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Evaluation of matrix effects using a spike recovery approach in a dilute-and-inject liquid chromatography–tandem mass spectrometry opioid monitoring assay

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ABSTRACT

Background: Liquid chromatography–tandem mass spectrometry has become the gold standard for quantitative analysis of compounds in human matrices. Introduction of these assays into clinical practice, where false positive and false negative results have substantial implications, requires careful attention to matrix effects. We describe an evaluation of matrix effects in human urine from a dilute-and-inject liquid chromatography–tandem mass spectrometric assay for the quantitative analysis of opioids and metabolites.
Methods: A spike-recovery approach was employed for each analyte in each sample. We examined the impact of spike-recovery for the 6 glucuronides measured in this assay and compared the analytes for which conventional stable isotope-labeled internal standards were used with the analytes for which analog internal standards were used.
Results: For analytes that had analog internal standards, up to 1.5% of negative samples failed our requirement of recovering at least 80% of the expected spiked concentration while passing all other quality control parameters.
Conclusions: Using spike-recovery as a quality control parameter decreases the rate of false negatives for compounds using analog internal standards, but does not have benefit for compounds with conventional stable isotope-labeled internal standards.

1. Introduction

Tandem mass spectrometry has become the gold standard for the quantitative analysis of small molecule analytes in biological matrices and is especially useful in chronic pain screening due to its superior sensitivity and specificity. Substantial work has been devoted to analyzing and describing methods to control for matrix effects in such assays, with extensive evaluation of extraction methods and sample preparation methods, chromatographic and mass spectrometric conditions, and varied strategies employing standard additions and internal standards [1]. Traditionally, matrix effects are assessed using a post-column infusion of analyte or a comparison of the response from analyte spiked into blank matrix with analyte in neat solution [2,3]. These evaluations are typically completed during assay validation but may not account for the full spectrum of potential matrix effects encountered in clinical samples. The use of stable isotope-labeled internal standards to compensate for matrix effects represents a robust approach for most assays, but can be limited by the availability of commercially available internal standards [4].

We recently introduced a liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for the quantitative analysis of twenty different opioids, including 6 glucuronide metabolites [5]. Three of these metabolites (codeine-glucuronide, hydromorphone-glucuronide and norbuprenorphine-glucuronide) did not have commercially available isotope-labeled internal standards when the assay was developed, so internal standards for other compounds with similar elution times were used as analogs, similarly to previous work with opioid glucuronide detection [6]. In addition to internal standards, a rigorous spike-recovery approach was performed, during which standard concentrations of all analytes were added to each sample to ensure recovery of each analyte in every negative specimen. This approach adds considerably to the time and effort required to perform the assay since there are 4 injections per sample. However, the effort may be warranted in chronic pain medication monitoring because the absence of a prescribed drug may represent diversion of the drug. In such a setting, a false negative result in the appropriate clinical context could have significant implications for a patient's future opioid prescriptions. Adopting a spike-recovery approach to QC has allowed us to analyze the utility of internal standard QC parameters in ensuring against false negatives as well as present a comprehensive review of matrix effects in human urine.

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2. Materials and methods

2.1. Chemicals and reagents

Morphine-3-β-D-glucuronide (M-018), morphine-6-β-D-glucuronide (M-096), hydromorphone-3-β-D-glucuronide (H-051), oxymorphone-3-β-D-glucuronide (O-030), codeine-6-β-D-glucuronide (C-087), norbuprenorphine-glucuronide (N-045), morphine-3-β-D-glucuronide-D3 (M-017), morphine-6-β-D-glucuronide-D3 (M-120), oxymorphone-D3-3-β-D-glucuronide (O-031), codeine-D6 (C-040), and norfentanyl-D5 (N-030) were from Cerilliant. Optima grade methanol and acetonitrile and HPLC grade water were from Fischer Scientific. Ammonium acetate was purchased from ICN Biomedicals and formic acid was from J.T. Baker. The working internal standard solution was prepared by diluting methanol stock solutions of the deuterated opioids into water to a final concentration of 50 ng/ml with the exception of fentanyl (1.25 ng/ml), morphine (100 ng/ml) and oxymorphone glucuronide (100 ng/ml). These standards were stored at −20 °C and were stable for 6 months. A spike solution was also prepared by diluting the analytes into blank urine to a final concentration of 1000 ng/ml.

2.2. Sample preparation

All samples were prepared in four different ways by diluting with an internal standard solution as described previously to create a 1:2 dilution (straight), a 1:2 dilution with spiked compounds (straight spiked), a 1:20 dilution (10-fold diluted), and a 1:20 dilution with spiked compounds (10-fold diluted spiked) [5]. Briefly, urine samples were centrifuged for 1 min at 17,000 rpm. A 100 μl volume of sample was then mixed 1:1 with the working internal standard solution to prepare the straight sample. A 95 μl aliquot of the sample was mixed with 5 μl of the spike solution and mixed 1:1 with internal standard solution to prepare the straight spiked sample. Alternatively, 10 μl of centrifuged urine sample was diluted with 90 μl of blank urine and then mixed 1:1 with the working internal standard solution to prepare the 10-fold diluted sample. A 95 μl aliquot of the 10-fold diluted sample (prior to internal standard addition) was mixed with 5 μl of the spike solution and then mixed 1:1 with internal standard solution to prepare the 10-fold diluted spiked sample.

2.3. LC–MS/MS analysis

Chromatographic separation was achieved using an Aquity UPLC T3 (2.1 × 50 mm, 3 μm) column with gradient elution (A: 2 mmol/l ammonium acetate, 0.1% formic acid; B: acetonitrile, 0.1% formic acid; flow rate of 0.2 ml/min, temperature of 35 °C). The gradient consisted of 4–13% B from 0–2.63 min, was held at 13% B until 4.18 min, and was then developed linearly to 90% B at 7.61 min, after which the column was re-equilibrated at initial conditions. Mass spectrometry was performed with a Waters Xevo TQMS tandem mass spectrometer with electrospray ionization in positive mode using multiple reaction monitoring (MRM) of the protonated molecular ion $[M + 1]^+$ of each analyte. Each of the glucuronide analytes was quantified relative to a 50 ng/ml calibrator (although reported as a positive or negative clinically) and two levels of quality control were run each day patient samples were analyzed. Peak integrations were reviewed manually and corrected when needed by a technologist for each of the 4 injections per sample.

2.4. Data analysis

A total of 2182 urine samples were considered for analysis, which spanned approximately 18 months of clinical opioid monitoring assays. The data were extracted from stored run data using a lab-developed software program called “smack” (available online at https://github.com/nhoffman/opiates), which was also used to perform the quality control calculations and output [7]. The data aggregated from “smack” were analyzed using the R computing environment [8].

To evaluate the impact of various quality control parameters on spiked samples, samples with no quantifiable peak area (i.e. calculated to have concentrations of 0 for the analyte of interest) in the straight (undiluted) and 10-fold diluted samples were selected for further analysis. The quality control parameters, which are listed in Supplementary Table 1, were devised during assay development as described previously: (1) minimum signal-to-noise (S/R) ratio of 8, (2) relative retention time (RRT, or retention time of analyte relative to internal standard) within 1% of the average retention time observed over 20 runs for analytes with conventional isotope-labeled internal standards or within 2% for analytes with analog internal standards, (3) confirmatory ion ratios (IR) within 2 times the coefficient of variation of controls, standards, and spiked specimens, and (4) internal standard peak areas representing greater than 80% of the peak areas observed during validation [5]. A failure of spike recovery was defined as a sample in which the less than 80% of the spiked amount of compound was recovered.

Initially, the Spearman correlation coefficient between the internal standard peak area and the analyte peak area for the undiluted spiked samples was calculated for the all samples in the data set negative for each analyte. Next, the quality control parameters described above were applied in parallel to the undiluted spiked and 10-fold diluted spiked samples for all compounds, and the number of samples failing each of the QC parameters individually was tallied. The number of samples failing multiple QC parameters was also determined. The only exception to this evaluation of QC parameters was internal standard peak area, which was evaluated on the straight and 10-fold diluted straight samples rather than the spiked samples. This was done to best represent the primary QC parameter that could be used to detect false negatives in the absence of spiked samples. Samples that failed each QC parameter on both dilutions (undiluted and 10-fold diluted) were tabulated to represent the failure rate. Fisher's exact test was used for all statistical comparisons of failure rates, including comparison of failure rates between undiluted and 10-fold diluted samples and comparison of failure rates for each QC parameter between analytes with analog internal standards and those with conventional internal standards.

3. Results

In total, 2182 samples of a dilute-and-inject LC–MS/MS opioid assay were evaluated to determine whether implementing a spike-recovery quality control parameter adds value in detecting evidence of ion suppression. Three of the glucuronides evaluated in this assay, codeine, hydromorphone, and norbuprenorphine, did not have adequate commercially available internal standards when the assay was designed and validated, so analog internal standards were utilized instead (stable isotope-labeled codeine, morphine-3-glucuronide, and norfentanyl, respectively). The analysis was focused on samples that contained no quantifiable peak area for the glucuronides of interest in the straight and 10-fold diluted samples. This allowed an evaluation of urine matrix effects on known concentrations of spiked compounds in a large number of clinical urine specimens. This subset of samples ($n = 643$, 1074, 2101, 1102, 1525, and 882 for codeine-6-glucuronide, hydromorphone-glucuronide, norbuprenorphine-glucuronide, oxymorphone-glucuronide, morphine-6-glucuronide, and morphine-3-glucuronide, respectively) was then examined for correlations between internal standard and standard addition peak areas as well as for the proportion of samples failing specific QC parameters.

For each of the samples negative for the glucuronide of interest, the peak area of each analyte in the spiked sample was plotted against the peak area of its internal standard (Fig. 1), and Spearman