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High-throughput liquid chromatography–tandem mass spectrometry determination of bupropion and its metabolites in human, mouse and rat plasma using a monolithic column

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Abstract

In the present work, a high-throughput LC/MS/MS method using a Chromolith RP-18 (50 mm × 4.6 mm) monolithic column was developed and partially validated for the determination of bupropion (BUP), an anti-depressant drug, and its metabolites, hydroxybupropion and *threo*-hydrobupropion (TB), in human, mouse, and rat plasma. A modern integrated liquid chromatograph and an LC/MS/MS system with a TurboIonSpray (TIS) interface were used for the positive electrospray selected reaction monitoring (SRM) LC/MS analyses. Spiked control plasma calibration standards and quality control (QC) samples were extracted by semi-automated 96-well liquid–liquid extraction (LLE) using ethyl acetate. A mobile phase consisting of 8 mM ammonium acetate-acetonitrile (55:45, v/v) delivered isocratically at 5 ml/min, and split post-column to 2 ml/min directed to the TIS, provided the optimum conditions for the chromatographic separation of bupropion and its metabolites within 23 s. The isotope-labeled D_6-bupropion and D_6-hydroxybupropion were used as internal standards. The method was linear over a concentration range of 0.25–200 ng/ml (bupropion and *threo*-hydrobupropion), and 1.25–1000 ng/ml (hydroxybupropion). The intra- and inter-day assay accuracy and precision were within 15% for all analytes in each of the biological matrices. The monolithic column performance as a function of column backpressure, peak asymmetry, and retention time reproducibility was adequately maintained over 864 extracted plasma injections.

Keywords: Bupropion; Hydroxybupropion; *threo*-Hydrobupropion

1. Introduction

Bupropion is an aminoketone used as antidepressant and non-nicotine aid to smoking cessation [1]. In humans, bupropion (BUP) is metabolized to hydroxybupropion (HB), the pharmacologically active main metabolite, and to *threo*-hydrobupropion (TB) and erythrohydrobupropion (EB) (Fig. 1) [2]. Studies indicate that the cytochrome P450 enzyme CYP2B6 is involved in the hydroxylation of bupropion [3,4].

BUP and its metabolites, HB, TB and EB, have been determined in human plasma by TurboIonSpray (TIS) LC/MS/MS in positive ion mode (unpublished work, GlaxoSmithKline, Research Triangle Park, NC, USA). The chromatographic conditions included a reversed-phase C4 HPLC column (150 mm × 4.6 mm), flow rate of 1.1 ml/min, and a mobile phase consisting of 2 mM ammonium acetate–acetonitrile (30:70, v/v). HB, BUP, and TB were eluted at 2.6, 3.1 and 4.3 min, respectively, under isocratic conditions at ambient temperature. Under these conditions, TB and EB co-elute and show the same SRM transitions. The results for TB and EB were reported as a sum using TB as the standard. Previous pharmacokinetic studies [1,5] showed that the concentrations of EB in human plasma are very low and contribute only a small portion of the overall combination peak. In high-throughput applications of drug discovery, it may not be necessary to chromatographically separate these two analytes. However, in those instances where it is important to quantify each analyte it may be necessary to develop a

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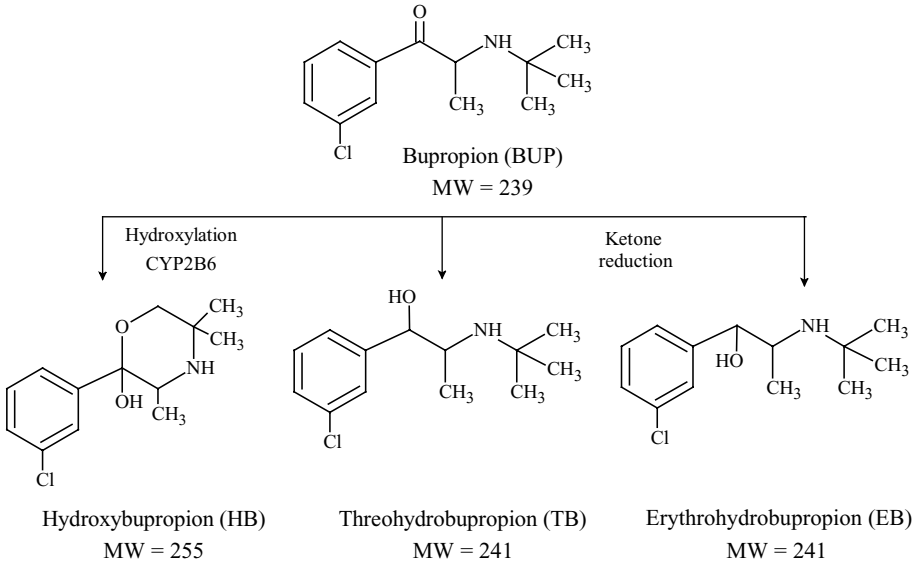


Fig. 1. Metabolism of bupropion in humans.

slower HPLC method that could affect a separation of each compound.

The introduction of monolithic silica HPLC columns combined with the high flow rate capability of the API 4000 TIS interface, and high-speed autosamplers have facilitated the development of high-throughput LC/MS/MS methods for the rapid determination of drugs and metabolites in biological matrices. Assay speed, sensitivity, selectivity, and reproducibility have been demonstrated using these new technologies [6–15]. Monolithic silica columns have been used as an alternative to particulate silica columns due to the low backpressure under high flow rates [16]. These columns are single rods of polymerized silica prepared by a sol–gel process. They contain a biporous structure that permits the use of high flow rates at considerably reduced backpressure. The mesoporous (13 nm) nature of these columns provides a large adsorption surface area for best interaction with the analytes, while the macroporous (2 μm) features decreases eluent flow resistance, consequently reducing analysis run time for high-throughput analysis [17,18].

In the present work, a high-throughput, sensitive LC/MS/MS method for the determination of BUP and its metabolites, HB and TB, in human, mouse, and rat plasma has been developed and partially validated using a monolithic silica HPLC column. The aims of this work were to determine BUP and its metabolites within 30 s run time, and to evaluate the monolithic column technology for such a bioanalytical application. The use of a Chromolith SpeedROD RP-18 (50 mm × 4.6 mm) monolithic column, operated at 5 ml/min, split post-column to 2 ml/min directed to the mass spectrometer, dramatically reduced analysis run time, allowing the separation of the analytes within 23 s under isocratic elution conditions. The monolithic column performance as a function of column backpressure, peak asymmetry, and retention time reproducibility was adequately maintained over 864 extracted plasma injections.

2. Experimental

2.1. Chemicals and reagents

Bupropion ($C_{13}H_{18}ClNO$, MW = 239), hydroxybupropion ($C_{13}H_{18}ClNO_2$, MW = 255), TB ($C_{13}H_{20}ClNO$, MW = 241), and the D_6-isotope-labeled internal standards [$^2H_6$]-bupropion (MW = 245) and [$^2H_6$]-hydroxybupropion (MW = 261) were kindly donated by GlaxoSmithKline (Research Triangle Park, NC, USA) as hydrochloride salts. Analytical reagent grade ammonium acetate, sodium carbonate, and sodium bicarbonate were purchased from Sigma (St. Louis, MO, USA), and hydrochloric acid was purchased from EM Science (Gibbstown, NJ, USA). HPLC grade acetonitrile and ethyl acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA), and NANOpure deionized water (Barnstead, Boston, MA, USA) was used to prepare all standard solutions. Three different lots of each human, mouse, and rat control plasma were purchased from Lampire Biological Laboratories (Pipersville, PA, USA). The human and animal control plasma contained (Na) EDTA and (K2) EDTA, respectively, as anticoagulants.

2.2. Equipment

A Tomtec Quadra 96 model 320 robotic pippetor (Hamden, CT, USA), an IKA-Schuttler MTS 4 microtiter four 96-well plate shaker (IKA-Werke GmbH & Co. KG, Staufen, Germany), Rainin electronic digital pipettes (Woburn, MA, USA), an Eppendorf 5810R centrifuge with a four 96-well plate rotor (Brinkmann Instrument, Westbury, NY, USA),

a water-bath (Precision Scientific, Chicago, IL, USA), a Mettler AE240 balance (Hightstown, NJ, USA), 1.2 ml polypropylene 96-well plates (Phenix Research Products, Hayward, CA, USA) and sealing mats (Matrix Technologies, Hudson, NH, USA), Costar 0.5 ml polypropylene 96-well assay blocks and SUN-SRI silicone mats (#502 345) (Wilmington, NC, USA), and a Harvard Apparatus Model 22 infusion pump (South Natick, MA, USA) were used.

2.3. Chromatographic conditions

A liquid chromatograph model LC-2010C integrated system, consisting of a high-speed autosampler, low-pressure mixing pump, mobile phase degasser and column oven (Shimadzu, Columbia, MD, USA), and a Chromolith SpeedROD RP-18 (50 mm × 4.6 mm) monolithic column (kindly donated by Merck, Darmstadt, Germany) were used for the chromatographic separation of BUP, TB, and HB. The optimum mobile phase composition consisted of 8 mM ammonium acetate–acetonitrile (55:45, v/v), delivered isocratically at 5.0 ml/min, and split post-column to 2.0 ml/min *via* a PEEK "T" connector to the mass spectrometer. The monolithic HPLC column was maintained at 40 °C, and the mobile phase was preheated to 40 °C using an integrated column mobile phase pre-heater.

2.4. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Concord, Ont., Canada), with a TIS interface operated in the positive ion mode, was used for the selected reaction monitoring (SRM) LC/MS analyses. The mass spectrometric conditions were optimized for BUP, HB, TB, D_6-BUP, and D_6-HB by infusing a 1.0 μg/ml standard solution containing all compounds in mobile phase at 10 μl/min using a Harvard infusion pump directly connected to the mass spectrometer. An additional tuning optimization was performed by continuously infusing the same standard solution at 10 μl/min *via* a "T" connector into the post-column mobile phase flow (2 ml/min). The TIS source temperature was maintained at 450 °C and the TIS voltage was set at 1500 V. The curtain gas was set at 10 (arbitrary units), the declustering potential (DP) at 40 V, and the nebulizer (GS1) and TIS (GS2) gases at 60 and 80 psi, respectively. Q1 full scan (*m/z* 100–600) and collision-induced dissociation (CID) mass spectra (*m/z* 50–300) were acquired for each analyte. For the SRM analyses, the CID gas was set at 6 (arbitrary units), and the collision energy was set at 18 eV for BUP, D_6-BUP, HB and D_6-HB, and 24 eV for TB. The following precursor → product ion transitions were used for the SRM analyses: BUP, *m/z* 240 → *m/z* 184; D_6-BUP, *m/z* 246 → *m/z* 184; HB, *m/z* 256 → *m/z* 238; D_6-HB, *m/z* 262 → *m/z* 244; TB, *m/z* 242 → *m/z* 168, with the dwell time set at 50 ms. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both Q1 and Q3 quadrupoles. Data were acquired using the Analyst 1.3 software (AB/MDS-Sciex, Concord, ON, Canada).

2.5. Sample preparation

Individual standard stock solutions of BUP (20 μg/ml), HB (50 μg/ml), and TB (20 μg/ml) were prepared in 0.01N HCl and further diluted with the same solvent in order to provide 1 and 0.1 μg/ml standard solutions each containing BUP, HB, and TB and internal standard solution containing 400 ng/ml of D_6-BUP and 135 ng/ml of D_6-HB was prepared in 0.01N HCl.

Calibration standards and quality control (QC) samples were prepared by manually spiking human, mouse, and rat control plasma with individual standard stock solutions and diluted standard solutions of the analytes. A representative control plasma lot from each species was chosen to prepare the calibration standards in duplicate. Calibration curves were constructed at 0.25, 0.5, 1.0, 5.0, 25, 100 and 200 ng/ml for BUP and TB, and 1.25, 2.5, 5.0, 25, 125, 500 and 1000 ng/ml for HB. Six replicates of QC samples at low (QC1), mid (QC2), and high (QC3) concentration levels of 0.75, 50 and 175 ng/ml for BUP and TB, and 3.75, 250 and 875 ng/ml for HB were prepared by individually spiking each of the three lots of human, mouse, and rat control plasma. A batch size of three 96-well plates was prepared daily on three consecutive days, each plate corresponding to one single species, and included two calibration standard curves, one at the beginning and one at the end, interspersed with QCs, blank and double blank samples.

2.6. Sample extraction

The analytes were extracted from human, mouse and rat plasma by semi-automated 96-well plate liquid–liquid extraction (LLE) [19]. The plasma samples (150 μl) were manually transferred into 1.2 ml polypropylene 96-well plates. Aliquots of 25 μl of the internal standard solution, containing D_6-BUP and D_6-HB, were added robotically to the 96-well plate, except to the double blank samples, using the Tomtec Quadra 96, followed by the addition of 150 μl of 0.6 M carbonate buffer. The plate was gently agitated for 1 min between additions of the reagents on a 96-well plate shaker. Aliquots of 550 μl of ethyl acetate were robotically added to the plate, the plate was sealed using a Matrix sealing mat, followed by vigorous agitation on a 96-well plate shaker for 20 min. The plate was centrifuged at 3000 rpm for 10 min at 4 °C. Aliquots of 375 μl of the supernatant were transferred robotically to a 0.5 ml polypropylene 96-well assay block, followed by the addition of 10 μl of 0.1N HCl. The organic solvent was evaporated to dryness on a water-bath at 50 °C under a gentle stream of nitrogen. The residues were reconstituted with 150 μl of mobile phase, the 96-well assay block was sealed with a SUN-SRI silicone mat, and the block was agitated on a 96-well plate shaker for 1 min. The assay blocks were then placed in the autosam-

pler, and aliquots of 20 μl were injected into the LC/MS/MS system.

2.7. Data analysis

Data were processed using the AB/MDS-Sciex Analyst 1.3 software. Method validation included determination of linearity (r) of the mass detector response over the analyte concentration range, and intra- and inter-day assay accuracy (percentage nominal concentration) and precision (%CV). The calibration curves (analyte peak area/IS peak area *versus* analyte concentration/IS concentration) were constructed using the least square linear regression fit ($y = a + bx$), and a weighting factor of $1/x^2$ was applied to the data. Acceptance criteria were established to be >0.98 for the calibration curve coefficient of correlation (r), and within ±15% of the nominal concentration and ≤15% CV for intra- and inter-day assay accuracy and precision, respectively. The deuterated internal standard D_6-BUP was used to calculate the concentrations of BUP and TB, while D_6-HB was used for HB.

2.8. Matrix ion suppression

Matrix ion suppression effects on the SRM LC/MS sensitivity were evaluated by the post-column analyte infusion experiment [20]. A standard solution containing 1.0 μg/ml of BUP, TB, HB, D_6-BUP, and D_6-HB in mobile phase was infused post-column *via* a "T" connector into the mobile phase flow (2 ml/min) at 10 μl/min employing a Harvard infusion pump (Fig. 2). Aliquots of 20 μl of extracted control plasma were then injected into the monolithic HPLC column by the Shimadzu autosampler, and SRM LC/MS chromatograms were acquired for each analyte.

2.9. Influence of the mobile phase flow rate on the SRM LC/MS sensitivity

To evaluate the effects of the mobile phase flow rate on the SRM LC/MS sensitivity, the mobile phase flow rate was maintained at 5 ml/min for fast chromatographic separation of the analytes and split post-column to 0.5, 1.0, 2.0, 3.0 ml/min via a "T" connector to the TIS interface. The mass spectrometric conditions were maintained constant throughout the experiment without compromising electrospray ionization performance. Aliquots of 20 μl of a standard solution containing 0.25 ng/ml of BUP, TB and HB in mobile phase were then injected into the monolithic column, and SRM LC/MS chromatograms were acquired for each analyte.

3. Results and discussion

3.1. SRM LC/MS

BUP and its metabolites, TB and HB, contain a secondary amine group that may be protonated in solution under the experimental chromatographic conditions. Therefore, electrospray ionization (ESI) in the positive ion mode was used for the SRM LC/MS analyses. The Q1 full scan (m/z 100–600) and collision-induced dissociation (CID) (m/z 50–300) mass spectra of BUP, TB, and HB are shown in Fig. 3A–F. The protonated molecules $[M + H]^+$ of BUP, TB, and HB were observed at m/z 240.2, m/z 242.2 and m/z 256.2, respectively (Fig. 3A–C). Fig. 3D–F shows the CID mass spectra of BUP, TB, and HB, respectively. The product ions at m/z 184.2 and m/z 186.2 are consistent with the loss of the C_4H_9 moiety of the molecules of BUP and TB, respectively, while the product ions at m/z 166.2 and m/z 168.2 are consistent with the loss of the $C_4H_{10}N$ moiety of the molecules of BUP and TB, respectively. The product ion at m/z 238.2 is consistent with the loss of H_2O from the molecule of HB. The most abundant product ions at m/z 184.2, m/z 168.2 and m/z 238.2 were chosen to monitor the precursor → product ion transitions of BUP, TB, and HB, respectively, in order to provide the highest sensitivity possible under the experimental conditions. The CID mass spectra of D_6-BUP showed product ions at m/z 184.2 and m/z 166.2 consistent with the loss of the $C_4H_3D_6$ moiety, and a single product ion at m/z 244.2 consistent with the loss of H_2O from the D_6-HB molecule (data not shown). The precursor → product ion transitions of m/z 246 → m/z 184 and m/z 262 → m/z 244 were chosen for the SRM analyses of D_6-BUP and D_6-HB, respectively.

Representative SRM LC/MS chromatograms of a double-blank plasma sample and a calibration standard at 0.25 ng/ml (BUP and TB) and 1.25 ng/ml (HB) in human plasma are shown in Fig. 4. BUP eluted at 0.38 min (23 s),

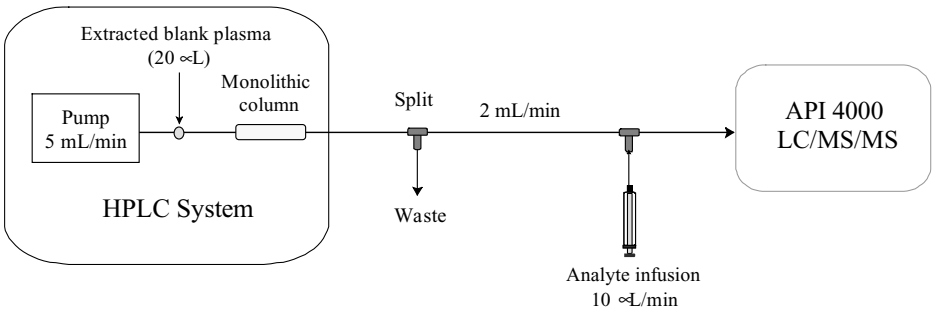


Fig. 2. Schematic representation of matrix ion suppression experiments.

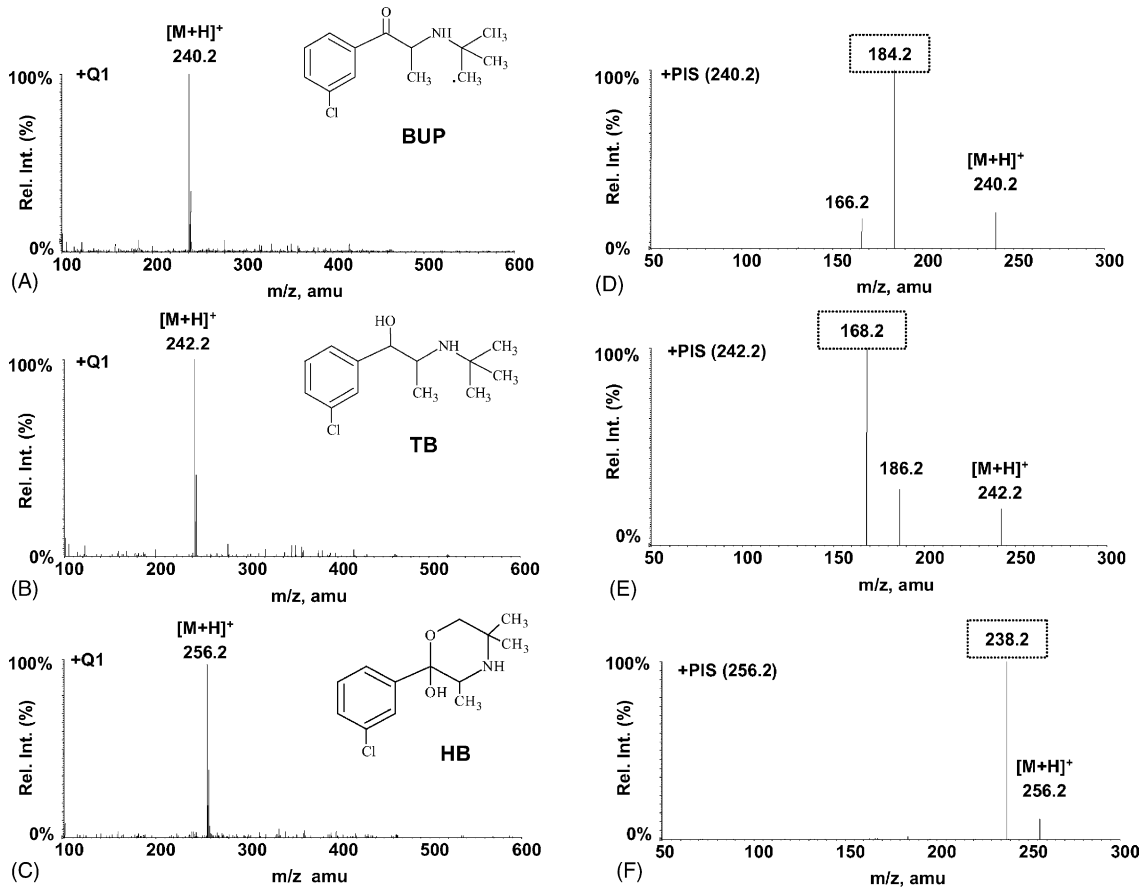


Fig. 3. Q1 full scan (*m*/*z* 100–600) (A–C) and CID (*m*/*z* 50–300) (D–F) mass spectra of BUP, TB, and HB. The protonated molecules $[M + H]^+$ of BUP, TB, and HB are shown at *m*/*z* 240.2, *m*/*z* 242.2 and *m*/*z* 256.2, respectively (A–C). The most abundant product ions at *m*/*z* 184.2, *m*/*z* 168.2 and *m*/*z* 238.2 were chosen to monitor the precursor → product ion transitions for BUP, TB and HB, respectively.

while TB and HB at 0.25 min (15 s) and 0.23 min (14 s), respectively. Similarly, an investigation into the extent of carryover following the loading of a high level standard (upper limit of quantification, ULOQ) was performed. Although a trace level of each analyte was detected in the double blank sample following this experiment (<0.02%), this was well below 20% of the LLOQ for even the highest level for HB and therefore considered acceptable for this application. No significant chromatographic interference was observed between analytes or from endogenous compounds. Preliminary experiments (data not shown) were carried out in order to determine the optimum chromatographic conditions for the elution of the analytes within 30 s run time, to reduce interference between analytes, and to minimize peak tailing without compromising peak shape and resolution. Peak interference from BUP (*m*/*z* 240 → *m*/*z* 184) was observed in the TB SRM transition channel (*m*/*z* 242 → *m*/*z* 168), presumably due to isotopic interference of ^{37}Cl. Although SRM provides high selectivity, there was a need for adequate chromatographic resolution between BUP and TB. The quantitative determination of EB was not carried out due to the lack of the respective standard. It is likely that under the chromatographic conditions used in the present work, both isomeric forms, TB and EB, co-elute. The SRM LC/MS method was linear for BUP and TB from 0.25 to 200 ng/ml, and for HB from 1.25 to 1000 ng/ml in all matrices (Fig. 5). The intra-day (Table 1) and inter-day (Table 2) assay accuracy (percentage nominal value) and precision (%CV) were within 15% for BUP, TB, and HB in each matrix.

To evaluate the analytical potential for the monolithic column technology for fast chromatographic separation of the analytes, three monolithic HPLC columns were evaluated, including Chromolith Flash RP-18 (25 mm × 4.6 mm), Chromolith SpeedROD RP-18 (50 mm × 4.6 mm), and Chromolith Performance RP-18 (100 mm × 4.6 mm) (Merck, Darmstadt, Germany). The shorter monolithic column provided the separation of the analytes within 30 s at a lower flow rate (3.5 ml/min). However, broaden peak tailing was observed for each analyte (data not shown). The longer monolithic column increased the retention time of the analytes by two-fold with the necessity of applying lower flow rates to maintain column back pressure within the acceptance limits (data not shown). The best chromatographic conditions as a function of analyte peak shape and chromatographic resolution, analyte peak intensity re-

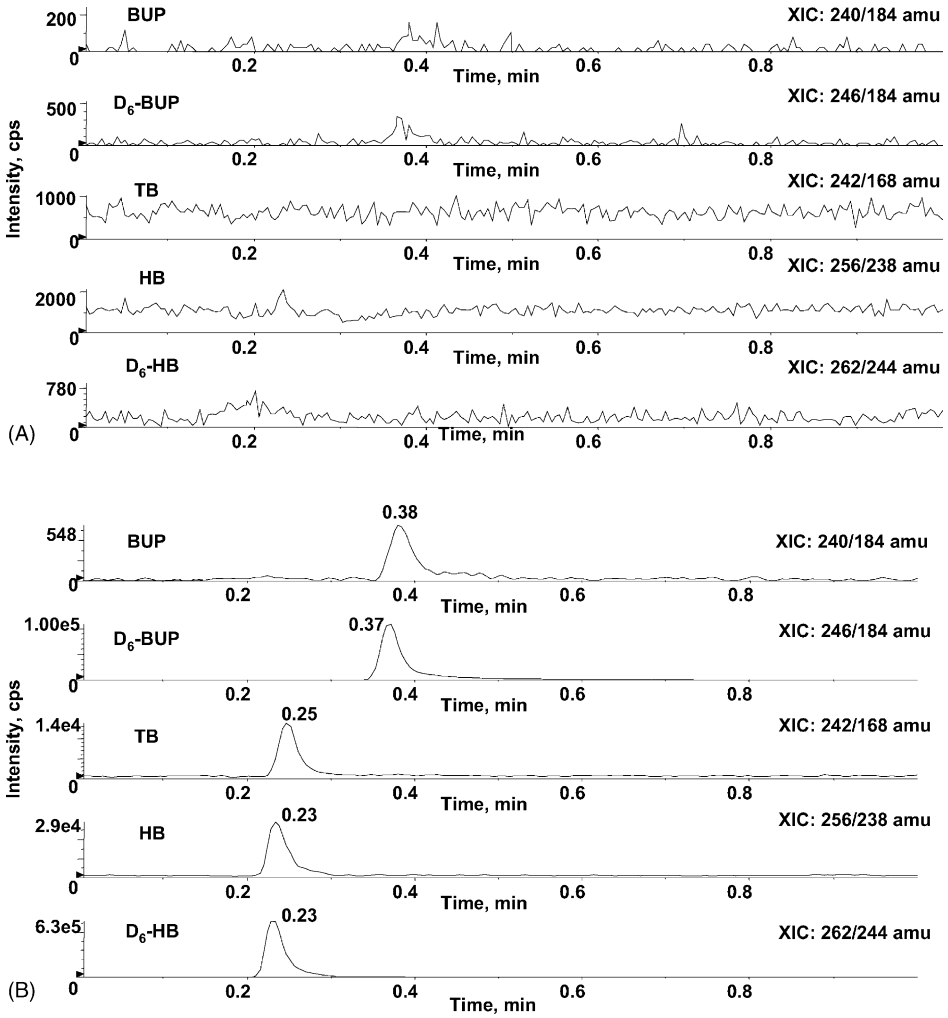


Fig. 4. Representative SRM LC/MS chromatograms of (A) extracted double blank human plasma, and (B) extracted human plasma spiked at the LOQ (0.25 ng/ml for BUP and TB, and 1.25 ng/ml for HB). The analytes were eluted under isocratic conditions using a Chromolith SpeedROD RP-18 (50 mm × 4.6 mm) monolithic column, $T = 40\,^{\circ}C$, mobile phase 8 mM ammonium acetate-acetonitrile (55:45, v/v) as mobile phase, and flow rate of 5 ml/min (split post-column to 2 ml/min to the TIS interface).

sponse (sensitivity), and analysis run time were achieved using a Chromolith SpeedROD RP-18 (50 mm × 4.6 mm) column maintained at 40 °C, and a mobile phase composition of 8 mM ammonium acetate–acetonitrile (55:45, v/v), delivered isocratically at 5 ml/min, and split post-column to 2 ml/min directed to the mass spectrometer. Chromolith HPLC columns are highly porous monolithic rods of polymerized silica produced by a "sol–gel" process [21]. The silica skeleton contains a bimodal pore structure with the macroporous features of approximately 2 μm in diameter, which confers high porosity to the column, and the mesoporous features of about 13 nm in diameter, which creates a large internal surface area for efficient adsorption of the analytes. The network of macropores allows the use of up to 9 ml/min eluent flow rates with low column backpressure, promoting rapid separation of compounds and, consequently, reducing analysis time [21]. These columns are reversed-phase C18-modified silica columns, endcapped for best chromatographic performance. The selectivity is comparable to conventional C18 silica columns, and the separation efficiency is better than 5 μm particle columns and equivalent to 3.5 μm columns [21]. The monolithic silica rods are encased in a PEEK plastic cover by a cladding process that ensures minimum void space around the monolithic rod. According to the manufacturer (column data sheet insert), Merck Chromolith columns are made to operate at pressures up to 120 bar and temperatures not exceeding 30 °C. In the present work, peak tailing was observed during the optimization of the chromatographic conditions. It was observed, however, that an increase in temperature from 30 to 40 °C improved peak shape for all analytes. Furthermore, the performance of the monolithic HPLC column was not sacrificed by maintaining the column at higher temperature (40 °C) over the time of the analyses. Although Chromolith columns are endcapped, it is possible that free silanols at the monolithic silica surface interact with the basic analytes,

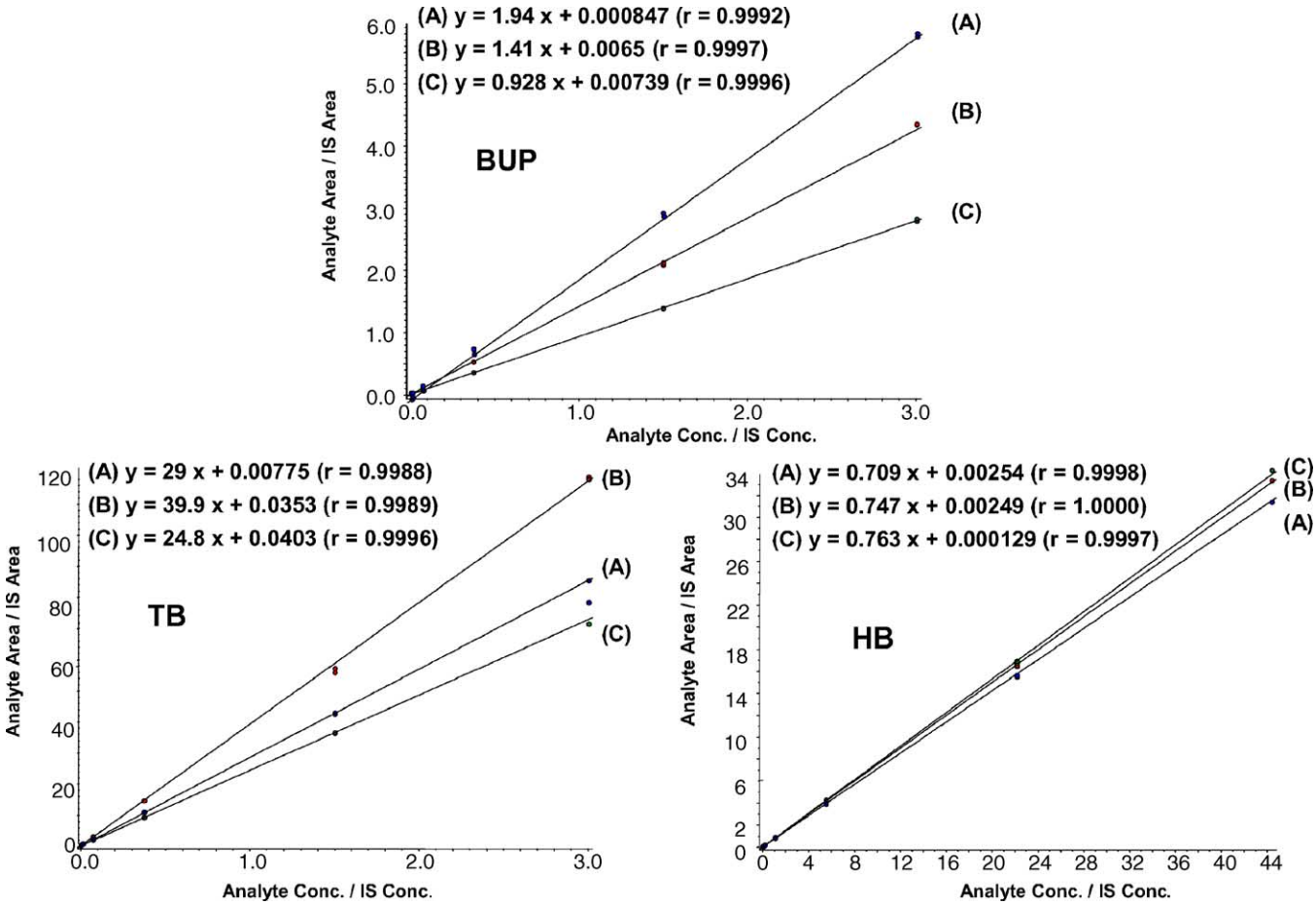
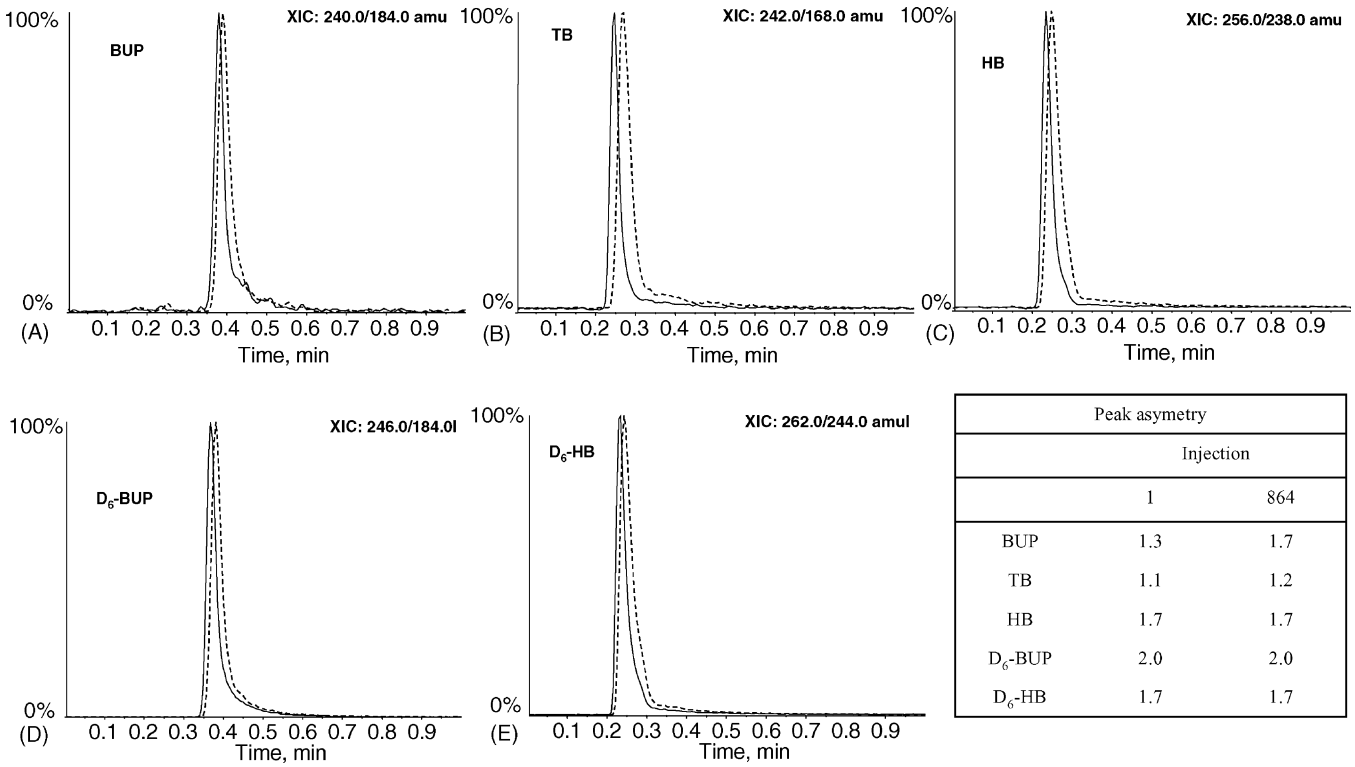


Fig. 5. Representative calibration curves ($n = 2$) for BUP, TB, and HB in human, mouse, and rat plasma.

consequently causing some peak tailing. It is also possible that unknown column voids contribute to this phenomenon. McCalley [22] concluded that monolithic silica RP columns give better peak shape for neutral compounds than for weak and strong bases. It was reported that peak tailing of strong bases increased by increasing the buffer pH values from acidic to pH 7. In the present work, the use of a mobile phase containing 55% 8 mM ammonium and 45% acetonitrile, apparent pH 6.3, provided the optimum conditions for fast chromatography of the analytes with minimized peak tailing. The monolithic column performance was evaluated over 864 injections of extracted plasma samples. The column backpressure increased from 104 to 123 bar, without significantly compromising chromatographic performance. The average retention times of the analytes, measured from the first to the last injection, was 0.38 min (5.9% CV), 0.26 min (3.7% CV), and 0.24 min (2.6% CV) for BUP, TB, and HB, respectively, and 0.37 min (1.8% CV) and 0.24 min (2.8% CV) for D_6-BUP and D_6-HB, respectively. Fig. 6A–E shows peak shape variation in human plasma for BUP (1 ng/ml), TB (1 ng/ml), HB (5 ng/ml), D_6-BUP (66.5 ng/ml), and D_6-HB (22.5 ng/ml) after 864 injections. In this work the peak tailing was assessed by measuring the asymmetry of the chromatographic peaks over the course of the analysis of almost 900 samples. The SRM LC/MS extracted ion chromatogram (XIC) of each compound showed a slight increase in peak broadening after 864 injections of extracted plasma, without compromising peak area measurement.

3.2. Matrix ion suppression

Fig. 7A–C shows representative post-column analyte infusion SRM LC/MS chromatograms in human, mouse, and rat plasma. The analytes were continuously infused post-column *via* a "T" connector into the mobile phase flow (2 ml/min) at 10 μl/min employing a Harvard infusion pump (Fig. 2), and aliquots of 20 μl of extracted control plasma were injected into the monolithic HPLC column. Comparable electrospray ionization suppression regions were observed for all analytes in all matrices. BUP, D_6-BUP, TB, HB and D_6-HB showed an ionization suppression region from 0.18 to 0.22 min. An ion suppression region from 0.30 to 0.35 min was also observed for HB and D_6-HB. Elution of the analytes was established in regions of minimum suppression (Fig. 7). Matrix endogenous components are likely the main cause for ion suppression effects during electrospray ionization [20,23,24]. The extent of the sup-



Peak asymetry		
	Injection	
	1	864
BUP	1.3	1.7
TB	1.1	1.2
HB	1.7	1.7
D_6-BUP	2.0	2.0
D_6-HB	1.7	1.7

Fig. 6. Peak shape variation (measured as peak asymmetry). SRM LC/MS XICs for (A) BUP (1 ng/ml), (B) TB (1 ng/ml), (C) HB (5 ng/ml), (D) D_6-BUP (66.5 ng/ml), and (E) D_6-HB (22.5 ng/ml) in spiked human plasma. (Continuous line: first injection, dotted line: after 864 injections.)

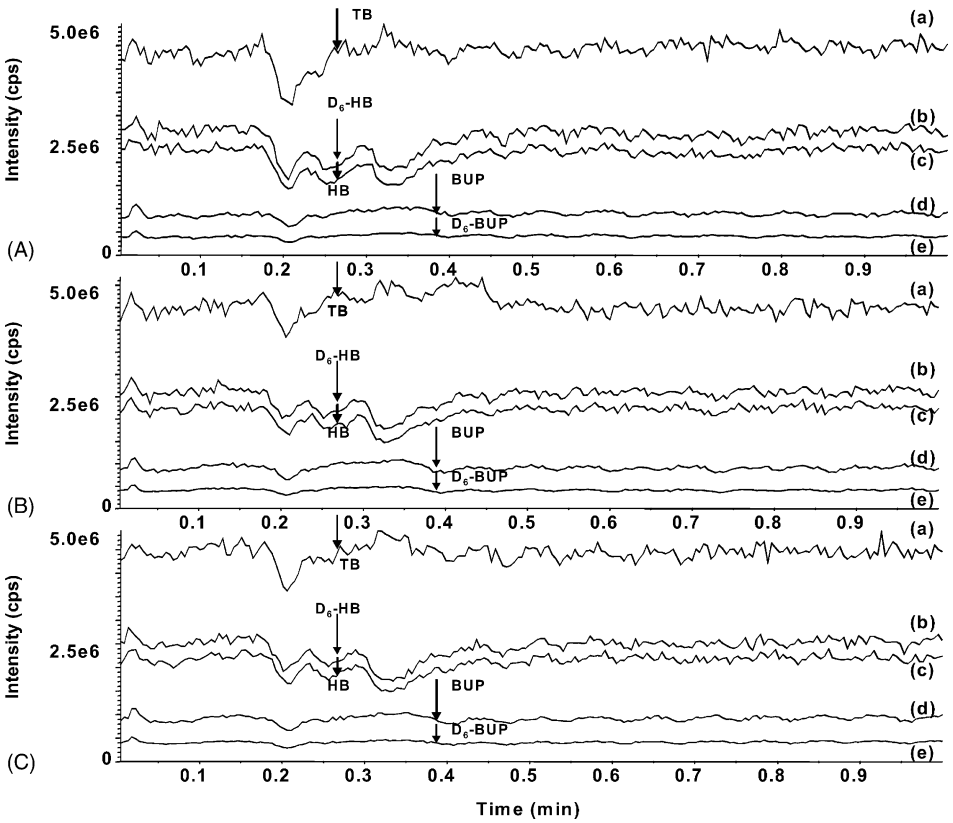


Fig. 7. Matrix supression interference studies. Representative post-column analyte infusion SRM LC/MS chromatograms for (A) human, (B) mouse, and (C) rat plasma. (a) TB (*m*/*z* 242 → *m*/*z* 168), (b) D_6-HB (*m*/*z* 262 → *m*/*z* 244), (c) HB (*m*/*z* 256 → *m*/*z* 238), (d) BUP (*m*/*z* 240 → *m*/*z* 184), (e) D^6-BUP (*m*/*z* 246 → *m*/*z* 184). Arrows show retention times for the analytes.

Table 1
Intra-day assay accuracy (percentage nominal concentration) and precision (%CV) ($n = 18$)

	Human			Mouse			Rat		
QC (ng/ml)	0.75	50	175	0.75	50	175	0.75	50	175
BUP									
Day 1									
CV (%)	9.6	1.6	0.5	12	2.8	0.7	12	1.5	0.8
Accuracy (%)	106	102	101	108	103	100	101	102	101
Day 2									
CV (%)	7.4	5.3	0.5	9.8	1.1	0.7	6.1	2.7	1.7
Accuracy (%)	101	98	100	105	102	100	101	101	100
Day 3									
CV (%)	4.9	5.1	2.1	0.8	2.8	0.7	1.5	1.3	0.9
Accuracy (%)	98	97	99	101	104	100	101	102	101
TB									
Day 1									
CV (%)	8.1	1.9	2.7	3.3	9.6	15	2.5	1.5	6.2
Accuracy (%)	100	103	99	98	101	89	101	103	98
Day 2									
CV (%)	0.5	2.1	2.2	2.9	1.0	3.6	2.1	1.7	2.1
Accuracy (%)	101	103	100	98	100	99	101	101	99
Day 3									
CV (%)	1.1	8.8	2.9	3.6	2.5	3.4	1.7	2.0	6.8
Accuracy (%)	101	104	99	99	100	97	100	102	98
QC (ng/ml)	3.75	250	875	3.75	250	875	3.75	250	875
HB									
Day 1									
CV (%)	1.1	0.3	0.1	8.0	0.4	0.2	5.0	1.8	0.9
Accuracy (%)	100	100	100	102	100	100	101	101	100
Day 2									
CV (%)	2.7	0.4	0.1	0.4	0.6	0.2	1.3	4.5	0.2
Accuracy (%)	99	100	100	100	100	100	100	101	100
Day 3									
CV (%)	0.3	0.3	0.2	4.7	0.5	0.2	3.0	2.9	0.5
Accuracy (%)	100	100	100	101	100	100	100	101	100

pression is dependent on the sample extraction procedure, and it is also compound dependent. Protein precipitation has the most severe effect on ion suppression compared to SPE and LLE. In the present work, although the analytes were extracted from plasma using 96-well LLE with ethyl acetate, ion suppression effects were still observed due to matrix components present in the reconstituted extracts. However, the low limits of quantitation achieved for each analyte in the matrices studied suggest that the matrix ion suppression effects were overcome by the use of D_6-isotope

Table 2
Inter-day assay accuracy (% nominal concentration) and precision (%C.V.) ($n = 54$)

	Human			Mouse			Rat		
QC (ng/ml)	0.75	50	175	0.75	50	175	0.75	50	175
BUP									
CV (%)	8.1	4.8	1.6	9.6	2.5	0.7	7.6	2.0	1.2
Accuracy (%)	102	99	100	105	103	100	101	102	100
TB									
CV (%)	4.6	5.3	2.6	3.2	5.6	11	2.1	1.9	5.4
Accuracy (%)	100	103	99	99	100	94	100	102	98
QC (ng/ml)	3.75	250	875	3.75	250	875	3.75	250	875
HB									
CV (%)	1.7	0.3	0.1	4.7	0.5	0.2	3.0	2.9	0.5
Accuracy (%)	100	100	100	101	100	100	100	101	100

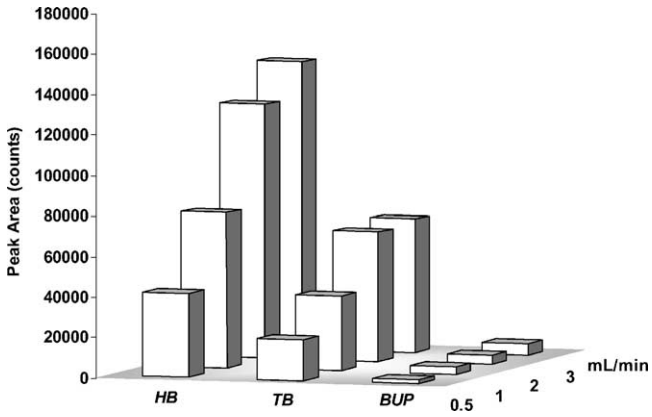


Fig. 8. Influence of the flow rate on the SRM LC/MS sensitivity. The mobile phase flow rate was maintained at 5 ml/min for fast chromatographic separation of BUP, TB, and HB, and split post-column to 0.5, 1.0, 2.0, 3.0 ml/min *via* a "T" connector to the TIS interface. Aliquots of 20 μl of a standard solution containing 0.25 ng/ml of BUP, TB and HB in mobile phase were then injected into the monolithic column, and SRM LC/MS chromatograms were acquired for each analyte.

labeled internal standards that underwent the same matrix suppression interference.

3.3. Influence of the mobile phase flow rate on the SRM LC/MS sensitivity

The influence of the mobile phase flow rate on the SRM LC/MS sensitivity was evaluated by splitting post-column the mobile phase flow rate from 5 ml/min to 0.5, 1, 2 and 3 ml/min to the TIS interface *via* a "T" connector. Flow rates of 4 and 5 ml/min were also diverted to the TIS source. However, although an optimization of the TIS source temperature and nebulizer (GS1) and TIS (GS2) gases was carried out for these relatively high flow rates, the mobile phase was poorly sprayed under these high flow rate conditions. Fig. 8 shows the effects of the mobile phase flow rate on the SRM LC/MS sensitivity. Under the experimental conditions employed, there was, approximately, a two-fold increase in peak area (counts) for all analytes by increasing the flow rate from 0.5 to 1 ml/min, and from 1 to 2 ml/min. A further increase from 2 to 3 ml/min slightly increased peak area of the analytes. Therefore, the mobile phase flow rate was split post-column from 5 to 2 ml/min via a "T" connector as a compromise between analyte sensitivity and performance of the TIS interface.

3.4. Reproducibility and accuracy at the lower limit of quantification (LLOQ) and recovery

The lower limit of quantitation is defined here as the lowest concentration on the calibration graph for which an acceptable accuracy of 100 ± 20% [(mean observed concentration/theoretical concentration) × 100] and a precision of 20% (R.S.D.) were obtained. The described assay has an LLOQ of 0.25 ng/ml for BUP and TB, and 1.25 ng/ml for HB in the plasma samples from each of the title species. Replicates of six analyses for each species and its corresponding LLOQ were performed and in all cases acceptable accuracy and precision were obtained at these LLOQs. Similarly, the QC1 studies described above met these same acceptance criteria. These data are consistent with those reported in previous studies (vide supra).

The extraction recoveries for each analyte at the three QC levels were determined by comparing the peak area ratios for each compound to that of the corresponding internal standard in samples that had been spiked with both analytes prior to extraction (pre-extraction) with samples to which both analytes had been added post-extraction. The internal standards were added to both sets of samples post extraction. For each analyte studied the recoveries were comparable to those reported previously [5].

4. Conclusions

A rapid, selective, and sensitive SRM LC/MS bioanalytical method has been developed and partially validated for the determination of bupropion and its metabolites, TB and hydroxybupropion, in human, mouse, and rat plasma. The use of a monolithic silica HPLC column combined with the high flow capability of the API 4000 allowed the chromatographic separation of BUP, TB, and HB within 23 s. The method was linear, accurate and precise over a concentration range of 0.25–200 ng/ml for BUP and TB, and from 1.25 to 1000 ng/ml for HB in all plasma samples. Although extracted matrix components contributed to some electrospray ionization suppression for each analyte, as observed by analyte post-column infusion experiments, the sensitivity of the method was adequate for all analytes in each biological matrix. The monolithic column performance was not significantly affected over 864 extracted plasma injections. The column back pressure increased approximately 20% from the first to the last injection, chromatographic peak shape was maintained, and the retention times of the analytes were reproducible within 5.9% CV. Although these columns showed a tendency for some peak tailing, they were very robust and gave reliable peak area reproducibility without compromising the determination of the analytes. The effect of the mobile phase flow rate on the SRM LC/MS sensitivity was observed by the increase in analyte peak area when the mobile phase flow rate was increased post-column to the mass spectrometer, supporting the concept of the TIS interface is mass flow sensitive.

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