Buprenorphine Detection in Urine Using Liquid Chromatography–High-Resolution Mass Spectrometry: Comparison with Cloned Enzyme Donor Immunoassay (ThermoFisher) and Homogeneous Enzyme Immunoassay (Immunalysis)

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A sensitive liquid chromatographic–high-resolution mass spectrometric (LC–HR-MS) assay for buprenorphine and its urinary metabolites has been developed that requires minimal sample preparation. The results obtained have been compared with those given by (i) cloned enzyme donor immunoassay (CEDIA) and (ii) homogeneous enzyme immunoassay (HEIA) in the analysis of patient urines submitted for buprenorphine analysis. Centrifuged urine (100 µL) was diluted with internal standard solution (25 µL) + LC eluent (875 µL), and 50 µL of the prepared sample were analyzed (Accucore Phenyl-Hexyl column). MS detection was in alternating positive and negative mode using heated electrospray ionization (ThermoFisher Q Exactive). Intra- and inter-assay accuracy and precision were 104–128 and <11%, respectively, at 5 µg/L. Limits of detection were 1.3 µg/L (buprenorphine, norbuprenorphine and buprenorphine glucuronide) and 2.5 µg/L (norbuprenorphine glucuronide). Immunoassay sensitivity and selectivity were 97 and 100% (HEIA) and 99 and 84% (CEDIA), respectively, compared with LC–HR-MS. In 120 patient urines, norbuprenorphine glucuronide was easily the most abundant analyte except when adulteration with buprenorphine had occurred. The median immunoreactive buprenorphine species present (unhydrolysed urine) were 7.5 and 13% for HEIA and CEDIA, respectively. However, codeine, dihydrocodeine, morphine and morphine-3-glucuronide did not interfere in the HEIA assay.

Introduction

Buprenorphine is a potent partial opioid receptor agonist, which is used in low doses (<1 mg/day) for pain relief and in higher doses (2–32 mg/day) for treating opioid addiction. Illicit use of buprenorphine has also been reported (1). Urinalysis to detect buprenorphine has also been reported (1). Urinalysis to detect buprenorphine after either low-dose therapy or in samples obtained several hours after suspected covert administration. The results obtained using this method have been compared with those given by (i) a validated implementation of the CEDIA buprenorphine assay and (ii) by a buprenorphine HEIA (Immunalysis) in the analysis of patient urines submitted for buprenorphine analysis.

Materials and reagents

Buprenorphine, norbuprenorphine and their respective 3-βα-glucuronides were supplied as 100 mg/L solutions (LGC Standards, Teddington, UK). Buprenorphine-D₄, norbuprenorphine-D₃, (+)-aminopropyl, (±)-amisulpride, quinine, methanol, acetonitrile, formic acid, hydrochloric acid and ammonium formate were used in low doses (2–32 mg/day) for pain relief and in higher doses for treating opioid addiction. Illicit use of buprenorphine has also been reported (1). Urinalysis to detect buprenorphine after either low-dose therapy or in samples obtained several hours after suspected covert administration. The results obtained using this method have been compared with those given by (i) a validated implementation of the CEDIA buprenorphine assay and (ii) by a buprenorphine HEIA (Immunalysis) in the analysis of patient urines submitted for buprenorphine analysis.
were from Sigma-Aldrich (Poole, UK). Codeine, dihydrocodeine, morphine and morphine-3β-glucuronide were from Cerilliant (Sigma–Aldrich). Water was deionized (18 mΩ, Elga, Marlow, UK). Analyte-free human urine (Liquichek Urine Toxicology Negative Control) was from Bio-Rad (Hemel Hempstead, UK).

Positive and negative mass calibration solutions for the LC–HR-MS instrument and CEDIA buprenorphine and opiates assay reagents were from ThermoFisher Scientific (Hemel Hempstead). HEIA buprenorphine assay reagents (Immunalysis) were supplied through Specialty Diagnostix (St. Albans, UK). Urinary buprenorphine external quality assessment (EQA) specimens (Drugs of Abuse in Urine Proficiency Testing Scheme) were from LGC Standards. Two milliliter autosampler vials were from Kinesis (Cambridgeshire, UK).

For LC–HR-MS, stock solutions (all 1,000 µg/L) of buprenorphine, norbuprenorphine, buprenorphine glucuronide, norbuprenorphine glucuronide, buprenorphine-D₄ and norbuprenorphine-D₃ were prepared by dilution of the solutions purchased with either methanol (buprenorphine and norbuprenorphine) or aqueous 0.1 mol/L hydrochloric acid. All stock solutions were stored at +2 to 8°C. Calibrator (all analytes 25 µg/L) and internal quality control (IQC; all analytes 5 µg/L) solutions were prepared from the stock solutions in analyte-free human urine. Calibrator and IQC solutions were stored in ~500 µL portions in 2-mL screw-cap polypropylene tubes (Alpha Laboratories, Eastleigh, UK) at +2 to 8°C until needed. The internal standard solution (buprenorphine-D₄ and norbuprenorphine-D₃, both 200 µg/L) was prepared by dilution of the stock solutions in deionized water. To investigate the cross-reactivity of buprenorphine, buprenorphine metabolites and the deuterated analogs in the immunoassays and to investigate interference in the LC–HR-MS assay, individual solutions of each analyte (5 µg/L) were prepared in analyte-free human urine and analyzed in replicate (N = 5). To investigate interference in the assays, separate solutions of codeine, dihydrocodeine, morphine and morphine-3-glucuronide (all 100 µg/L) and of quinine, amisulpride and sulpiride (all 1.0 g/L) were prepared in analyte-free human urine and analyzed in quintuplicate. For all solutions prepared in analyte-free urine, the appropriate volume of stock solution was evaporated to dryness under a stream of compressed air prior to reconstitution in urine.

**Analytical procedures**

**Liquid chromatographic–high-resolution mass spectrometry**

An Aria Transcend™ TLX-II system (ThermoFisher Scientific) consisting of four Acquity™ 1250 pumps, valve interface module and CTC PAL autosampler was used with a Q Exactive™ mass spectrometer (ThermoFisher Scientific). Instrument control was performed using TraceFinder™ software (version 3.0, ThermoFisher Scientific). System eluents were: (A) 10 mmol/L aqueous ammonium formate containing 0.1% (v/v) aqueous formic acid, (B) 0.1% (v/v) formic acid in methanol:acetonitrile (1:1, v/v). The total eluent flow rate was 0.3 mL/min. Centrifuged urine (100 µL) and internal standard solution (25 µL) were added to autosampler vials and diluted with eluent A (875 µL) using an automated dilutor (Hamilton 530C). Prepared samples (50 µL) were injected onto an Accucore Phenyl-Hexyl column (2.6 µm a.p.s., 100 × 2.1 mm i.d., ThermoFisher Scientific) maintained at 40°C (CO-2067, Jasco).

MS detection was carried out in alternating positive and negative modes using heated electrospray ionization [spray voltage 3.2 kV; temperatures: vaporizer 350°C, capillary 320°C, auxiliary, sheath and sweep gases 10, 55 and 0 (AU), respectively, S-lens voltage 70 V]. The total analysis time was 14 min. Full-scan MS data were acquired using a resolution setting of 35,000, defined as full width at half maximum at m/z 200, with a scan range of 100–1,200 m/z (Orbitrap settings: maximum injection time 100 ms, automatic gain control (AGC) target 3 × 10⁶ ions). Alternate all-ion fragmentation (AIF) MS² scans were collected to confirm peak identity following higher-energy collisional dissociation (HCD) in the HCD cell [collision gas nitrogen, stepped normalized collision energy 35 V (±50%)]. The settings for the MS² data were: resolution 17,500, scan range 80–1,200 m/z, maximum injection time 50 ms and AGC target 1 × 10⁵ ions.

For all post-acquisition data processing, peak areas were generated by filtering full-scan data using a mass extraction window of ±5 ppm based on theoretical m/z values (external mass calibration carried out on alternate days using positive and negative ion mass calibration solutions).

The calibrator, IQCs and a matrix blank were included at the beginning and end of each batch analysis. Peak area ratios (buprenorphine and buprenorphine glucuronide to buprenorphine-D₄ and norbuprenorphine and norbuprenorphine glucuronide to norbuprenorphine-D₃) were measured and used to construct single-point calibration curves.

Qualitative analyte identification criteria were: (i) LC retention time within ±30 s (including co-elution with the internal standards for buprenorphine and norbuprenorphine), (ii) monoisotopic m/z value of the parent ions within ±5 ppm and (iii) the presence of at least one product ion in the AIF MS² scans. The additional presence of [M−H]⁻ ion within ±5 ppm for the glucuronides may be used as a further confirmatory parameter but was not relied upon for analyte identification.

**LC–HR-MS method validation**

Intra- and inter-assay accuracy (% nominal concentration) and precision (%RSD) at 5 µg/L were assessed for each analyte through replicate analysis (N = 5) on the same day and singlicate
analysis on 5 consecutive days, respectively. The limit of detection (LoD) was ascertained through successive serial dilution (1 : 1, v/v, with analyte-free urine) of the IQC solution and was based upon the concentration at which the signal-to-noise ratio was >3, and the CV was <20% (N = 5). To investigate ion suppression, analyte-free urine from six independent sources was analyzed. These samples were prepared and analyzed as described except that deionised water was added in place of internal standard, and the detector response for each analyte was monitored whilst a methanolic solution containing all analytes (250 µg/L, 10 µL/min) was infused by syringe postcolumn. To further investigate matrix effects, solutions containing all analytes and internal standards (25 µg/L) were prepared in (i) analyte-free human urine from 10 independent sources, (ii) Bio-Rad analyte-free human urine and (iii) deionized water. Prepared solutions were diluted (1 : 9, v/v) with eluent A and analyzed in triplicate. The ratio of the peak area of each analyte to that of the relevant internal standard in the analyte-free urine samples was compared with that in (i) deionized water and (ii) Bio-Rad analyte-free human urine. Carryover was assessed through consecutive analysis of a urine sample containing low (L) and high (H) analyte concentrations in the order L, L, L, H, H, L, L, L (L: all analytes 5 µg/L and H: all analytes 1,000 µg/L). Solutions of the other drugs tested for cross-reactivity were analyzed in quintuplicate.

Immunoassays

Immunoassays were performed according to the manufacturer’s instructions using an Olympus AU640 analyzer (Beckman Coulter). Both assays were calibrated as necessary using the calibrators supplied [buprenorphine calibration range 0, 5, 20, 50 and 75 µg/L (CEDIA) and 0, 5, 10, 20 and 40 µg/L (HEIA)]. The supplied quality control (QC) solutions (buprenorphine 3 and 7 µg/L for CEDIA, and 3.75 and 6.25 µg/L for HEIA) were analyzed twice daily. The sample volumes were 6.5 and 8 µL, and the detection wavelengths were 570 and 340 nm, for CEDIA and HEIA, respectively.

HEIA method validation

Intra- and interday precision (%RSD) and accuracy were measured by replicate analysis (N = 5) of the negative QC, cutoff calibrator (5 µg/L) and positive QC solutions on the same day and on different days (mean of five replicates, over five consecutive days), respectively. The LoD was taken as the minimum buprenorphine concentration required to produce an absorbance change greater than two standard deviations of the mean response for the negative calibrator, ascertained through replicate analysis (N = 20) of the negative calibrator. Linearity of the assay was assessed by successive serial dilutions (1 : 1, v/v) of the high calibrator (40 µg/L) using the negative calibrator. Carryover assessment was as described for the LC–HR-MS assay. Prepared solutions of buprenorphine, buprenorphine metabolites, deuterated analogs and the other drugs tested were analyzed in replicate (N = 5).

Patient and EQA samples

Anonymized urine samples (N = 40 each category) submitted for buprenorphine assay were categorized for analysis by HEIA and LC–HR-MS based on CEDIA values as follows: “negative” (<5 µg/L), “equivocal” (5–50 µg/L) and “positive” (>50 µg/L). All samples were stored at +2 to 8 °C and analyzed by all methods within 1 week of receipt. Drugs of Abuse in Urine PT Scheme EQA specimens (2010–2013, stored at −20 °C after initial analysis) were analyzed as available.

Results and Discussion

LC–HR-MS method validation

Intra- and inter-assay accuracy and precision are summarized in Table I. The LoD was 1.3 µg/L for buprenorphine, norbuprenorphine and buprenorphine glucuronide and 2.5 µg/L for norbuprenorphine glucuronide. Typical chromatograms are shown in Figure 2. Typical mass accuracy was <2 ppm for all analytes (Supplementary data, Table S1). No carryover was observed (Supplementary data, Table SII). Column life was >500 injections. With the exception of buprenorphine glucuronide, no significant matrix effects were observed (Supplementary data, Table SII). For buprenorphine glucuronide, the mean peak area ratio to buprenorphine-D4 was significantly greater (131–222%) in urine samples compared with deionized water suggesting ion enhancement. A co-eluting endogenous substance (typical measured [M−H]** m/z 345.1555) was identified in all urine samples including the Bio-Rad analyte-free urine, and modification of the LC gradient (to 1 h total analysis time) to resolve buprenorphine glucuronide from this substance eliminated the enhancement.

HEIA method validation

Precision and accuracy are summarized in Table II. Linearity is detailed in Supplementary data, Table SIV. The LoD for
buprenorphine was 0.6 μg/L. No carryover was observed. Cross-reactivity towards buprenorphine and metabolites, the deuterated analogs and some potentially interfering compounds [(mean measured concentration/nominal concentration) × 100%] are listed in Supplementary data, Table SV. Cross-reactivity with the buprenorphine and norbuprenorphine glucuronides was relatively low. Codeine, dihydrocodeine, morphine, morphine-3-glucuronide, 6-monoacetylmorphine, morphine-3-glucuronide, quinine, amisulpiride and sulpiride did not interfere at the concentrations tested.

**External quality assurance samples**

Urine from a patient prescribed 600 mg/day sulpiride (LGC Standards Proficiency Testing, sample number 275) gave an apparent buprenorphine result (CEDIA) of 12 μg/L. The same sample was negative (buprenorphine <0.6 μg/L) when analyzed using HEIA and was also negative for buprenorphine, norbuprenorphine and their glucuronides by LC–HR-MS.

A urine sample containing 1.1 and 8.4 μg/L of buprenorphine and norbuprenorphine, respectively (LGC Standards Proficiency Testing, sample number 107-3), gave a negative result when analyzed by CEDIA, whereas a positive result was obtained by HEIA. LC–HR-MS revealed a norbuprenorphine concentration of 6.9 μg/L.

**Patient results**

The results obtained on analysis of the patient urines are summarized in Table III (full data given in Supplementary data, Table SVI). Using HEIA, the sensitivity and selectivity values for buprenorphine were 97 and 100%, respectively, whereas with CEDIA the sensitivity and selectivity were 99 and 84%, respectively. A high opiate concentration (>2,000 μg/L, CEDIA opiate assay result) was recorded in all samples that gave rise to a false-positive CEDIA buprenorphine result. Using LC–HR-MS, 6-monoacetylmorphine, morphine, morphine-3-glucuronide, codeine and codeine glucuronide were detected in six samples and dihydrocodeine and dihydrocodeine glucuronide were detected in the remaining sample. Of the two false negatives given by the HEIA assay, only norbuprenorphine glucuronide at low concentration (28 and 29 μg/L, respectively) was present, suggesting that buprenorphine had not been taken recently.

LC–HR-MS analysis revealed very high concentrations of buprenorphine in the absence of any buprenorphine glucuronide species in three patient urines, which suggested buprenorphine adulteration. Both immunoassays cross-react with buprenorphine, and hence gave positive results for these samples. Immunoassays are unable to differentiate between buprenorphine and metabolites, and thus sample adulteration will be missed using these techniques. In one buprenorphine adulterated patient urine, the concentrations (LC–HR-MS) of buprenorphine, norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide were ~27,600, 17, <1.3 and <2.5 μg/L, respectively. By way of comparison, the corresponding concentrations in a sample from a patient prescribed buprenorphine (Subutex, 2 mg/day) were 13, 61, 136 and 856 μg/L, respectively. LC–HR-MS analysis also revealed a relatively high proportion of norbuprenorphine and norbuprenorphine glucuronide compared with buprenorphine and buprenorphine glucuronide in one patient sample. No clinical details were available.

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**Figure 2.** Typical extracted ion chromatograms, from analysis of the IQC solution showing all analytes ([M + H]^+ ion m/z ± 5 ppm). For the two glucuronides that also ionize in negative mode, the TIC filtered for [M − H]^- ion m/z ± 5 ppm is shown (shaded black peak).

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**Table II**

HEIA Assay: Summary Accuracy and Precision Data

<table>
<thead>
<tr>
<th></th>
<th>Intraday (N = 5)</th>
<th>Interday (N = 5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Neg. QC</td>
<td>Cutoff calibrator</td>
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<tr>
<td>Nominal (μg/L)</td>
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<td>5.00</td>
</tr>
<tr>
<td>Mean measured</td>
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<td>5.06</td>
</tr>
<tr>
<td>RSD (%)</td>
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<td>4.6</td>
</tr>
<tr>
<td>Accuracy (% nominal)</td>
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<td>101</td>
</tr>
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</table>

**Table III**

Comparison of CEDIA and HEIA with LC–HR-MS in the Analysis of 120 Patient Urines Submitted for Buprenorphine Assay

<table>
<thead>
<tr>
<th>LC–HR-MS</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEDIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73</td>
<td>1*</td>
</tr>
<tr>
<td>Negative</td>
<td>72*</td>
<td>38</td>
</tr>
<tr>
<td>HEIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73</td>
<td>2**</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>45</td>
</tr>
</tbody>
</table>

*Norbuprenorphine 3.3 μg/L, buprenorphine glucuronide 8.2 μg/L and norbuprenorphine glucuronide 26 μg/L.

**Table V**

Comparison of CEDIA and HEIA with LC–HR-MS in the Analysis of 120 Patient Urines Submitted for Buprenorphine Assay
for this patient, but the result may suggest induction of CYP3A4, either through coadministered drugs or physiological changes, e.g., pregnancy (16).

In 75 patient samples where buprenorphine and/or buprenorphine metabolites were detected, the proportions of each species (LC–HR-MS data converted to nanomoles per liter) present were calculated (Figure 3). The median (range) percentage of buprenorphine, norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide found were 0.1 (0–7), 7.2 (0–47), 13 (1.7–34) and 78 (44–94)%, respectively (N = 72, buprenorphine adulterated samples excluded). The percentages of buprenorphine and norbuprenorphine (the species detected by the HEIA method), and of buprenorphine and norbuprenorphine glucuronide (the species detected by CEDIA) were then summed to give the “total immunoreactive species” for each immunoassay (Supplementary data, Figure S2). As a proportion of the total buprenorphine species present, the immunoreactive buprenorphine species contributed (median, range) 7.5 (0–54) and 13 (2.5–36)% for HEIA and for CEDIA, respectively.

Norbuprenorphine glucuronide was the most prevalent analyte detected in patient urine samples, and neither immunoassay cross-reacts significantly with this analyte. As a result, the immunoassay methods detect on average <15% of the total buprenorphine species present in unhydrolysed urine. The cutoff of 5 μg/L “total buprenorphine” advocated for immunoassay methods (3, 4) is on average equivalent to a cutoff of 50 μg/L total buprenorphine species.

General Discussion
The LC–HR-MS method developed acquires full-scan data, as opposed to tandem mass spectrometry (MS-MS) methods that only capture preselected data. One advantage this offers is the ability to reprocess data acquired to search for compounds that were not targeted initially. Choosing a more selective sample preparation method (e.g., liquid–liquid extraction) would limit the possibility of retrospectively identifying analytes, especially polar metabolites. Utilizing a “dilute and shoot” method is simple and inexpensive and ensures that as much information as possible is retained for possible future interrogation (17).

On the other hand, use of “dilute and shoot” does give the possibility of matrix effects, and significant (on average 170%) enhancement of the buprenorphine glucuronide response was observed in urine as compared with deionized water, an effect only partially compensated for by preparing calibrators in analyte-free urine because the concentration of the matrix component responsible for the problem varied between samples. As the method is qualitative this ion enhancement was not thought to be a serious issue. For quantitative work, use of stable isotope labeled buprenorphine glucuronide, if available, as the internal standard for buprenorphine glucuronide may compensate for the observed effect.

Finally, the measurement of free naloxone in addition to free buprenorphine has been suggested as an indicator of sample adulteration via addition of Suboxone (buprenorphine and naloxone mixture) to urine samples (15). Retrospective analysis of the patient data from this study showed that naloxone was present in one of the three samples where buprenorphine had clearly been added to the specimen (urinary naloxone concentration ≈4,000 μg/L by comparison with the results of the analysis of a calibrator prepared at 1,000 μg/L naloxone in analyte-free urine and assayed 55 days after the original sample analysis). It would be possible to look for naloxone metabolites in the same analysis if confirmation of adherence to Suboxone was deemed important.
Conclusions
The LC–HR-MS method developed is simple, sensitive and robust, and can detect the presence of buprenorphine and norbuprenorphine glucuronides in urine without prior hydrolysis when buprenorphine and norbuprenorphine themselves are undetectable. The ability to detect the glucuronides is valuable in identifying sample adulteration with buprenorphine on the one hand, and extends the window of detection for buprenorphine administration in unhydrolysed urine on the other, which could be valuable if covert buprenorphine administration is suspected. The results given by the HEIA buprenorphine assay were largely in agreement with the LC–HR-MS results and were superior to those given by CEDIA in that opiates did not interfere.

Supplementary data
Supplementary data are available at Analytical Toxicology Journal online.

Acknowledgments
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References