Quantification of Buprenorphine, Norbuprenorphine and 6-Monoacetylmorphine in Urine by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Monitoring pain management medications and illicit drugs in urine is commonly used to assess patient compliance. Previously, we developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to measure 19 analytes important for pain management. In the current report, we validated this method for two additional drugs, buprenorphine and heroin. For buprenorphine, we quantified both the parent drug and its major metabolite, norbuprenorphine. For heroin, we monitored its unique metabolite, 6-monoacetylmorphine (6-MAM). Urine samples were subjected to enzymatic hydrolysis prior to turbulent flow online extraction and LC-MS/MS analysis. No matrix effect or interference was found. Lower limits of quantifications were 9.7, 9.6, and 4.9 ng/mL for buprenorphine, norbuprenorphine and 6-MAM, respectively. Within the linear range, analytical recovery was 80.5-113.0% for all analytes. Intra-assay and total coefficient of variations were between 0.2% and 10.3%. This method demonstrated consistent patient results (n=40) with the independent LC-MS/MS methods offered by two other laboratories. Percentage of glucuronide conjugation of 6-MAM varied from 0 to 45% in 8 patient urine samples positive for 6-MAM. In conclusion, we have successfully expanded current pain management panel to include buprenorphine and heroin with high sensitivity, specificity, and precision.

Keywords: LC-MS/MS; Buprenorphine; Norbuprenorphine; Heroin; 6-Monoacetylmorphine

Introduction

Monitoring the use of prescribed pain medications and illicit drugs is routinely used to assess the compliance of patients enrolled in pain management programs [1]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been increasingly used due to the high specificity and sensitivity, relatively simple sample preparation, and ability of analyzing a large panel of analytes simultaneously [2]. Previously, we developed an LC-MS/MS method to monitor the usage of 15 drugs important for pain management [3]. In the current study, our goal was to add two more drugs, buprenorphine and heroin, to this panel to meet the clinical needs. Buprenorphine is an opioid medication used to treat chronic pain, and it has less potential for abuse when combined with naloxone, an opioid antagonist [4]. Heroin is a commonly abused street drug in the US with increasing trend among persons aged 12 or older [5].

In humans, buprenorphine is metabolized to norbuprenorphine. Both the parent drug and the metabolite undergo extensive conjugation with glucuronide prior to excretion in urine [6]. A hydrolysis step is needed when measuring total concentrations of buprenorphine and norbuprenorphine [7,8]. Enzymatic hydrolysis of these conjugates is commonly used, and the choice of glucuronidase and incubation conditions has significant impact on the hydrolysis efficiencies [9]. Heroin is rapidly metabolized (half-life is 3 minutes in plasma) first to 6-monoacetylmorphine (6-MAM), and further to morphine. Although the morphine to codeine concentration ratio can be helpful in differentiating heroin users from codeine users, presence of 6-MAM in urine indisputably confirms heroin use [10]. Presence of conjugated 6-MAM in urine has been indicated, [11] but its relative amount has not been reported. In this study, we aimed to measure buprenorphine, norbuprenorphine, and 6-MAM in urine after enzymatic hydrolysis. We intended to use the same sample preparation and analytical methods that we previously developed [3].

Materials

Standard solutions of buprenorphine, norbuprenorphine, 6-MAM, buprenorphine glucuronide, norbuprenorphine glucuronide, buprenorphine-d4, norbuprenorphine-d3, 6-MAM-d3, were from Cerilliant (Round Rock, TX). Deuterium-labeled standards were used as internal standards (IS). Drug-free urine (Liquichek urine toxicology negative control, Bio-Rad, Hercules, CA) was used to prepare calibrators (25, 50, 250, 500, and 1000 ng/mL). Glucuronidase used for hydrolysis was isolated from Patella vulgata (Sigma-Aldrich, St. Louis, MO). All other reagents were the same as our previous report [3].

Methods

Sample preparation including enzymatic hydrolysis, on-line extraction with turbulent flow chromatography and analytical methods were described in our previous report [3]. In this work, a 2-channel multiplexed LC system (Aria TLX2 from ThermoFisher Scientific, Waltham, MA) was used, and the analytical time between injections was reduced from 23 to 12 minutes. The mass spectrometer (TSQ Ultra from ThermoFisher Scientific) was operated in the positive electrospray ionization mode for all the added analytes and IS. Multiple reaction monitoring (MRM) transitions for each analyte are listed in (Table 1). Method validation was performed using the same protocol.
Results and Discussion

Buprenorphine, norbuprenorphine and 6-MAM were readily detected without any change to our previous sample preparation and HPLC methods [3]. The measurement of the original 19 analytes was not affected. In November 2011, propoxyphene was withdrawn from US market due to its serious toxicity to the heart. Since then, we had found that the positive rate of propoxyphene declined sharply, and the same trend has been reported by others [12]. We stopped monitoring propoxyphene use in September 2012. The chromatogram of the same specimens directly onto the analytical column. The mean recovery (300 ng/mL, n=3) onto turbulent column with those from injecting the remaining analytes and the 3 newly added ones is shown in (Figure 1).

The efficiency of enzymatic hydrolysis was estimated using blank urine samples spiked with known amounts (450 ng/mL) of glucuronide-conjugated standards, and complete hydrolysis (>95%) was achieved for both buprenorphine and norbuprenorphine within 1 hour of incubation at 60°C. In practice the hydrolysis time was kept at 16-20 hours to ensure the complete hydrolysis of codeine-glucuronide [3]. The recovery of on-line turbulent flow extraction was assessed by comparing the peak areas from injecting spiked blank urine specimens (300 ng/mL, n=3) onto turbulent column with those from injecting the same specimens directly onto the analytical column. The mean recovery was determined to be 16.4%, 95.9% and 84.7% for buprenorphine, norbuprenorphine, and 6-MAM, respectively. The relative recovery after correction by IS varied from 97.9% to 114.5%. No obvious matrix effect was observed in the post-column T infusion experiments in which 6 negative patient samples (3 males and 3 females) were injected while a constant infusion of the analytes was introduced (Figure 2). No interference was found in five commercial urine controls which were Lypohechcurine toxicology control level 3 (Bio-Rad), Lypohechc quantitative urine controls (both normal and abnormal, Bio-Rad), MAS Urichem TRAK liquid assayed urine controls (Levels 1 and 2, as our previous report [3]. Major assay characteristics included matrix effects, interference, analytical measurement range (AMR), carryover, precision, and method comparison. Usage of the leftover patient samples in this study was approved by the Institutional Review Board of Cleveland Clinic.
was serially diluted with the same blank urine pool. The resulting spiking a pool of blank patient urines with high levels of the analytes

ThermoFisher Scientific). In the AMR study, a specimen prepared by spiking a pool of blank patient urines with high levels of the analytes was serially diluted with the same blank urine pool. The resulting specimens were processed and analyzed in triplicate. The lower limit of quantification (LLOQ) was determined by lowest concentration tested with <20% CV and 100 ± 20% accuracy. The LLOQ was found to be 9.7, 9.6, and 4.9 ng/mL for buprenorphine, norbuprenorphine and 6-MAM, respectively (Table 1). Carryover was assessed by extracting two levels (low and high) of spiked patient samples in triplicate and by running each set in the sequence of low-high-low, where low2 is a reinjection of low1. Analyte concentration in the low sample was 10-20 ng/mL. There would be no carryover if the difference between low1 and low2 was within 20% of low, and that the mean of low was within 3 standard deviations of the mean of low1. Analyte concentration of the passing high sample was determined after dilution and is listed in (Table 1). Precision was evaluated based on CLSI EP10-A3 guideline at three concentration levels, run twice a day over 5 days. The total CV was 3.3-13.3% across the concentration levels tested (Table 1). Measurement of buprenorphine and norbuprenorphine was compared with an LC-MS/MS method offered by NMS Labs (Willow Grove, PA) using 40 leftover patient samples. Buprenorphine and norbuprenorphine were detected by both methods in all 40 samples. As shown in (Figures 3a and 3b), a positive bias was observed for both buprenorphine (+25.2%) and norbuprenorphine (+15.3 %). Interestingly, such a bias was not noticed in a later comparison using negative urine samples (n=5) spiked with unconjugated buprenorphine and norbuprenorphine (Table 2). Therefore, the bias was hypothetically due to differences in hydrolysis efficiencies. Measurement of 6-MAM was compared with an independent LC-MS/MS method (ARUP, Salt Lake City, UT) using 40 leftover patient samples. Seven samples with low concentrations (5.0, 7.4, 9.0, 10.0, 12.2, 14.4, and 21.0 ng/mL by this method) were not quantifiable by the ARUP method. Quantitative comparison of the remaining 33 samples is shown in (Figure 3c). The obvious positive bias (+28.8%) was likely due to the lack of a hydrolysis step in the ARUP method. In order to estimate the percentage of glucuronide-conjugated 6-MAM in patient urine, we analyzed eight 6-MAM positive samples (68-877 ng/mL) with and without performing the hydrolysis step to determine the total and free 6-MAM concentrations. The percentage of conjugated 6-MAM was calculated as [total-free]/total, and varied from 0 to 45%. To the best of our knowledge, this is the first report of conjugated 6-MAM was calculated as [total-free]/total, and varied from 0 to 45%. To the best of our knowledge, this is the first report

Table 2: Method comparison using patient specimens spiked with unconjugated buprenorphine and norbuprenorphine. Five negative patient samples were spiked with different concentrations of buprenorphine and norbuprenorphine, and analyzed by the reported method (CCF) and an LC-MS/MS method offered by NMS Labs (Willow Grove, PA). The measured concentrations and the percent differences are shown.

<table>
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<th>CCF NMS</th>
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<td>15.7</td>
<td>89</td>
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Figure 3: Method comparison. Leftover patient samples were split and analyzed by the reported method and an independent LC-MS/MS method. Results obtained from both methods were quantitatively compared using Deming regression (left panels). The determined slope, Y-intercept, and correlation coefficient (r) are shown. Numbers in parenthesis are 95% confidence intervals. Bias was analyzed using Bland-Altman plot (right panels) in which dashed line represents mean percentage bias, and dotted lines represent ± 2SD.

Thermofisher Scientific. In the AMR study, a specimen prepared by spiking a pool of blank patient urines with high levels of the analytes was serially diluted with the same blank urine pool. The resulting specimens were processed and analyzed in triplicate. The lower limit of quantification (LLOQ) was determined by lowest concentration tested with <20% CV and 100 ± 20% accuracy. The LLOQ was found to be 9.7, 9.6, and 4.9 ng/mL for buprenorphine, norbuprenorphine and 6-MAM, respectively (Table 1). Carryover was assessed by extracting two levels (low and high) of spiked patient samples in triplicate and by running each set in the sequence of low-high-low, where low2 is a reinjection of low1. Analyte concentration in the low sample was 10-20 ng/mL. There would be no carryover if the difference between low1 and low2 was within 20% of low1, and the mean of low was within 3 standard deviations of the mean of low1. Analyte concentration of the passing high sample was determined after dilution and is listed in (Table 1). Precision was evaluated based on CLSI EP10-A3 guideline at three concentration levels, run twice a day over 5 days. The total CV was 3.3-13.3% across the concentration levels tested (Table 1). Measurement of buprenorphine and norbuprenorphine was compared with an LC-MS/MS method offered by NMS Labs (Willow Grove, PA) using 40 leftover patient samples. Buprenorphine and norbuprenorphine were detected by both methods in all 40 samples. As shown in (Figures 3a and 3b), a positive bias was observed for both buprenorphine (+25.2%) and norbuprenorphine (+15.3 %). Interestingly, such a bias was not noticed in a later comparison using negative urine samples (n=5) spiked with unconjugated buprenorphine and norbuprenorphine (Table 2). Therefore, the bias was hypothetically due to differences in hydrolysis efficiencies. Measurement of 6-MAM was compared with an independent LC-MS/MS method (ARUP, Salt Lake City, UT) using 40 leftover patient samples. Seven samples with low concentrations (5.0, 7.4, 9.0, 10.0, 12.2, 14.4, and 21.0 ng/mL by this method) were not quantifiable by the ARUP method. Quantitative comparison of the remaining 33 samples is shown in (Figure 3c). The obvious positive bias (+28.8%) was likely due to the lack of a hydrolysis step in the ARUP method. In order to estimate the percentage of glucuronide-conjugated 6-MAM in patient urine, we analyzed eight 6-MAM positive samples (68-877 ng/mL) with and without performing the hydrolysis step to determine the total and free 6-MAM concentrations. The percentage of conjugated 6-MAM was calculated as [total-free]/total, and varied from 0 to 45%. To the best of our knowledge, this is the first report on the proportion of glucuronide-conjugated 6-MAM in urine. It is noteworthy that the majority of 6-MAM positive samples (37 out 40) contained both morphine (7,000-90,000 ng/mL) and codeine (80-4,500 ng/mL), and their morphine to codeine ratios were greater than 10.

This pattern is consistent with heroin use [10].

Conclusion

Buprenorphine, norbuprenorphine, and 6-MAM have been successfully added to an existing LC-MS/MS test panel for pain management service. The quantification of these analytes was proven sufficient for clinical use with high sensitivity, specificity, and precision. We have analyzed over 3000 specimen in the past 6 months without any problems, and successfully passed the College of American Pathologists proficiency tests.

References


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