A High Resolution Accurate Mass Approach for the Quantitation of Buprenorphine and Paroxetine in Rat Plasma

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Overview

Purpose: To demonstrate a High Resolution Accurate Mass (HRAM) approach for the quantitation of Buprenorphine and Paroxetine in the presence of protein precipitated rat plasma.

Methods: Sample analysis was performed using Single Ion Monitoring (SIM) with generic mass spectrometer source conditions and chromatographic separation utilizing ultra high performance chromatography (UHPLC).

Results: Sample analysis demonstrated a lower limit of quantitation (LOQ) of 10pg/mL for both analytes, a linear signal response of more than four orders of magnitude, a coefficient of variance of less than ten percent for all replicate injections.

Introduction

As potential leads for successful drug candidates move through the drug discovery process there is a need for quantitative in vitro and in vivo analysis at each step of the journey. The requirement for quantitative assays that provide sensitivity, ruggedness, and linear response has remained a constant but as mass spectrometer technology has progressed over time other considerations also play an important factor in meeting the needs of these assays. Ease of use, simplified method development, and troubleshooting tools, now play an important factor when choosing the most appropriate technology for a particular assay and the corresponding method development.

Quantitation by high resolution accurate mass (HRAM) delivers reliable results for analyses at extremely low concentration levels and in complex matrices . HRAM enables direct analysis and monitoring of the ionized target analyte through SIM scanning, without the need to improve selectivity through fragmentation or multiple reaction monitoring (MRM). HRAM analysis at a resolution setting of 50,000 (FWHM at m/z 200) or greater has been routinely demonstrated to effectively resolve interferences present in complex matrixes such as plasma and honey.[1,2] Data collection in SIM mode also provides additional information regarding possible interferences near the target analyte m/z. This additional information can be highly beneficial in method development, method optimization, and troubleshooting while eliminating the need for additional injections or complex scan experiments. Here we examine the quantitative performance of the Thermo Scientific™ Q Exactive™ Focus mass spectrometer for the analysis of Buprenorphine and Paroxetine in rat plasma.

Methods

Sample Preparation

Crashed plasma stock solutions were prepared using an Acetonitrile (ACN) crash at a ratio of 3:1, ACN to plasma. The resulting solution was centrifuged at 10,000rpm for 10 minutes. The supernatant was removed and added to an equivalent volume of water to make the final crashed plasma stock solution. Stock solutions of Buprenorphine and Paroxetine at 1mg/mL were diluted in the crashed plasma stock to concentration ranges of 10pg/mL to 25,000pg/mL and 10pg/mL to 100,000pg/mL respectively. Isotopically labeled Buprenorphine-D4 and Paroxetine-D6 were added at each concentration level as an internal standard to produce a final internal standard concentration of 1ng/mL. All reagents were obtained from Cerilliant Corporation, Round Rock, Texas, at 1mg/mL in methanol.

Liquid Chromatography

Chromatographic separation was achieved using Thermo Scientific™ Vanquish™ UHPLC System. Samples were injected (5uL) onto a 2.1 x 100mm, 1.9um Thermo Scientific™ Hypersil Gold aQ column. Gradient elution was accomplished using water + 0.1% formic acid (FA) (A) and acetonitrile + 0.1% formic acid (FA) (B), with a 6 minute gradient at a flow rate of 500uL/min (Table 1). Total run time including column equilibration was approximately 8 minutes.

Mass Spectrometry

Compounds were analyzed utilizing a Thermo Scientific™ Q Exactive Focus™ MS with heated electrospray ionization (HESI-II). Generic source conditions suitable for a 500uL/min LC flow rate were applied for all data collection (Table 2). Data was acquired in selected ion monitoring (SIM) mode with an external mass calibration and collected and using Thermo Scientific™ Xcalibur data acquisition software.

Data Analysis

All data was processed utilizing Thermo Scientific™ Xcalibur processing data . All chromatographic integration was accomplished using automated processing settings. No manual integration or smoothing was applied to any chromatographic data.

Time (min)	Flow rate (uL/min)	%A	%B
0	500	95	5
0.1	500	95	5
0.5	500	70	30
4.5	500	67	33
5	500	5	95
6.0	500	5	95
6.1	500	95	5
8.0	500	95	5

Table 1. LC gradient method utilized for sample analysis.

HESI Source Settings	Value
Spray Voltage (V)	4000
Vaporizer temperature (°C)	450
Capillary Temperature (°C)	350
Sheath Gas Pressure (Arb)	45
Aux Gas Pressure (Arb)	15
Ion Sweep Gas Pressure (Arb)	1

MS Scan Settings	Valu
Scan Type	SIM
Resolution	70,00
AGC Target	2.00E+
IT Fill Time (ms)	260

Table 2. Mass Spectrometer settings utilized for sample analysis.

Results

Quantitative Results

The main goals for any rugged quantitative assay are reaching the lowest possible limit of quantitation, providing a linear response, and maintaining good reproducibility. To evaluate the performance and reproducibility of the LC/MS method, calibration curves were analyzed with replicates of n=6. Linearity and reproducibility were calculated across the working range of the curve. The limit of quantitation (LOQ) was defined as the lowest concentration level that is both within <20% difference of the linear fit and <20% RSD for each group of replicate concentration points. Using the above criteria, the LOQ was determined to be 10pg/mL for both Buprenorphine and Paroxetine. (Figure 1) A linear response was observed across four orders of magnitude for Buprenorphine and Paroxetine ranging from 10pg/mL to 100ng/mL (Figure 2).

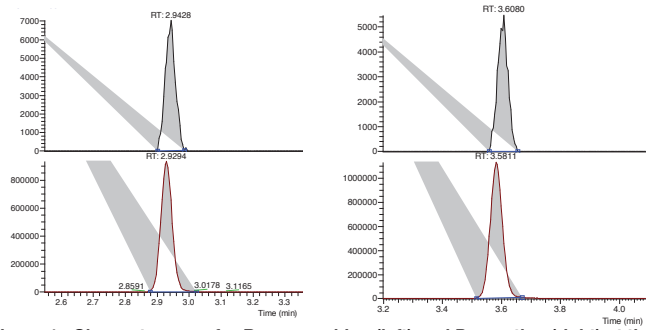

Figure 1. Chromatograms for Buprenorphine (left) and Paroxetine (right) at the LOQ of 10pg/mL. Target analyte is displayed at 10pg/mL in the top chromatogram. The internal standard chromatogram at 1ng/mL is displayed below. No smoothing was applied during the chromatographic peak integration and data processing procedure.

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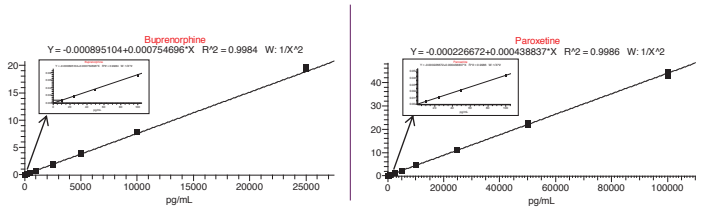

Figure 2. Calibration Curve for Buprenorphine and Paroxetine. Linear regression was performed using 1/x2 weighting.

A linear response was observed across four orders of magnitude for Buprenorphine and Paroxetine ranging from 10pg/mL to 25,000 and 10pg/mL to 100,000pg/mL respectively (Figure 2).

The analytical scan speed of the instrument was evaluated at the LOQ for each sample. The number of scans across each analyte peak at the LOQ was assessed to ensure adequate scan speed in conjunction with the sample peak width produced by the chromatographic method. The chromatographic peak width for each analyte ranged between 3.6 and 4.2 seconds at the base, providing 11-15 scans for the target analytes at the LOQ (Figure 3).

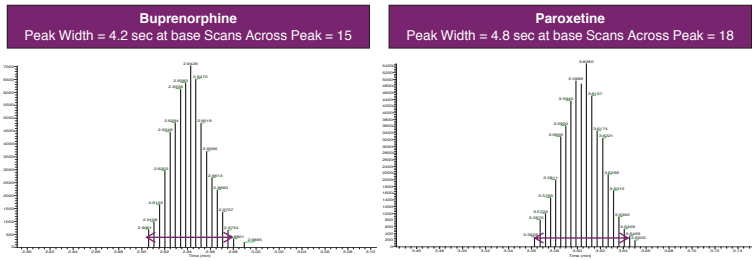

Figure 3. Evaluation of chromatographic peak width vs scan speed for Buprenorphine (left) and Paroxetine (right) the LOQ of 10pg/mL

The number of scans recorded across the width of each analyte peak correlates with the instrument specification of an analytical scan speed of 3Hz at the 70,000 resolution setting. Analysis of the prepared sample set at each concentration level demonstrated results that met or exceeded all of the previously described acceptance criteria indicating a high level of performance across the working range of the assay (Table 3)

Buprenorphine				
Standard Conc.Level (pg/mL)	Mean Calc Amount	AVG %Diff	Area Ratio % CV	Excluded Replicates
10	10.3	3.2%	4.4%	0
25	23.7	5.1%	1.9%	0
50	47.7	4.6%	1.5%	0
100	98.9	1.1%	1.5%	0
250	242	3.4%	1.0%	0
500	497	0.6%	0.6%	0
1000	1011	1.1%	0.7%	0
2500	2501	0.05%	0.8%	0
5000	5159	3.2%	1.0%	0
10,000	10413	4.1%	0.8%	0
25,000	25794	3.2%	0.9%	0

Paroxetine				
Standard Conc.Level (pg/mL)	Mean Calc Amount	AVG %Diff	Area Ratio % CV	Excluded Replicates
10	10.3	2.7%	8.6%	0
25	23.7	5.3%	1.8%	0
50	48.4	3.3%	1.2%	0
100	101	1.3%	1.5%	0
250	245	2.1%	1.0%	0
500	507	1.4%	0.6%	0
1000	1030	3.0%	0.5%	0
2500	2474	1.0%	1.2%	0
5000	5122	2.4%	0.7%	0
10,000	10286	2.9%	0.4%	0
25,000	24702	1.2%	0.6%	0
50,000	49916	0.2%	1.2%	0
100,000	99385	0.6%	1.2%	0

Table 3. Statistical Results for Buprenorphine and Paroxetine Replicates. Note: AVG %Diff represents the average difference from the linear regression fit of the calibration curve for each set of replicates at a given concentration point.

The spectra of target analytes at the LOQ can be used to verify method quality, determine the suitability of a given resolution setting, and help identify interferences that may inhibit sensitivity and reproducibility. Spectra from injections made at the LOQ for each compound were evaluated to identify unresolved interferences. At the resolution setting of 70,000 (FWHM at m/z 200) both compounds were adequately resolved from all co-eluting interferences (Figure 4). As seen in the figure, although interferences are present during the elution of each compound, the target analyte m/z was baseline resolved from nearby interferences at the 70,000 resolution setting. Additionaly the mass extraction window of +/- 5ppm used to generate the chromatographic peaks used for quantitation is illustrated in the figures. This narrow extraction window allows greater assay confidence and robustness by ensuring that signal contributing to the chromatographic peak area is resulting from the target analyte itself and not the nearby interferences. A narrow mass extraction windows and the resulting XIC do not provide analytical value without a high level of scan to scan mass accuracy across the entire analyte peak. Scan to scan mass accuracy can also offer an important indicator for indentifying potential analyte interferences as well for accessing overall assay performance. A loss in mass accuracy can provide an indication of an unresolved or co eluting interference and can be implemented as a quality control measure in validated analyses.

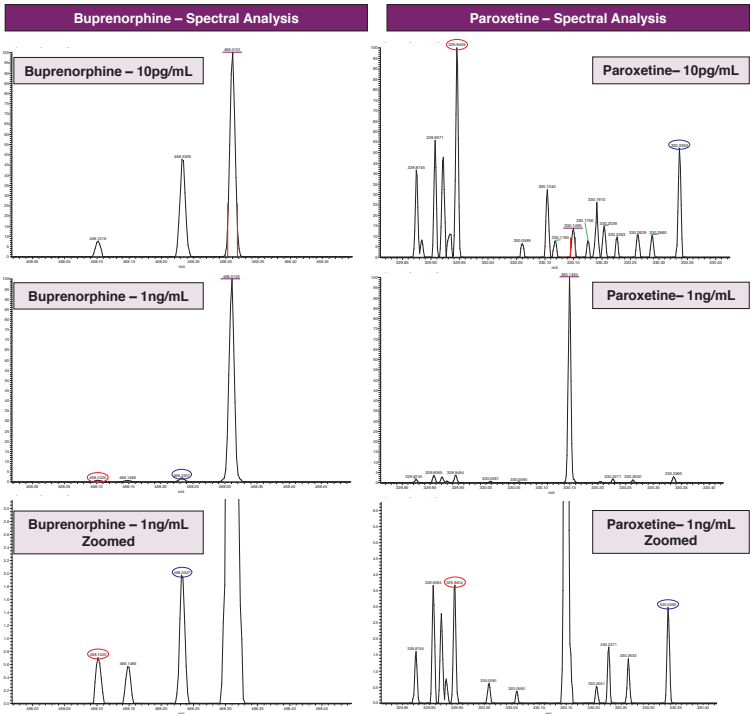

Figure 4. Spectra analysis of Buprenorphine and Paroxetine at 10pg/mL and 1ng/mL in rat plasma. The exact m/z for the target analyte has been underlined in each spectra and the 5ppm mass extraction window used to generate chromatographic peaks has been outlined. Common interferences across each sample concentration have been circled. All interferences present in the sample were demonstrated to be baseline resolved at the 70,000 resolution setting.

Here we examine the scan to scan mass accuracy of the Q Exactive Focus with an external mass calibration for Buprenorphine and Paroxetine at assay LOQ of 10pg/mL (Figure 5). The mass accuracy for each scan across the analyte peaks was demonstrated to be less than 3ppm for both Buprenorphine and Paroxetine. The high level of mass accuracy from scan to scan allows the utilization of a narrow mass extraction window providing robust and reproducible results at low analyte concentration levels while in the presence of a biological matrix. Evaluation of the scan to scan mass accuracy provides a method development and troubleshooting tool that is unique to HRAM and the Orbitrap mass analyzer.

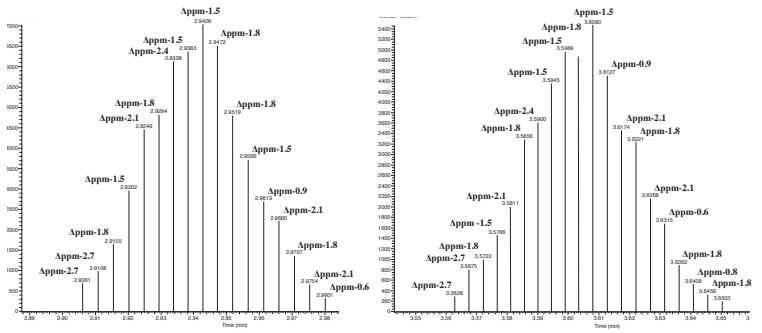

Figure 5. Zoomed view of Buprenorphine at 10pg/mL (LEFT) and Paroxetine at 10pg/mL (Right). The figure illustrates the scans measured across the peak and the corresponding mass accuracy. Mass accuracy is displayed in parts per million. (ppm)

Chromatographic reproducibility is an import factor for determining assay quality and ruggedness. Consistent and reproducible signal response at the assay LOQ eliminates the need for manual chromatographic peak integration and improves the overall data confidence and the assay as a whole. Here we examine the chromatographic reproducibility of Buprenorphine and Paroxetine at the assay LOQ of 10pg/mL. Each analyte was analyzed in replicates of n=6. The chromatogram for each replicate can be seen in figure 6.

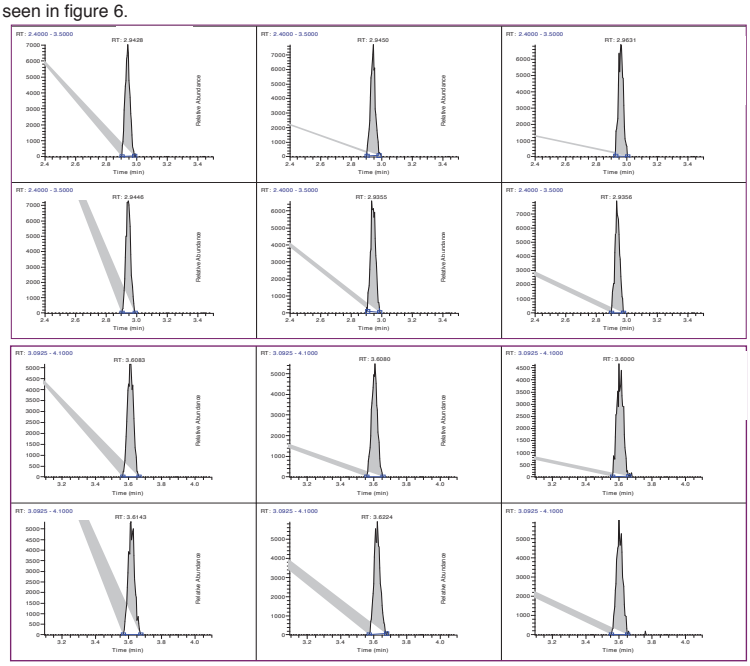

FIGURE 6. Replicate injections of Buprenorphine (Top) and Paroxetine (bottom) at the assay LOQ of 10pg/mL. Method integration settings where implement for all chromatogram integration and no manual integrations were performed. Additionally no smoothing was applied for any chromatographic peak generation.

Conclusion

In this experiment we described a method for the quantitation of Buprenorphine and Paroxetine. The LOQ was determined to be 10 pg/mL for each compound. The signal response across the concentration range of the assay was determined to be linear across 4 orders of magnitude, from 10 pg/mL to 100 ng/mL. Evaluation of the analytical scan speed determined that 11-15 scans were generated for chromatographic peak widths ranging between 3-4 seconds at the base. Additionally the spectrum of each analyte was examined near the target m/z to identify possible interferences. At the resolution setting of 70,000, all interferences present in the sample matrix were adequately resolved from the analyte of interest. Also the scan to scan mass accuracy at the LOQ was evaluated demonstrating a Δppm of less than +/- 5ppm for each scan across the analyte peak. These data interrogation options offer valuable method development and troubleshooting tools that provide greater confidence in the analytical method and quantitative results. Overall the Q Exactive Focus mass spectrometer was determined to be very well suited for robust and reproducible quantitation at very low concentration levels in biological matrixes.

References

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2. Henry H. et al., Rapid Commun. Mass Spectrom. 2012, 26, 499–509

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