20.0	7.0
Phospholipids	168.0	168.0	0.05	90.0	7.0
	153.0	153.0	0.05	90.0	7.0

modes. However, detection in the positive ionization mode is more efficient. In the negative ionization mode, acyl chain fragments (*sn*-1 and *sn*-2 fatty acid residues), characteristic of the head group or its fragments (m/z 168 $(HPO_4CH_2CH_2N+(CH_3)_2)^-$ for phosphatidylcholine and common fragments for all glycerophospholipids such as m/z 153 $(CH_2C(OH)CH_2HPO_4)^-$, m/z 79 (PO_3^-) and m/z 97 $(H_2PO_4^-)$) could be detected.[7–17] Both m/z 168 and m/z 153 fragment ion peaks were detected in plasma extracts and neat solutions of either phosphatidylcholine isolated from chicken eggs or synthetic lysophosphatidylcholine Figure 2. Phosphatidylcholine and lysophosphatidylcholine eluted at approximately 5.5 minutes, other peaks that were observed in plasma extracts between 2.5 and 3.5 minutes and may have been due to elution of other types of phospholipids, Figure 3. Another fragment ion was observed at m/z 93, which has not been previously reported. This ion may have been due to fragmentation of the phosphocholine head group, fatty acyl chain fragment, or may have been due to a degradation product of the phospholipids.

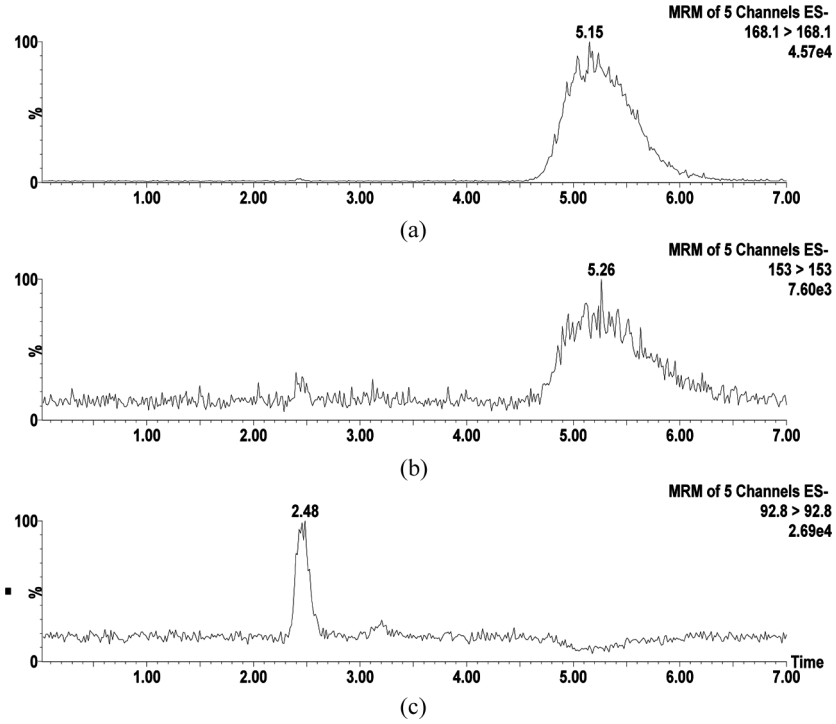

Figure 2. LC/MS/MS chromatograms of 250 µg/mL Phosphatidylcholine solution (A) m/z 168 → 168 and (B) m/z 153 → 153.

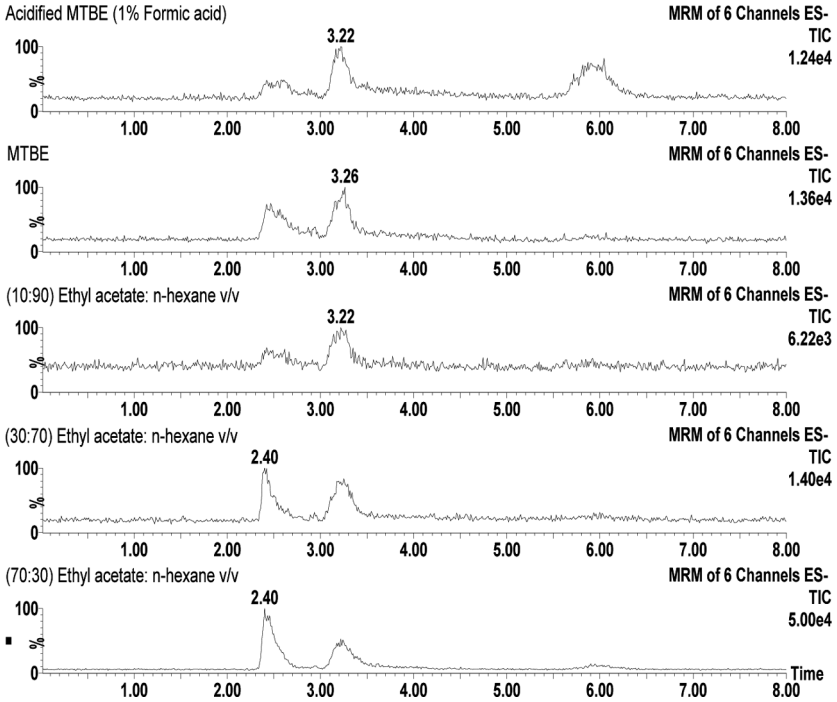

Figure 3. Comparison of different solvents for the extraction of the phospholipids from human plasma using 1 mL of each solvent and 50 µL 1.0 N HCl for all solvents except for the acidified MTBE (1% Formic acid).

Method Development for the Analysis of Ibuprofen

Many approaches have been described to remove phospholipids from biological samples to minimize matrix ionization effects using solid phase extraction.[2,4,5] A simple liquid-liquid extraction procedure is described in this paper and the selectivity of various organic solvents for removing phospholipids from ibuprofen in plasma samples is evaluated. (Methyl tertiary butyl ether (MTBE), acidified MTBE (1% formic acid), ethyl acetate: n-hexane (10:90), (30:70), and (70:30) v/v were evaluated for ibuprofen extraction recovery and phospholipid removal capability, using the peak area responses of the phospholipid peaks as indicators for extraction selectivity Figure (3). Comparable ibuprofen recoveries were obtained using (10:90), (30:70), or (70:30) ethyl acetate:n-hexane v/v Figure 4 (a). However, (10:90) ethyl acetate: n-hexane v/v provided the lowest responses for the phospholipid peaks Figure 4 (b). The phosphatidylcholine peak at 5.5 minutes was

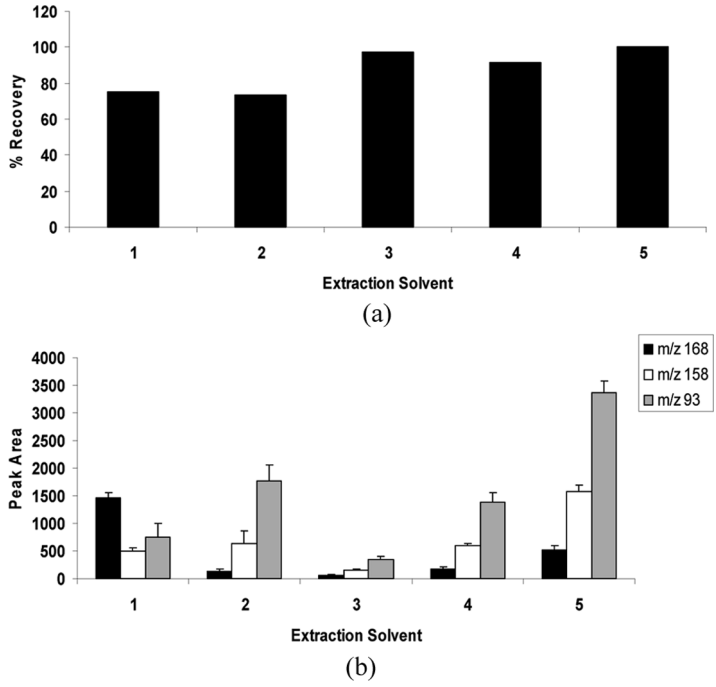

Figure 4. (a) Absolute recovery of 50 ng/mL ibuprofen, (b) Phospholipids peak are responses after liquid–liquid extraction using 1 mL of (1) Acidified MTBE (1% Formic acid), (2) MTBE, (3) ethyl acetate:n-hexane (10:90) v/v, (4) ethyl acetate:n-hexane (30:70) v/v, and (5) ethyl acetate:n-hexane (70:30) v/v.

completely absent in blank plasma extracts after liquid-liquid extraction and the lowest responses for other phospholipids peaks that eluted from 2.5–3.5 minutes was obtained. (10:90) ethyl acetate: n-hexane v/v was found to be the best liquid-liquid extraction solvent for this study.

Monolithic columns provide many advantages over conventional HPLC columns such as high separation efficiency, allowing use of high flow rates and fast analysis time.[18] A monolithic silica column was used in this work. Mobile phase additives such as 10 mM ammonium acetate, 2 mM ammonium formate, 0.1% formic acid, and 0.1% acetic acid in different proportions of methanol, water, and acetonitrile using either isocratic or gradient flow at different flow rates were evaluated. An isocratic method using a mobile phase consisting of 20% 2.0 mM ammonium formate in methanol and 80% acetonitrile at a 0.5 mL/min flow rate was found to yield optimal retention times, good peak shape, and good sensitivity for ibuprofen and its labeled internal standard Figure 5.

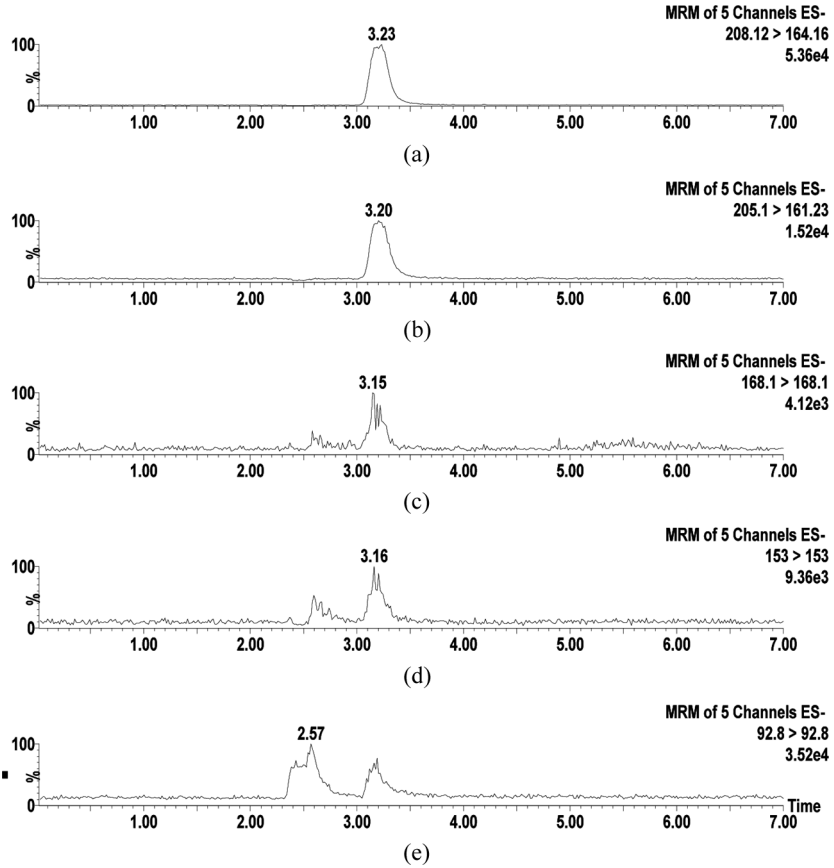

Figure 5. Chromatograms of (a) 500 ng/mL Ibuprofen-d_3, m/z 208 → 164, (b) 150 ng/ml Ibuprofen, m/z 205 → 161, (c) m/z 168 → 168, and (d) m/z 153 → 153, using an isocratic mobile phase consisting of 20% (2.0 mM) ammonium formate in methanol and 80% acetonitrile v/v at a 0.5 mL/min flow rate.

Post-Column Infusion Experiment

Post-column infusion experiments were conducted to determine matrix ionization effects for ibuprofen and its labeled internal standard that resulted from phospholipids using both blank plasma extracts and standard solutions of phosphatidylcholine and lysophosphatidylcholine. A post-column infusion experiment using blank plasma extracts showed sharp ion suppression (approximately 85%) for ibuprofen and its labelled internal standard (at 2.5 minutes) Figure 6. By adjusting HPLC conditions, ibuprofen, and its labelled internal standard were eluted at approximately 3.2 minutes and away from the sharp ion suppression window. Post-column infusion

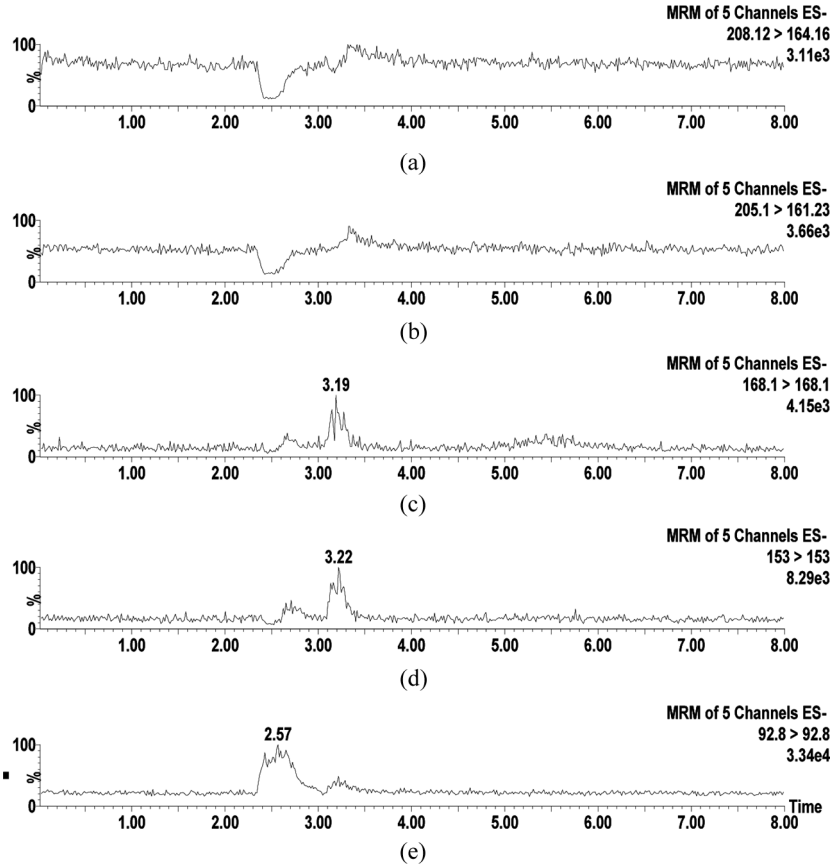

Figure 6. Matrix ionization effects with post-column infusion (250 ng/mL, 10 μL/min) of (a) Ibuprofen-d_3, m/z 208 → 164, (b) Ibuprofen, m/z 205 → 161, and LC/MS/MS analysis of extracted plasma, (c) m/z 168 → 168, and (d) m/z 153 → 153.

experiments using either 500 μg/mL phosphatidylcholine solution or 500 μg/mL lysophosphatidylcholine showed suppression widows that correlated in time with the elution of phospholipids at approximately 5.5 minutes Figure 7. This suppression window could not be observed in post-column infusion experiment using blank plasma extracts because of the absence of this peak in extracted blank plasma after liquid-liquid extraction using (10:90) ethyl acetate:n-hexane v/v.

Validation

The resulting LC/MS/MS method for determination of ibuprofen in human plasma was validated with a total run time of seven minutes.

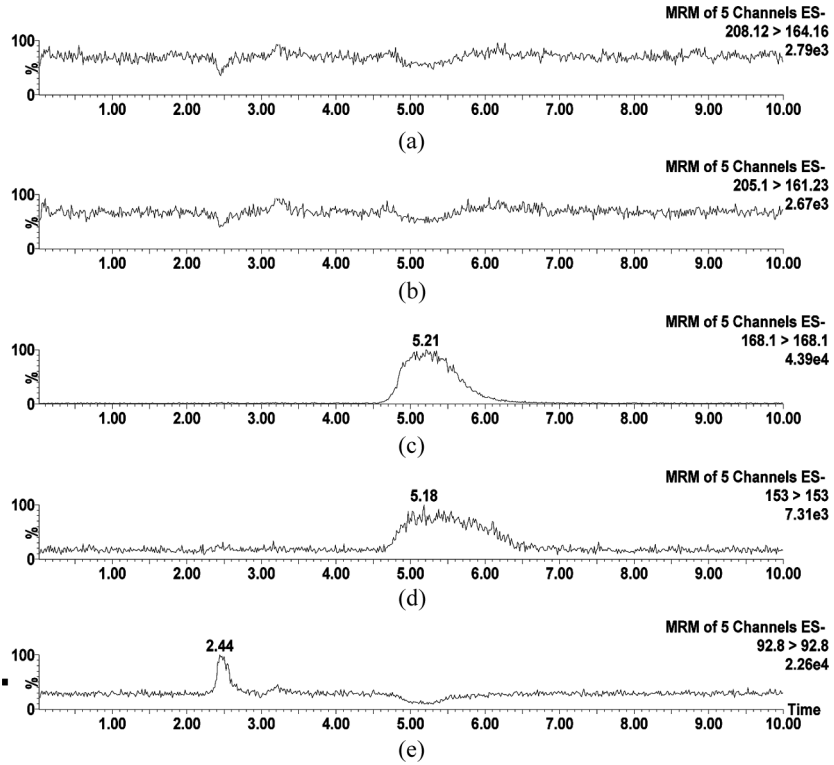

Figure 7. Matrix ionization effects with post-column infusion (250 ng/mL, 10 μL/min) of (a) Ibuprofen-d_3, m/z 208 → 164, (b) Ibuprofen, m/z 205 → 161, and LC/MS/MS chromatograms of 500 μg/mL Phosphatidylcholine solution, (c) m/z 168 → 168, and (d) m/z 153 → 153.

Linearity was obtained over the concentration range of 50–10,000 ng/mL for ibuprofen using linear regression weighted by inverse concentration squared. The lower limit of quantitation (LLOQ) was 50 ng/mL. The means of the calibration parameters, slope ± standard deviation (SD), intercept ± standard deviation (SD), and correlation coefficient, were 0.0375 ± 0.01, 0.0016 ± 0.00002, and 0.9961, respectively. The percent relative standard deviation (%RSD) of back calculated standards was less than 7.2% for all standards of ibuprofen. The percent difference from nominal concentration (%DFN) was less than 3.8% for all standards of ibuprofen. Six blank plasma samples from different individuals were analyzed for interference at the retention times of ibuprofen and its labeled internal standard. Five out of six 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 ibuprofen and ibuprofen-d_3.

Inter- and intra-assay precision and accuracy were calculated from quality control samples at three concentration levels over three validation days as shown in Table 2. The overall inter-assay precision (measured as percent relative standard deviation, %RSD) was less than 9.3% and the intra-assay precision was less than 10.0%. The inter-assay accuracy (measured as percent difference from nominal, %DFN) was less than −1.9% and the intra-assay accuracy was less than 1.0%.

The inter-assay precision at the lower limit of quantitation (LLOQ) was 12.14% and the intra-assay precision of the (LLOQ) was 6.02%. The inter-assay accuracy of the (LLOQ) was −1.96% and the intra-assay accuracy of the (LLOQ) was 1.92%.

The extraction recoveries of ibuprofen at 150, 900, and 8000 ng/mL were 83.57, 72.09, and 71.51% (% RSD, 8.99), respectively.

Freeze/Thaw stability was evaluated over three Freeze/Thaw cycles from −20°C to room temperature, using quality control (QC) samples (n = 6) with concentrations of 150 and 8000 ng/mL of ibuprofen. Samples for cycle one were frozen for at least 24 hours, then each consecutive cycle was frozen for at least 12 hours before thawing at room temperature. Bench stability was investigated by removing (QC) samples (n = 6) with concentrations of 150 and 8000 ng/mL of ibuprofen from −20°C storage, thawing to room temperature, and allowing them to sit for four hours before starting analysis. Post-preparative stability was determined by storing the samples after preparation in the autosampler at approximately 4°C for 24 hours. Acceptable results (within 15% of expected) were obtained for all stability experiments.

Table 2. Precision and accuracy calculated from quality control (QC) samples of ibuprofen using negative ESI mode

	Quality control samples (ng/mL)		
	150	900	8000
Inter-run precision and accuracy (n = 12)			
Mean	147.46	894.50	7852.37
SD	13.69	47.83	384.85
%RSD	9.28	5.35	4.90
%DFN	−1.69	−0.61	−1.85
Intra-run precision and accuracy (n = 6)			
Mean	150.09	906.59	7950.86
SD	14.98	58.78	538.26
%RSD	9.98	6.48	6.77
%DFN	0.06	0.73	−0.61

CONCLUSION

A sensitive and selective LC/MS/MS method for determination of ibuprofen in human plasma optimized for avoidance of matrix effects through monitoring phospholipids in the negative ESI mode was developed and validated. The total run time was 7 minutes. A simple liquid-liquid extraction method using 10:90 ethyl acetate:n-hexane v/v at acidic pH was found to be effective. The phosphatidylcholine peak was completely absent in the blank plasma extracts after liquid-liquid extraction. We found that monitoring phospholipids as markers for endogenous matrix components and as indicators for sample matrix effect selectivity, was useful during method development to select the most appropriate extraction solvents and the most appropriate chromatographic conditions.

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Received April 1, 2008
Accepted June 11, 2008
Manuscript 6361