Analysis of Buprenorphine in Whole Blood Using Liquid Chromatography-Tandem Mass Spectrometry

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With buprenorphine use on the rise it has become more important than ever for the forensic laboratory to be capable of analyzing for it. Described is the approach used by the Georgia Bureau of Investigation for the screening and confirmation of buprenorphine in whole blood by liquid chromatography-tandem mass spectrometry (LC–MS-MS), along with case reviews for the first 2 months of method implementation. Screening by LC–MS-MS is capable of identifying buprenorphine cases at concentrations as low as 1–2 μg/L. Confirmatory testing is performed on both indicatively screened samples and cases where buprenorphine is specifically requested. Confirmatory analysis by LC–MS-MS has a limit of detection and limit of quantification of 0.75 μg/L with estimated uncertainties of 7.2% at 1 μg/L, 3.5% at 10 μg/L and 4.8% at 20 μg/L based on a 95% confidence interval, with the highest percent coefficient of variation being 3.7% for the 1 μg/L level. Since its implementation, the laboratory has reported out nine cases for buprenorphine. Seven of those cases were detected by the initial screen and two were identified by a specific request for buprenorphine. The cases’ average concentration was 4.25 μg/L with a mode of 3.1 μg/L.

Introduction

Buprenorphine is a synthetic partial opioid agonist that comes in a transdermal version, an injectable version, and two sublingual formulations, one with buprenorphine being the only active ingredient and the other a combination of buprenorphine and naloxone to prevent patients from dissolving pills and injecting them (1, 2). Available since 1985, buprenorphine has increased in use across the USA for its applications in pain management clinics and management of opioid withdrawal (Figure 1) (3–5). Along with the increased lawful use, there has also been an increase in abuse to the point where it is now more likely than methadone to appear in law enforcement drug seizures tested by forensic laboratories in the USA (6, 7). With a potency up to 40 times that of morphine and central nervous system-depressant properties that may cause drowsiness, dizziness, confusion, fatigue and blurred vision, the analysis in a forensic laboratory is essential (3, 8). Buprenorphine’s analysis to this point has primarily been focused in the urine, serum or plasma with some publications about its analysis in whole blood, the standard matrix for forensic applications (9–13). With buprenorphine use on the rise and more requests coming into the laboratory for its testing, the Georgia Bureau of Investigation has developed a simple procedure for the general screening and confirmatory analysis of buprenorphine in whole blood using liquid chromatography-tandem mass spectrometry (LC–MS-MS). In this paper, these processes are described along with a brief summary of case concentrations.

Materials and methods

Chemicals and reagents

Buprenorphine and buprenorphine-d4 were purchased from Cerilliant (Round Rock, TX, USA). Methanol (HPLC grade), acetic acid (A.C.S. plus), methylene chloride (HPLC grade), isopropanol (HPLC grade), ammonium hydroxide (A.C.S. plus), potassium phosphate monobasic and formic acid (88% w/v) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium formate (99.4%5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Negative blood was screened for drugs of abuse and therapeutic drugs prior to use. Mobile phase A was 0.1% formic acid and ~15 mM ammonium formate in Fisher Optima grade water and mobile phase B was 0.1% formic acid and ~15 mM ammonium formate in Fisher Optima grade methanol.

Sample preparation/analysis

Cases that are received by the laboratory are screened by LC–MS-MS using methods described in previous literature (14–16). Briefly, 1 mL of whole blood is processed through a precipitation procedure that is then divided into two aliquots for screening. One aliquot receives a six-panel CEDIA® screen for benzodiazepines, barbiturates, opiates, amphetamines, cocaine and THC. The second aliquot receives an ~130 drug panel screen using LC–MS-MS. Buprenorphine was added to this general LC–MS-MS screen, although it reaches only the threshold for triggering an enhanced product ion scan (EPI) producing a full mass spectrum at 1–2 μg/L. Initial LC–MS-MS screening of buprenorphine is accomplished by using a nonselective transition that has higher sensitivity to aid in identifying cases for confirmation testing; confirmation testing is done using a more selective transition to ensure the reliability of analysis (Table 1). The screening transition is considered nonselective due to the transition occasionally being triggered by an unidentifiable substance, which is distinguishable from buprenorphine by mass spectrum (Figure 2). The initial screen is sufficient to identify certain cases that come into the laboratory, in particular those of moderate to high concentrations. The confirmation testing subsequently described is further performed on the samples that show an indication of buprenorphine from the screen, or in cases where it is specifically requested for analysis by the submitting agency.

Confirmatory testing is conducted using solid-phase extraction where initially 2 mL of 0.1 M phosphate buffer is added to a 2 mL aliquot of blood followed by 20 μL (2 mg/L) of buprenorphine-d4. Next, samples are mixed and then centrifuged for 10 min at 2000 rpm. Samples are then applied to a UCT Clean Screen® DAU mixed-mode column that was preconditioned with 2 mL methanol, 2 mL deionized water and 1 mL 0.1 M phosphate buffer. Next, the column is washed with 3 mL deionized
water, 3 mL 1 N acetic acid and 3 mL methanol. The column is subsequently air dried under vacuum for at least 10 min at 15 inches Hg, and the drug elution is performed using 3 mL of 80/20/2 methylene chloride/isopropanol/ammonium hydroxide. Finally, the eluent is evaporated to dryness and reconstituted in 100 µL of 1:1 mobile phase A/mobile phase B (17).

**Instrumentation**

Buprenorphine testing was performed on an Applied Biosystems QTRAP™ 3200 (Carlsbad, CA, USA), with electrospray ionization in positive mode. Chromatography was accomplished by injecting 20 µL of specimen on a MetaSil Basic RP (3 µm, 50 × 2.0 mm) column at a temperature of 30°C. Separation was achieved by gradient elution using mobile phase A (A) and mobile phase B (B) on a Perkin Elmer series 200 auto-sampler, micro-pumps and column oven (Waltham, MA, USA). The gradient conditions were initially

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM transition (m/z)</th>
<th>Declustering potential (DP) (V)</th>
<th>Entrance potential (EP) (V)</th>
<th>Collision entrance potential (CEP) (V)</th>
<th>Collision energy (CE) (V)</th>
<th>Collision exit potential (CXP) (V)</th>
<th>Dwell time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine (screen)</td>
<td>468→55</td>
<td>86</td>
<td>10</td>
<td>22</td>
<td>77</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>468→396</td>
<td>86</td>
<td>10</td>
<td>22</td>
<td>49</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Buprenorphine-d4</td>
<td>472→400</td>
<td>91</td>
<td>10.5</td>
<td>22</td>
<td>51</td>
<td>4</td>
<td>25</td>
</tr>
</tbody>
</table>

**Figure 1.** Structure of buprenorphine $\text{MW} = 467.6$ (C$_{29}$H$_{41}$NO$_4$).

**Figure 2.** Mass spectrum of buprenorphine and unidentified peak.
95% (A) and 5% (B) for 1 min decreasing to 5% (A) and 95% (B) at 17 min and resetting to the initial conditions for a final 2 min producing a total run time of 20 min. The retention time for buprenorphine using these settings was 10.8 min. Instrumental parameters for quantitative analysis were set for multiple reaction monitoring (MRM), and the parameters for qualitative analysis were set to perform an enhanced product ion scan (EPI) for full mass spectral identification to a library match internally generated by the Georgia Bureau of Investigation (Figure 2). Owing to an institutional requirement, two separate analyses of a compound for reporting, the qualitative and quantitative analyses are performed on two separate aliquots of sample.

**Validation**

Buprenorphine and buprenorphine-d₄ were infused on the instrument to determine the optimal parameters (Table 1). Validation was performed by investigating limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy, linearity, recovery, matrix effects, selectivity and stability.

**LOD and LOQ**

The LOD was established to be 0.75 µg/L; with the criteria that the method produce a qualitative full mass spectrum identification when compared with an internal library reference, and produce a signal-to-noise ratio of greater than 3:1. The LOQ was determined to be 0.75 µg/L, the concentration in which the signal-to-noise ratio proved to be greater than 10:1 for the MRM transition.

**Precision and accuracy**

The variability was determined for the lowest, middle and the highest point of a calibration curve that ranged from 1 to 20 µg/L. The variability was estimated to be 7.2% at 1 µg/L, 3.5% at 10 µg/L and 4.8% at 20 µg/L based on a 95% confidence interval, with the highest percent coefficient of variation (%CV) being 3.7% for the 1 µg/L level. Variability and %CV were determined using a series of fortified control sample analyses (N = 30).

**Linearity**

A seven-point calibration curve with concentrations ranging from 1 to 20 µg/L was incorporated, and the coefficient of determination ($r^2$) was 0.997.

**Recovery**

Analyzing blood samples fortified at a concentration of 10 µg/L, the percent recovery ranged from 60 to 80% with an average of 69% recovery.

![Figure 3](https://academic.oup.com/jat/article-abstract/37/8/495/778739)
Matrix effects

Investigation of ion suppression or enhancement by post-column infusion of buprenorphine provided visualization of the effect of interfering species on the ionization of buprenorphine. This displayed areas of the gradient affected by the matrix and demonstrated no real effect at the retention time that buprenorphine elutes off the column (Figure 3).

Selectivity

An interference study was performed to determine method specificity. A total of 64 drugs that included analytes of similar molecular weight, retention time and structure were analyzed under the screen and the confirmatory LC–MS-MS methods. No drugs were found to interfere with the buprenorphine transitions. While there is an unidentifiable peak that occasionally triggers the transition for buprenorphine in the general screen, it is capable of being distinguished from buprenorphine by mass spectrum as demonstrated by Figure 2.

Stability

The stability of extracted calibrators and controls were investigated on the day of the extraction and after 7 days of storage at room temperature. There was a 10% average increase in internal standard compensates for any changes in the sample over time. The quantitative values of the two analyses were found to be within 3%, indicating that the internal standard response ranging from 1 to 18%, probably due to evaporation during storage. The average percent recoveries from whole blood of 69%.

Conclusions

The described method has proven its capacity for the screening and confirmatory analysis of buprenorphine in whole blood, capable of detecting and quantitating buprenorphine as low as 0.75 μg/L. Confirmatory analysis has also proven to be precise and accurate showing estimated uncertainties to be 7.2% at 1 μg/L, 3.5% at 10 μg/L and 4.8% at 20 μg/L based on a 95% confidence interval, with the highest %CV being 3.7% for the 1 μg/L level. It has shown to have little matrix effects and average percent recoveries from whole blood of 69%.

Since the methods implementation, the Georgia Bureau of Investigation has seen nine cases in only a 2-month period of time. All but two of those cases were detected by the initial screen. With a combination of screen results and further testing of buprenorphine-requested samples, a large percent of the buprenorphine cases that come into the laboratory should now be confirmed increasing the thoroughness of reports by the laboratory.

References


Table II

Summary of Buprenorphine Case Results

<table>
<thead>
<tr>
<th>Subject</th>
<th>Concentration, μg/L</th>
<th>Request</th>
<th>Screen</th>
<th>Other drugs present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>x</td>
<td></td>
<td>Amphetamine lower than the lowest calibrator of 50 μg/L</td>
</tr>
<tr>
<td>2</td>
<td>0.96*</td>
<td>x</td>
<td></td>
<td>Diazepam 120 μg/L</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>x</td>
<td></td>
<td>Clonazepam 13 μg/L</td>
</tr>
<tr>
<td>4</td>
<td>2.6</td>
<td>x</td>
<td></td>
<td>Methylenedehydramepam 19 ng/mL</td>
</tr>
<tr>
<td>5</td>
<td>3.1</td>
<td>x</td>
<td></td>
<td>Methyclothalamine 120 μg/L</td>
</tr>
<tr>
<td>6</td>
<td>2.7</td>
<td>x</td>
<td></td>
<td>Alprazolam 57 μg/L</td>
</tr>
<tr>
<td>7</td>
<td>4.8</td>
<td>x</td>
<td></td>
<td>THC-COOH® 57 ng/mL</td>
</tr>
<tr>
<td>8</td>
<td>4.6</td>
<td>x</td>
<td></td>
<td>THC-COOH® 31 ng/mL</td>
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<tr>
<td>9</td>
<td>1.7</td>
<td>x</td>
<td></td>
<td>Alprazolam 59 μg/L</td>
</tr>
</tbody>
</table>

*Lower than the lowest calibrator of 1 μg/L.
®11-Nor-delta-9-tetrahydrocannabinol-9-carboxylic acid.


