Quantitation of Opiates to Low ng/mL Levels in Urine for Forensic Use Using an Affordable, High-Resolution, Accurate-Mass Mass Spectrometer

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Key Words
Q Exactive Focus, opiates, morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, TraceFinder, forensic toxicology, drugs of abuse, PRM, parallel reaction monitoring

Goal
To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer as a quantitative platform for HPLC-MS analysis of opiates in human urine to low ng/mL levels for forensic toxicology.

Introduction
Forensic toxicologists need an economical instrument capable of both screening a large number of compounds and quantifying smaller panels to industry-established limits. Here we present a method for quantitation of six opiates—morphine, codeine, hydromorphone, hydrocodone, oxymorphone, and oxycodone—in human urine down to low ng/mL levels. This work was performed on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer.

Methods
Sample Preparation
Samples were processed by enzymatic hydrolysis followed by urine dilution. Briefly, an aliquot of urine was spiked with stable-isotope-labeled internal standards and incubated with β-glucuronidase enzyme. The resulting mixture was centrifuged and further diluted before an aliquot was analyzed by gradient HPLC and a Q Exactive Focus MS. Calibrators and controls were prepared by spiking compounds into blank synthetic urine in the range of 1 to 5000 ng/mL.

Liquid Chromatography
Gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with OAS autosampler (Figure 1). Mobile phases consisted of 10 mM ammonium acetate with 0.1% formic acid in water and methanol (Fisher Chemical brand) for solvents A and B, respectively. The column used was a Thermo Scientific™ Accucore™ PFP, 2.6 μm particle size, 50 x 2.1 mm fused core (p/n 17426-052130). The gradient was run from 0 to 70% mobile phase B over 3.3 minutes followed by a column wash at 100% B and re-equilibration to starting conditions. The total run time was 5.3 minutes.

Mass Spectrometry
Compounds were detected on a Q Exactive Focus MS equipped with a Thermo Scientific™ Ion Max™ source and a heated electrospray ionization (HESI-II) sprayer. Data was acquired in parallel reaction monitoring (PRM) mode. In this mode, a single precursor ion is selected in the quadrupole with an isolation width of 3.0 m/z and fragmented in the HCD cell. The resulting MS/MS product ions are detected in the Orbitrap detector at a resolution of 35,000.

Method Evaluation
The method precision and accuracy were evaluated by running a calibration curve and quintuplicate replicates of quality controls on three different days. Additionally, internal-standard response was assessed in 58 donor samples obtained from a collaborator laboratory and compared to a sample prepared in water to determine matrix effects.
**Data Analysis**

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. Two product ions were selected as the quantifying and confirming ions for each compound. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification and ion ratio confirmation. Because the entire MS/MS spectrum was collected, multiple confirming ions could be chosen. Figure 2 shows a representative MS/MS spectrum for oxymorphone, highlighting the quantifying and confirming ions with corresponding reconstructed chromatograms.

**Results**

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20%, ion ratios within defined tolerance (tolerance dependent upon actual ratio), and quality controls within 20% RSD as well as meeting the above two requirements. Using these criteria, limits of quantitation for codeine, oxycodone, and oxymorphone were determined to be 2.5 ng/mL. For morphine, hydrocodone, and hydromorphone, the limit was 5 ng/mL. Tables 1 and 2 show the inter- and intra-assay statistics, respectively, for quality controls for all compounds in this method. Limited matrix effects were observed. The average recovery across 58 donor urine samples obtained from a collaborator laboratory ranged from 69% to 81% for the six internal standards evaluated. Figure 3 shows a combined chromatogram for analytes at their respective LOQs, and Figure 4 shows chromatograms for each compound with confirming ion ratio at its LOQ. Figure 5 shows representative calibration curves for all compounds. Figure 6 shows representative chromatograms with ion ratio confirmation for donor samples.
<table>
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<tr>
<th></th>
<th>Codeine</th>
<th>Hydrocodone</th>
<th>Hydromorphone</th>
<th>Morphine</th>
<th>Oxycodone</th>
<th>Oxymorphone</th>
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<td><strong>5 ng/mL</strong></td>
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<td>% RSD</td>
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Table 1. Inter-assay precision and bias.

Figure 3. Chromatograms extracted from MS^2 spectra obtained from a confirmation PRM experiment for six opiates at their respective LOQs (2.5 ng/mL for codeine, oxycodone, and oxymorphone, and 5 ng/mL for hydrocodone, hydromorphone, and morphine) in hydrolyzed and diluted urine.
Figure 4. Chromatograms showing quantifying and confirming ions with ion ratio at LOQ for each compound in this method.

- **Codeine LOQ = 2.5 ng/mL**
- **Hydrocodone LOQ = 5 ng/mL**
- **Hydromorphone LOQ = 5 ng/mL**
- **Morphine LOQ = 5 ng/mL**
- **Oxycodone LOQ = 2.5 ng/mL**
- **Oxymorphone LOQ = 2.5 ng/mL**
Figure 5. Representative calibration curves from PRM data for six opiates.
Figure 6. Extracted ion chromatogram from donor sample obtained in confirmation PRM experiment.

- **Codeine in Donor D2, 12.1 ng/mL**
- **Hydrocodone in Donor F1, 60.6 ng/mL**
- **Hydromorphone in Donor D3, 37.1 ng/mL**
- **Morphine in donor F3, 217 ng/mL**
- **Oxycodone in Donor D1, 4.38 ng/mL**
- **Oxymorphone in Donor M2, 698 ng/mL**
Conclusion
The Q Exactive Focus MS accurately quantitated all six opiates tested to the low ng/mL level in human urine. This new instrument gives forensic laboratories a single versatile platform capable of both screening large panels and quantitative confirmation of specific panels that provides performance with value.

References

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