Assessment of Tandem Mass Spectrometry and High Resolution Mass Spectrometry for the Analysis of Bupivacaine in Plasma

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Abstract

Triple quadrupole mass spectrometers coupled with high performance liquid chromatography are workhorses in quantitative bioanalyses. It provides substantial benefits including reproducibility, sensitivity and selectivity for trace analysis. Selected Reaction Monitoring allows targeted assay development but data sets generated contain very limited information. Data mining and analysis of non-targeted high-resolution mass spectrometry profiles of biological samples offer the opportunity to perform more exhaustive assessments, including quantitative and qualitative analysis. The objectives of this study was to test method precision and accuracy, statistically compare bupivacaine drug concentration in real study samples and verify if high resolution and accurate mass data collected in scan mode can actually permit retrospective data analysis, more specifically, extract metabolite related information. The precision and accuracy data presented using both instruments provided equivalent results. Overall, the accuracy was ranging from 106.2 to 113.2% and the precision observed was from 1.0 to 3.7%. Statistical comparisons using a linear regression between both methods reveal a coefficient of determination (R^2) of 0.9996 and a slope of 1.02 demonstrating a very strong correlation between both methods. Individual sample comparison showed differences from -4.5% to 1.6% well within the accepted analytical error. Moreover, post acquisition extracted ion chromatograms at *m/z* 233.1648 ± 5 ppm (M-56) and *m/z* 305.2224 ± 5 ppm (M+16) revealed the presence of desbutyl-bupivacaine and three distinct hydroxylated bupivacaine metabolites. Post acquisition analysis allowed us to produce semi-quantitative evaluations of the concentration-time profiles for bupicavaine metabolites.

Introduction

Drug discovery and development requires analytical methods that can provide comprehensive qualitative exploration, rapid profiling, and high precision and accuracy quantitation [Kaufmann *et al*., 2015; Ramanathan *et al.*, 2011]. The objective is to provide faster, comprehensive and more accurate answers to many different questions imbedded in the drug discovery and development process [Pritchard *et al*., 2003]. This is intimately related to our capacity to explore complex biological samples with greater depth. For two decades now, most bioanalytical methods relied on unit mass resolution mass spectrometers (i.e. Triple Quadrupole and Hybrid Quadrupole Linear Ion Trap mass spectrometers) [Ramanathan *et al.*, 2011]. Liquid chromatography attached to tandem mass spectrometry (HPLC-MS/MS) has revolutionized the analysis of drug and metabolites in complex biological matrices [Lee and Kerns, 1999]. This technique replaced older instruments, where less selective detectors were used (e.g. ultraviolet, fluorescence, electrochemical). The significant improvement in selectivity led to less matrix related interferences and consequently improved signal to noise ratio and reduced analytical error [Cairns, 2011; Shrivastava and Gupta 2011, Mulvana, 2010]. Many significant benefits where obtained with the adoption of HPLC-MS/MS and users accepted limitations associated with selected reaction monitoring methods (SRM).

High resolution quantitative and qualitative analysis in drug discovery and development may provide a wider range of applications compared to traditional analysis performed on unit mass resolution instruments (i.e. Triple Quadrupole and Hybrid Quadrupole Linear Ion Trap mass spectrometers) [Ma and Chowdhury, 2013; Wagner *et al*., 2013]. Non-targeted pharmaceutical drug analyses using a linear ion trap high-resolution mass spectrometer (LTQ-Orbitrap) was recently investigated and the authors shown the system capability for quantitative and qualitative

analysis performed in a single experiment [Zhang *et al*., 2009]. However, the authors used a relatively low-resolution setting for the Orbitrap instrument (i.e. 15,000 FWHM) most likely due inadequate scan rate for quantitative analysis. During the development of a bioanalytical method, it is important to recognize that a detection system needs to satisfy several conditions; primarily, it must be sensitive and specific to allow the analysis of trace amounts [Tamvakopoulos, 2007]. The analytical methods need to allow the quantitative determination and comparisons of drug, metabolites or other molecules among samples by simultaneous or sequential detection with acceptable figure of merits [Ye et al., 2009]. The analytical strategy used in bioanalysis needs to minimize the fluctuation in data measurements that might mask or reduce the precision of the determinations and consequently reduce the sensitivity of the assay [Cairns, 2011]. It is well known that technical and instrumental errors can be a significant source of variation [Friedman, 2012; Shrivastava and Gupta 2011, Mulvana, 2010]. Thus, the analytical strategy used may hamper the ability to adequately determine drug and metabolite concentrations in biological matrices for further analysis (e.g. pharmacokinetics and toxicokinetics). The quantitative performance of Triple Quadrupole (QqQ) and Hybrid Quadrupole Linear Ion Trap (QqLIT) mass spectrometers instruments are very well established. However, high resolution mass spectrometer, such as a hybrid Quadrupole-Orbitrap mass spectrometer is generally not used for routine quantitative drug and metabolites analysis despite offering major advantages in mass resolution (up to 280,000 FWHM) and mass accuracy (< 2 ppm). These figures of merit can provide substantial analytical advantages for qualitative analysis [Backfisch *et al*., 2014; Dinger *et al*., 2014; Wohlfarth *et al*. 2013]. However it is important to adequately test the precision and accuracy and compare it with a more traditional approach [Yoneyama *et al*. 2014]. One major issue with Orbitrap instruments was associated with slow scan rate at higher resolution setting resulting in fewer data point being collected. This was a significant source of data variation

affecting quantification. Newer Orbitrap instruments present improve scan rate at high-resolution settings allowing, possibly, significant improvement. In our case, we compared results generated with a state of the art Thermo Scientific TSQ Quantiva Triple Quadrupole Mass Spectrometer (QqQ) with a Thermo Scientific Q-Exactive Plus Orbitrap Mass Spectrometer operating at a resolution of 70,000 FWHM. The objectives of this study was to (1) test method precision and accuracy, (2) statistically compare bupivacaine drug concentration in real study samples and (3) verify if operating in scan mode at high resolution (HR) and accurate mass (AM) can actually permit retrospective data analysis, more specifically, extract metabolite related information.

Materials and Methods

Chemicals and Reagents

Bupivacaine was obtained from Galenova (St-Hyacinthe, Canada) as a USP standard. d_9-Bupivacaine was obtained from Toronto Research Chemicals (Toronto, Canada). Methanol, acetonitrile, formic acid and water were purchased from Fisher Scientific (NJ, USA). Ten mL of cat plasma was obtained from the *Faculté de médecine vétérinaire de l'Université de Montréal* donor group to perform method evaluations.

Plasma sample preparation

Using a protein precipitation as sample preparation technique, bupivacaine was extracted from cat plasma. Five hundred µL of internal standard solution (5.0 ng/mL of d_9-bupivacaine in methanol) was added to an aliquot of 25µL of plasma sample. The sample was vortexed for approximately 5 seconds and let stand for a period of 10 minutes, then centrifuged at 12,000 *g* for 10 minutes. The supernatant was transferred to an injection vial for analysis.

Chromatographic conditions

An isocratic mobile phase was used with a Thermo Scientific Aquasil C18 column (100 x 2.1 mm I.D., 5 μm) heated at 40°C. The mobile phase conditions consisted of acetonitrile and 10 mM ammonium formate in type 1 water (pH 3.0) at a ratio of 40:60, respectively. The flow rate was fixed at 300 μL/min and bupivacaine and its internal standard eluted at 2.0 minutes. Two μL of the extracted sample was injected and the total run time was set at 3.0 minutes.

Mass spectrometric conditions

The Thermo Scientific TSQ Quantiva Triple Quadrupole mass spectrometer (San Jose, CA, USA) was interfaced with the Thermo Scientific UltiMate 3000 XRS UHPLC system (San Jose, CA, USA) using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode, using selected reaction monitoring (SRM). The SRM transitions were set to 289 → 140 and 298 → 149 for bupivacaine and d_9-bupivacaine respectively. In order to optimize the MS/MS parameters, a standard solution of bupivacaine was infused into the mass spectrometer. The following parameters were obtained. Nitrogen was used for the sheath and auxiliary gases and was set at 50 and 15 arbitrary units. The HESI electrode was set to 3500 V and the capillary temperature was set to 350°C. Argon was used as collision gas at a pressure of 2.5 mTorr and the collision energy was set to 22 eV for bupivacaine and d_9-bupivacaine. Total cycle time was set at 250 msec. Peak width of Q1 and Q3 were both set at 0.7 FWHM.

Thermo Scientific Q-Exactive Plus Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced with the UHPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode but operating in scan mode at high-resolution, and accurate-mass (HR/AM). Identical source parameters were used. The scan range was set to *m/z* 100-500. Data was acquired at a resolving power of 70,000 (FWHM), resulting to a scanning

rate of < 0.75 scans/sec when using automatic gain control target of $3.0x10^6$ and maximum ion injection time of 100 msec. Post acquisition high resolution extracted ion chromatograms were generated using exact masse of targeted compounds ± 5 ppm. For comparison, please note that the QqQ instrument operated in a typical selected reaction monitoring (SRM) mode and the Quadrupole-Orbitrap mass spectrometer operated in scan HR/AM mode. The analytical range used was from 5 to 5 000 ng/mL and QC samples were prepared at concentrations of 5, 15, 250 and 3000 ng/mL to test method precision and accuracy. Standards and QCs were prepared in cat EDTA plasma. The precision and accuracy were determined using three individual runs including six replicate of each QC level and a standard curve prepared independently for both instruments tested.

Study samples

Plasma samples from a previous pharmacokinetics (PK) study were used for comparison purposes [Benito *et al*. 2015]. Briefly, eight healthy adult female cats weighing 3.2 ± 0.7 kg were studied after owners' written consent and institutional approval. General anesthesia was induced with propofol IV and maintained with isoflurane. Intraperitoneal bupivacaine 0.5% (2 mg kg^{-1}) administration was performed and venous blood was drawn from the jugular vein (catheter 20G x 2 mL) at 0, 2, 5, 10, 15, 20, 30, 60, 120 and 240 minutes after bupivacaine administration. Plasma bupivacaine concentrations were determined using QqQ instrument operating in SRM mode and a Quadrupole-Orbitrap mass spectrometer operating in scan HR/AM mode.

Data analysis and regression

All regression analyses were performed with Prism (6.0f) GraphPad software (La Jolla, CA, USA) using the nonlinear curve-fitting module with an estimation of the goodness of fit. Statistical analyses and method comparison were also performed with Prism (6.0f).

Results and Discussion

Tandem Mass Spectrometry

Precursor ion and product ion mass spectra for bupivacaine and d_9-bupivacaine were obtained in positive ion mode. The precursor ion spectra of bupivacaine and d_9-bupivacaine showed an intense signal for the protonated molecules ($[M+H]^+$) at *m/z* 289.2 and *m/z* 298.3, respectively (Fig. 1A and Fig. 1B). The product ion spectrum of bupivacaine and d_9-bupivacaine has predominant fragment ions at *m/z* 140.0 and *m/z* 149.0 (Fig. 1C and Fig. 1D). The mass transition in SRM mode was set for best sensitivity and selectivity at *m/z* 289.2 → 140.0 and 298.3 → 149.0 for bupivacaine and d_9-bupivacaine respectively. The product ion spectra were compatible with the molecular structures. Quadrupole-Orbitrap HR/AM spectra (i.e 100 - 500 *m/z*) were acquired and protonated molecules ($[M+H]^+$) at *m/z* 289.2268 and *m/z* 298.2832 were observed (Fig. 1E and Fig. 1D). The monoisotopic mass of bupivacaine ($C_{18}H_{29}N_2O^+$) is 289.2274 and d_9-bupivacaine ($C_{18}H_{20}d_9N_2O^+$) is 298.2839. The observed masses are deviating by 2.1 ppm and 2.3 ppm respectively which is within the instrument specifications. The high-resolution extracted ion chromatograms were generated using 289.2268 ± 5 ppm and m/z 298.2832 ± 5 ppm.

Calibration Curve Analysis

A linear regression (weighted 1/concentration) produced the best fit for the concentration–detector relationship. The regression model used was determined using the sum of the squares of

the deviations [Beaudry, 1999]. By convention, the regression line is considered to properly fit the calibration set when the sum of squares of the deviations is minimized. The concentrations were determined based on the peak area ratio of bupivacaine and its deuterated analogue d_9-bupivacaine. The calculated coefficients of correlation (r) were better than 0.9993 for an analytical range set from 5 to 5000 ng/mL in cat EDTA plasma. Both instrument provided equivalent linearity.

Precision and Accuracy

The reproducibility of the method was evaluated by analyzing minimally six replicates of cat EDTA plasma fortified with bupivacaine at the nominal concentration of 5, 15, 250 and 3000 ng/mL. The precision and accuracy results are displayed in Table 1. The precision and accuracy data presented using a QqQ instrument in SRM mode or a Quadrupole-Orbitrap mass spectrometer in full scan HR/AM mode and post acquisition extracted ion chromatograms provided equivalent results. Overall, the accuracy was ranging from 106.2 to 113.2% and the precision observed was from 1.0 to 3.7%. Thus, results obtained from both approaches demonstrated that acceptable precision and accuracy results were achieved and were compatible with generally accepted criteria in bioanalysis [CDER and CVM, 2001]. Representative chromatograms are shown in Fig 2. The LLOQ chromatograms reveal an intense signal in SRM mode using a QqQ instrument. The signal to noise ratio observed was ≈ 30 following analysis onto a Quadrupole-Orbitrap mass spectrometer in full scan HR/AM mode using post acquisition extracted ion chromatogram. Both approaches provided adequate sensitivity for our bupivacaine study, but there is a tradeoff in sensitivity when using Quadrupole-Orbitrap mass spectrometer in scan HR/AM mode. The LLOQ was set to 5 ng/mL for this method since all the study samples shown concentration well above the current 5 ng/mL. Indeed, the QqQ instrument in SRM mode

could be optimized to achieve a much lower LLOQ if required for a targeted pharmacokinetic assay. The Quadrupole-Orbitrap mass spectrometer was acquiring data at a high-resolution setting (70 000 FWHM) and the resulting scan rate was 0.75 sec/scan allowing the collection of sufficient data points to obtain reproducible quantitation based on peak area ratios (e.g. drug and internal standard). The resolution and resulting scan rate should be carefully chosen based on the chromatographic condition, particularly when using Ultra High Performance Liquid Chromatography (UHPLC) to assure adequate data sampling. The minimum number of data points for each chromatographic peak should be eight or above to reduce instrumental error. A careful assessment of instrument sensitivity is required to assure a good fit for purposes. As illustrated in Fig 2., extracted blank cat plasma samples did not show any interference from endogenous substances at the mass transition monitored (*m/z* 289.2 → 140.0) or at the post acquisition extracted ion chromatogram at *m/z* 289.2268 ± 5 ppm.

Comparison of analytical methods

Assessment of the equivalence in results generated by different analytical methodologies is important especially when methods are used in a regulated laboratory [Yoneyame *et al*., 2014]. This is particularly important in pharmaceutical sciences since a large proportion of the studies are conducted under GLP. Methodology changes are implemented for both scientific and technical reasons during a preclinical or clinical study. Thus, it is important to demonstrate the adequacy of using data generated by distinct methods as part of a single study. Study samples (n = 80) were extracted and analyzed using a QqQ instrument operating in SRM mode and a Quadrupole-Orbitrap mass spectrometer operating in full scan HR/AM mode. Using a linear regression, the analysis of paired measurements generated by both methods was assessed as shown in Fig. 3. The linear regression quantifies goodness of fit and the R^2 was 0.9996 and the

slope was 1.02 demonstrating a very strong correlation between both methods. Individual sample comparison showed differences from -4.5% to 1.6% well within the accepted analytical error [CDER and CVM, 2001]. The data demonstrated very good performance of the quantification method using HR/AM scan mode when compared to a state-of-the-art SRM assay. Targeted triple quadrupole SRM assays are very sensitive and quantitative, but they do not provide the benefit of comprehensive qualitative exploration or the possibility to re-visit data for further interrogation. Profiling samples using Quadrupole-Orbitrap mass spectrometer could allow the detection and confirmation of drug metabolites with high resolution and accurate mass.

Profiling study samples

The analysis of study samples using a Quadrupole-Orbitrap mass spectrometer in HR/AM scan mode allows scientists to extensively explore the resulting data. A majority of drugs have lipophilic properties and in most cases require biotransformation to be excreted [Yan and Caldwell, 2001]. Most biotransformation products result in a more polar substance and consequently are more easily excreted in urine and/or the biliary system. Hence the exposure of the animal (or human) to the compound is reduced and the potential toxicity may be reduced if the metabolites are pharmacologically inactive. The metabolic fate of a drug is an important factor contributing to its efficacy, toxicity, distribution and excretion. Metabolism is divided into two phases (i.e. phase 1 and phase 2). Phase 1 metabolic transformation includes oxidation, reduction and hydrolysis and phase 2 products are typically referred to as conjugated metabolites (i.e. glucuronide, sulphate, glutathione, cysteine, and methyl derivatives). Most of the metabolism is occurring in the liver because of its position, blood supply and function. However, other organs and tissues possess some metabolic activity and in some cases may be more important than the liver. As outlined before, biotransformation products often result in a change in mass that can be

predicted [Kostiainen *et al*., 2003]. Bupivacaine metabolism data are sparse and to our knowledge, no data were reported in cat. Two main metabolites were previously identified; desbutyl-bupivacaine and hydroxylated bupivacaine in other animal species [Pere *et al*., 1991; Gantenbein *et al*., 2000]. The monoisotopic mass of desbutyl-bupivacaine ($C_{14}H_{21}N_2O^+$) is *m/z* 233.1648 and the monoisotopic mass of the hydroxylated bupivacaine ($C_{18}H_{29}N_2O_2^+$) is *m/z* 305.2224. As shown in Figure 4, post acquisition extracted ion chromatograms at *m/z* 233.1648 ± 5 ppm (Fig. 4B) and *m/z* 305.2224 ± 5 ppm (Fig. 4C) revealed the presence of desbutyl-bupivacaine and three distinct hydroxylated bupivacaine metabolites. Moreover, as illustrated in Figure 4, using targeted extracted ion chromatograms, we can concentration-time profiles for bupicavaine and its metabolites allowing semi-quantitative evaluations performed post acquisition. The metabolic fate of a drug is very important and the analysis of these cat samples permitted the identification of several metabolites with high accuracy and enabled the generation of concentration-time profiles that allowed us to better understand the drug elimination and exposition in a single injection without prior knowledge. High resolution and accurate mass acquisition strategies on high-resolution mass spectrometers permit data analysis that are not possible with SRM based method. These advanced acquisition strategies may have significant impacts in the development of quantitative and qualitative methods used in pharmaceutical drug discovery and development, clinical research and diagnostic, food safety, environmental analysis, forensic toxicology, and basic research.

Conclusion

Triple quadrupole mass spectrometers have been the workhorse for quantitative bioanalytical laboratories over the last two decades. It still delivers the best sensitivity and allows multiplexing using SRM mode. The introduction of high-resolution mass spectrometers driven for routine

analysis may modernize analytical strategies used in bioanalysis. These instruments have the sensitivity, speed, mass accuracy and resolution to deliver comprehensive qualitative and high-resolution quantitation in complex biological matrices within a single injection. Moreover, data collected can be further analyzed as shown with the current example with the discovery of bupivacaine metabolites post sample analysis. We believe that the scope of high-resolution mass spectrometry in bioanalysis reaches far beyond the example shown in this paper and can enable analysis in pharmaceutical drug discovery and development, life science research, clinical research, toxicology and food and environmental safety that was not previously achievable using a single platform with a generic approach.

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Table 1. Determination of the precision and accuracy for the analysis of bupivacaine in feline plasma by HPLC-MS/SRM and HPLC-MS/HR/AM

Instrumental Method	Concentration (ng/mL)	Intra (n=6) Mean ± SD (ng/mL)	% CV	% NOM	Inter (n=18) Mean ± SD (ng/mL)	% CV	% NOM
HPLC-MS/SRM							
	5.00	5.60 ± 0.08	1.4	112.1	5.41 ± 0.16	3.0	108.1
	15.0	16.3 ± 0.2	1.5	108.8	16.4 ± 0.3	1.6	109.2
	250	276 ± 3	1.0	110.6	272 ± 5	1.9	108.9
	3000	3211 ± 57	1.8	107.0	3245 ± 60	1.8	108.2
HPLC-MS/HR/AM							
	5.00	5.33 ± 0.12	2.2	106.6	5.31 ± 0.19	3.7	106.2
	15.0	16.6 ± 0.4	2.3	110.4	16.4 ± 0.3	1.6	109.0
	250	283 ± 3	1.0	113.2	276 ± 6	2.1	110.5
	3000	3210 ± 38	1.2	107.0	3259 ± 53	1.6	108.6

Figure legends

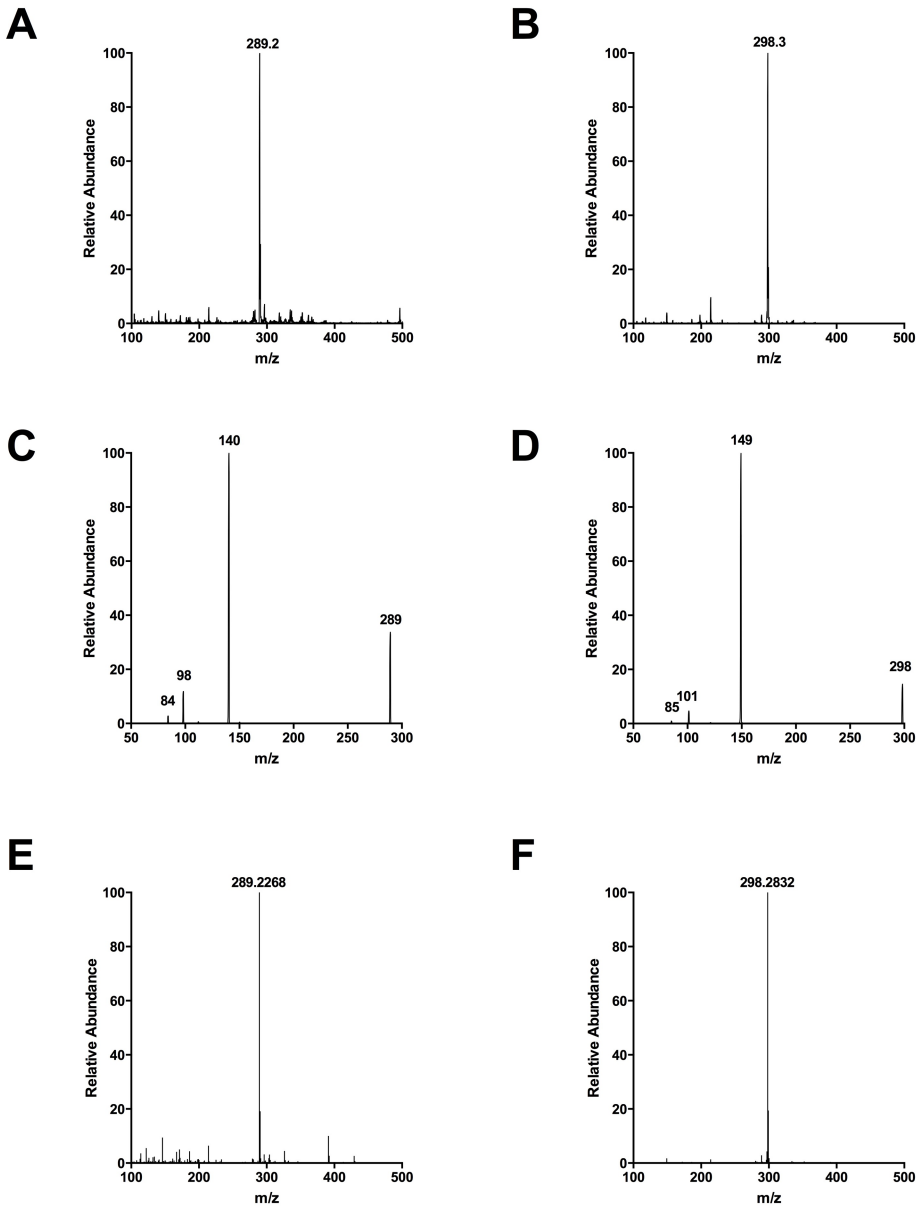
Figure 1. Positive mode ESI-MS and ESI-MS^2 spectra of bupivacaine and d_9-bupivacaine generated following a direct infusion on a triple quadrupole mass spectrometer and a hybrid quadrupole-orbitrap mass spectrometer. (A) and (B) are precursor ion spectra showing singly charged bupivacaine and d_9-bupivacaine ions at *m/z* 289.2 and 298.0 obtained on a triple quadrupole mass spectrometer. (C) and (D) are product ion spectra generated by CID on a triple quadrupole mass spectrometer and subsequently used for the analysis in HPLC-MS/SRM mode. (E) and (F) are precursor ion spectra generated with a hybrid quadrupole-orbitrap mass spectrometer showing singly charged bupivacaine and d_9-bupivacaine ions at *m/z* 289.2268 and 298.2832

Figure 2. Reconstructed ion chromatograms for bupivacaine. (A) HPLC-MS/SRM extracted ion chromatogram of transition *m/z* 289.2 → 140.0 of an extracted blank sample (red line), an extracted LLOQ plasma sample (blue line) and an extracted PK sample (green line). (B) HPLC-MS/HR/AM extracted ion chromatogram of *m/z* 289.2268 (± 5 ppm) for an extracted blank sample (red line), an extracted LLOQ plasma sample (blue line) and an extracted PK sample (green line).

Figure 3. Regression analysis for the assessment of the agreement between both mass spectrometric approaches for PK measurements.

Figure 4. Overlay HPLC-MS/HR/AM extracted ion chromatograms for the drug and two metabolites. (A) Time overlays HPLC-MS/HR/AM extracted ion chromatograms for bupivacaine at *m/z* 289.2268 (± 5 ppm). (B) Time overlays HPLC-MS/HR/AM extracted ion chromatograms for desbutyl-bupivacaine at *m/z* 233.1648 (± 5 ppm). (C) Time overlays HPLC-MS/HR/AM extracted ion chromatograms for the hydroxylated bupivacaine at *m/z* 305.2224 (± 5 ppm).

Figure 1.


A
289.2
Relative Abundance
m/z
B
298.3
Relative Abundance
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C
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289
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84
Relative Abundance
m/z
D
149
298
101
85
Relative Abundance
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E
289.2268
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F
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Relative Abundance
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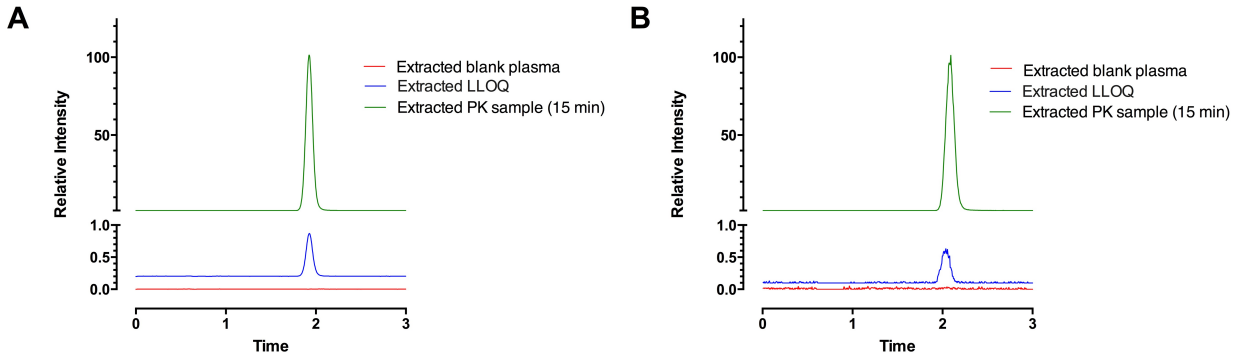
Figure 2.


A
B
Relative Intensity
100
50
1.0
0.5
0.0
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1
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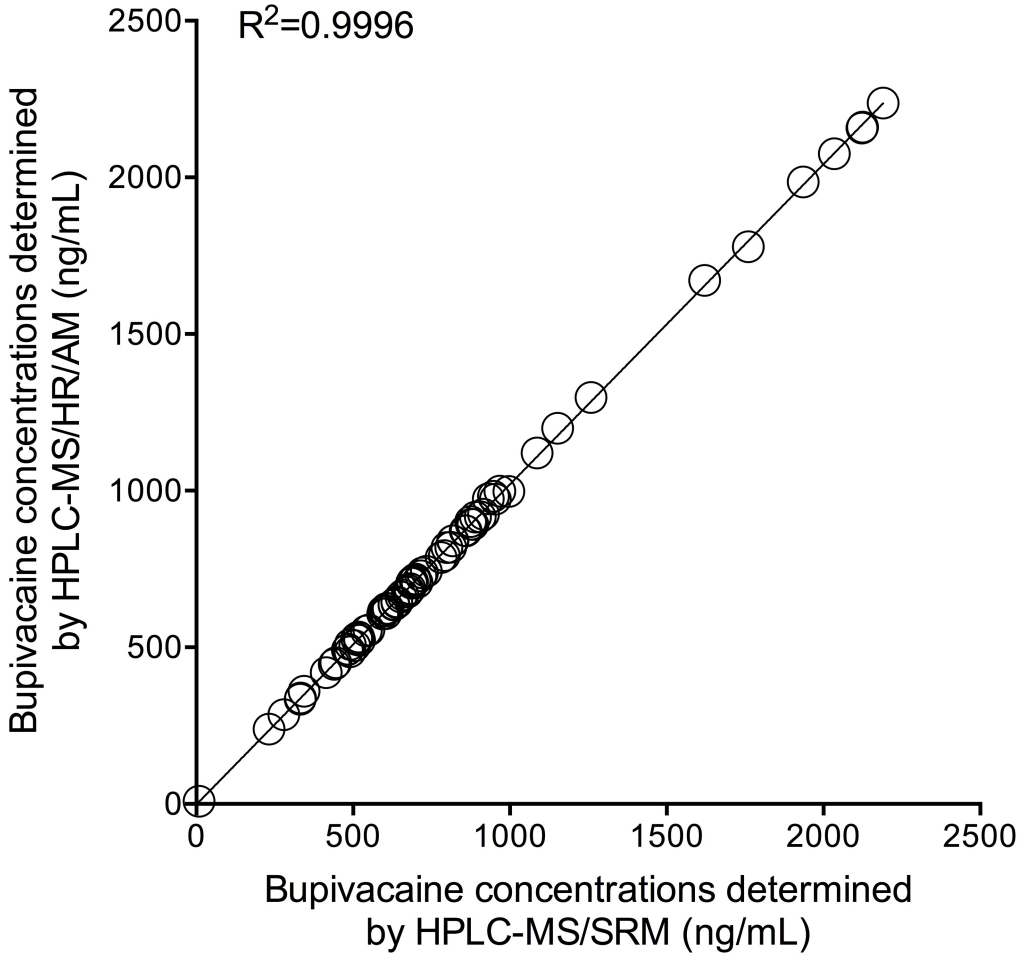
Time
Extracted blank plasma
Extracted LLOQ
Extracted PK sample (15 min)

Figure 3.


R2=0.9996
2500
2000
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1000
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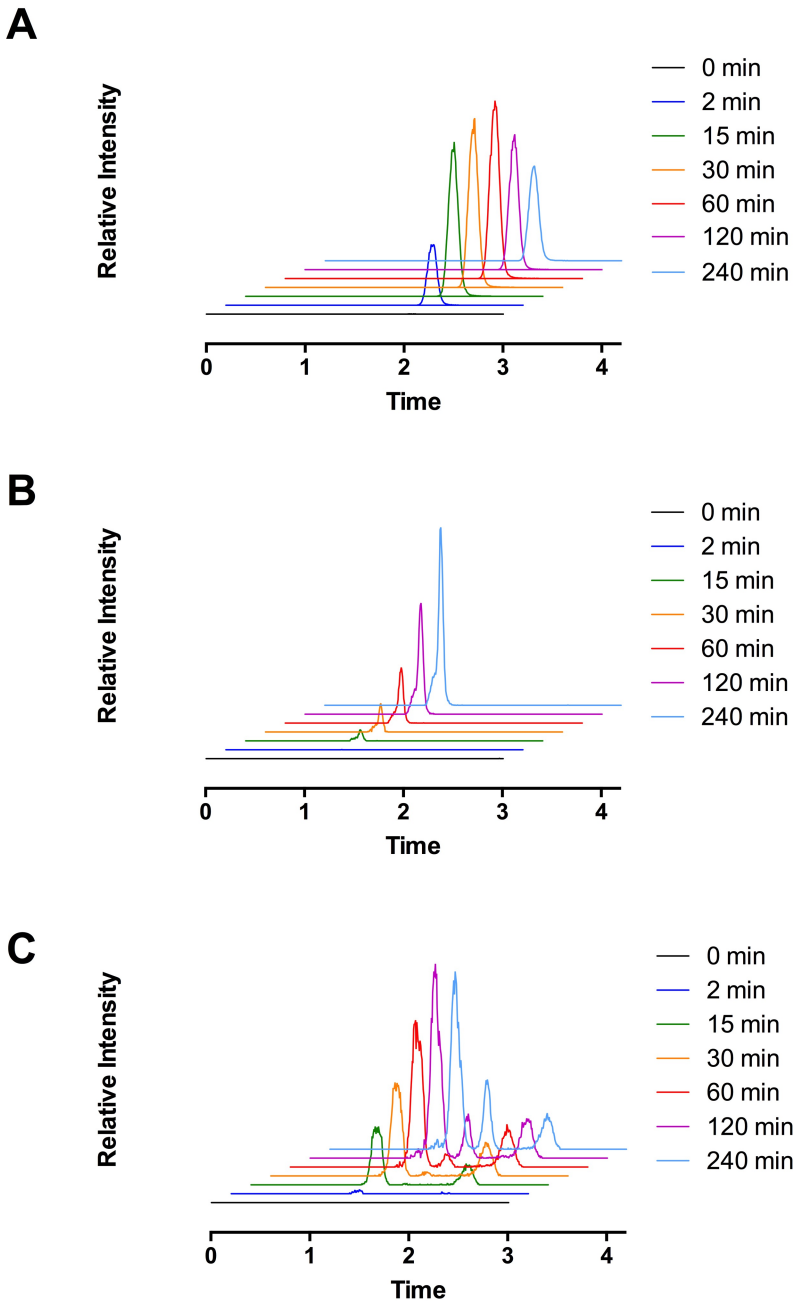
0
0
500
1000
1500
2000
2500
Bupivacaine concentrations determined by HPLC-MS/HR/AM (ng/mL)
Bupivacaine concentrations determined by HPLC-MS/SRM (ng/mL)

Figure 4.


A
Relative Intensity
0 min
2 min
15 min
30 min
60 min
120 min
240 min
0
1
2
3
4
Time
B
Relative Intensity
0 min
2 min
15 min
30 min
60 min
120 min
240 min
0
1
2
3
4
Time
C
Relative Intensity
0 min
2 min
15 min
30 min
60 min
120 min
240 min
0
1
2
3
4
Time