Journal of Analytical Toxicology Advance Access published April 26, 2016

Journal of Analytical Toxicology, 2016, 1–7
doi: 10.1093/jat/bkw026
Article

OXFORD

Article

Development and Validation of a Novel LC-MS/MS Opioid Confirmation Assay: Evaluation of β-glucuronidase Enzymes and Sample Cleanup Methods

He S. Yang†, Alan H. B. Wu, and Kara L. Lynch*

Department of Laboratory Medicine, University of California San Francisco, San Francisco General Hospital, 1001 Potrero Avenue NH 2M16, San Francisco, CA 94110, USA

*Author to whom correspondence should be addressed. Email: kara.lynch@ucsf.edu

†Present address: Department of Special Chemistry and Toxicology, Quest Diagnostics, 27027 Tourney Rd, Valencia, CA 91355, USA.

Abstract

With the rise in the use and misuse of prescription opioids, there is an increasing need for the confirmed identification of opioid analgesics in toxicology laboratories. The goals of this study were to (i) systematically evaluate the hydrolysis efficiency of four β-glucuronidase enzymes under optimized condition; (ii) evaluate compound recovery, matrix effects and precision of three protein precipitation plates and (iii) develop and validate a qualitative liquid-chromatography mass spectrometry (LC–MS/MS) assay to identify 13 opioids in urine. A recombinant β-glucuronidase exhibited the best overall hydrolysis efficiency for seven opioid glucuronide conjugates compared with β-glucuronidase from red abalone, *Escherichia coli* and *Patella vulgata*. One of the protein precipitation plates tested exhibited overall better recovery of the opioids and lower ion suppression compared with the other two plates. An ESI positive mode LC–MS/MS assay for qualitative opioid analysis was developed and validated. Linearity, LOD, precision, matrix effect, recovery, carryover and interference of the method were evaluated. Sixty-two patient samples were analyzed by both a legacy GC–MS opioid method and the LC–MS/MS method, and 22 samples were analyzed by the LC–MS/MS and an LC–MS/MS reference method. The results of the comparisons showed good concordance. Overall, we described an efficient sample preparation procedure for a sensitive qualitative opioid confirmation assay in urine.

Introduction

The prescription of opioids has increased dramatically in the past 20 years mainly due to the rise in the diagnosis and treatment of chronic pain (1). Prescription opioids are also commonly abused and used illicitly (2–4). Therefore, clinicians monitor their patients to ensure compliance and deter drug diversion. The standard of care for monitoring is to perform a urine drug screen using immunoassay methodologies followed by confirmation of preliminary positive by chromatographic separation and mass spectrometric detection (1). In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has largely supplanted gas chromatography mass spectrometry (GC–MS) as the gold standard for opioid confirmatory testing (5, 6). Unlike LC–MS/MS, GC–MS is not amenable to the detection of polar and nonvolatile compounds without performing a time-consuming sample preparation, including hydrolysis, derivatization and sample cleanup. Therefore, LC–MS/MS provides a superior alternative to GC–MS in pain management and toxicology testing laboratories because of the ease and speed of sample preparation (7).

A major metabolic pathway of opioids involves conjugation of a glucuronide group to increase their hydrophilicity and polarity for excretion (8). A significant proportion of opioids, such as morphine, codeine, hydromorphone, oxymorphone, buprenorphine and norbuprenorphine, is detected as glucuronide metabolites in urine. A previous study showed

that in 56 patient urine samples, 43% were positive for morphine glucuronide conjugates only, whereas 11% were positive for the parent drug only (7). While methods have been published in which parent drugs and glucuronide metabolites were simultaneously separated within one run, these assays required the use of an ultra-high pressure liquid chromatography (UHPLC) and relatively long gradients (9). Most other assays employed acid or enzyme hydrolysis to cleave the glucuronide linkage and measure total drug concentration. While acid hydrolysis is efficient in converting glucuronide metabolites to free drug, it destroys 6-monoacetylmorphine (6-MAM), a unique metabolite of heroin (10). Therefore, various sources of β-glucuronidase, such as *Escherichia coli*, *Helix pomatia*, *Patella vulgata* and abalone, have been utilized in opioid hydrolysis, with each exhibiting different hydrolysis efficiency and optimal conditions (11–13). However, so far few publications have systematically compared hydrolysis efficiency of different β-glucuronidase enzymes for opioid analysis (11, 12, 14).

Protein precipitation provides a fast and efficient approach for sample cleanup and removal of β-glucuronidase enzymes in urine following hydrolysis and improves sensitivity and precision of the LC–MS/MS method (15). There are a variety of protein and/or phospholipid removal 96-well plates that are currently on the market. In a review of the literature, no reported studies were found that compare the extraction recovery, matrix effect and precision of different protein precipitation plates for opioid sample preparation in urine. Therefore, the objectives of this study were to conduct a comprehensive evaluation of the performance of four different β-glucuronidase enzymes and three protein precipitation plates and optimize the sample preparation workflow for a qualitative opioid confirmation assay. In addition, this work aimed to develop and validate a qualitative LC–MS/MS method to replace a GC–MS opioid confirmation assay in which sample preparation was very time-consuming and laborious (11, 16).

Materials and methods

Chemicals and reagents

LC–MS/MS grade water, methanol and acetonitrile were obtained from Honeywell Burdick & Jackson Research Chemicals (Muskegon, MI, USA). Formic acid, ammonium formate, sodium citrate, sodium acetate, potassium phosphate monobasic and dibasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following analytical standards were purchased from Cerilliant Corporation (Round Rock, TX, USA): morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, 6-MAM, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), fentanyl, norfentanyl, buprenorphine, norbuprenorphine, morphine-3-β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G), codeine-6-β-D-glucuronide (C6G), hydromorphone-3-β-D-glucuronide (H3G), oxymorphone-3-β-D-glucuronide (O3G), buprenorphine-glucuronide (BG), norbuprenorphine-glucuronide (NBG), morphine-d6 and fentanyl-d5. IMCSzyme™ recombinant β-glucuronidase (>50,000 units/mL, catalog # 04-E1F-050) and its proprietary buffer were obtained from Integrated Micro-Chromatography System (Columbia, SC, USA). β-Glucuronidase from ocean-fresh Red abalone Haliotis rufescens entrails (BG100™) was obtained from Kura Biotech (Puerto Varas, Chile) as a solution containing 100,000 Fishman units/mL β-Glucuronidase and 8,000 Fishman units/mL sulfatase. Also, 140 U/mL (equivalent to 35,000 Fishman units/mL) recombinant β-glucuronidase from *E. coli* (EBG) was obtained from Kura Biotech. β-Glucuronidase from limpets (*P. vulgata*, catalog # G8132-100KU) was purchased from Sigma-Aldrich in lyophilized powdered (100,000 units dissolved in 2 mL acetate buffer). The Supelco 96-well protein precipitation plate (catalog # 55263-U), Impact™ protein precipitation plate (part # CEO-7565) and Isolute PLD+ protein precipitation plate (part # 121-5202) were obtained from Sigma-Aldrich, Phenomenex (Torrance, CA, USA) and Biotage AB (Uppsala, Sweden), respectively. Certified drug-free human urine was purchased from Utak Laboratories (Valencia, CA, USA).

Calibrators, controls and patient samples

The calibrator working solution of 13 opioids at a concentration of 100 µg/mL (except buprenorphine, fentanyl, norfentanyl and 6-MAM at 10 µg/mL) was purchased from Cerilliant. The working solution was used to prepare calibrators in human drug-free urine at concentrations of 10, 25, 50, 100, 250, 500, 750, 1,000 and 2,000 ng/mL (1, 2.5, 5, 10, 25, 50, 75, 100 and 200 ng/mL for buprenorphine, fentanyl, norfentanyl and 6-MAM). Two levels of opioids, at 80 and 120% of the cutoffs value, were purchased in a custom-made lyophilized quality control material from Utak Laboratories and were reconstituted with 3 mL reagent grade water. Two hydrolysis controls, with one containing M3G, C6G and O3G and the other containing M6G, H3G, BG and NBG, were prepared by fortifying each compound into certified drug-free urine at a concentration of 1 µg/mL. Two internal standards, fentanyl-d5 and morphine-d6, were prepared at a concentration of 1 mg/mL each.

Patient urine samples submitted to our laboratory for routine urine toxicology screening were initially tested by a CEDIA Opiate Immunoassay (Microgenics, Fremont, CA, USA) and CEDIA Oxycodone Immunoassay on an Advia 1800 chemistry analyzer (Siemens, Malvern, PA, USA). The cutoffs of the CEDIA opiate and oxycodone immunoassay were 300 and 100 ng/mL, respectively. Positive screening results for opiates and/or oxycodone were reflexed to a qualitative GC–MS (Agilent Technologies, Santa Clara, CA, USA) confirmatory assay which detected morphine, codeine, hydromorphone, hydrocodone, oxymorphone and oxycodone (11, 16). The GC–MS method cutoff was 100 ng/mL for each compound. Sixty-two patient urine samples that tested positive for one or more opioid compounds by the GC–MS assay were also analyzed by the LC–MS/MS assay. In addition, 22 patient urine samples that were tested positive for 6-MAM, buprenorphine/norbuprenorphine, methadone/EDDP and fentanyl/norfentanyl by a reference LC–MS/MS method were also analyzed by our LC–MS/MS assay. The reference method does not use a cutoff but rather calls a drug positive if a peak is identified and meets reporting criteria. This study was approved by the University of California San Francisco Committee on Human Research who deemed that patient consent was not required.

Hydrolysis conditions

The optimal buffer conditions and temperature used for each β-glucuronidase enzyme, as suggested by the manufacturers, were as follows: proprietary buffer at pH 6.8, 55°C for IMCSzyme; 0.2 M sodium citrate buffer at pH 4.8, 68°C for BG100; 0.2 M potassium phosphate buffer at pH 6.8, 46°C for EBG and 0.2 M acetate buffer at pH 5.0, 65°C for *P. vulgata*. First, the hydrolysis efficiency for each β-glucuronidase at two different enzyme concentrations was assessed with the urine hydrolysis controls to determine the optimal dose of each enzyme. These concentrations were selected based on the manufacturers' recommendations for each enzyme. Then, 5 and 10 KU/mL of IMCSzyme, 10 and 20 KU/mL of BG100, 10.5 and 17.5 KU/mL of EBG and 5 and 10 KU/mL of *P. vulgata* were added to 200 µL of the hydrolysis controls containing the internal standards (fentanyl-d5 and morphine-d6) and incubated for 1 h. Second, the hydrolysis efficiency for each β-glucuronidase at its own optimal pH, temperature and concentration (IMCSzyme 10 KU/mL, BG100 20 KU/mL, EBG 10.5 KU/

mL and *P. vulgata* 10 KU/mL) was compared. Lastly, the hydrolysis efficiency of the recombinant IMCSzyme enzyme was evaluated with 0.1, 1 and 10 µg/mL of the urine hydrolysis controls. Hydrolysis efficiency was calculated by dividing the total free drug released after hydrolysis by the amount of glucuronide fortified into the hydrolysis controls (1 µg/mL each) prior to hydrolysis and extraction.

Opioid extraction

Following hydrolysis, 100 µL of the urine samples was transferred to a protein precipitation plate. Then, 300 µL of acetonitrile was added to each well and mixed with the sample thoroughly for cleanup and removal of the enzyme. A vacuum was applied, and flow-through was diluted 1:5 in mobile phase A (10 mM ammonium formate). Compound extraction recovery, matrix effects and within-run precision were assessed for the three commercially available protein precipitation plates listed above.

Liquid chromatography and mass spectrometry

Separation of 13 opioids was performed using an Agilent (Santa Clara, CA, USA) high-performance liquid chromatography (HPLC) with a Kinetex 2.6 µm Phenyl-Hexyl 100 Å, 50 × 4.6 mm LC column. The column was used at 30°C with a gradient elution at a flow rate of 0.7 mL/min. Mobile phase A consisted of 10 mM ammonium formate, and mobile phase B consisted of 0.1% formic acid in methanol. The gradient started at 20% B was linearly increased from 20 to 40% B from 0 to 0.7 min, then increased from 40 to 100% B from 0.7 to 4.5 min, held at 100% B to 6 min, and re-equilibrated at the initial condition for 2 min. The injection volume was 10 µL. A 5500 QTrap (ABSciex, Redwood City, CA) was utilized with an electrospray ionization source operated in the positive mode. The source parameters included curtain gas of 20 psi, ion spray voltage of 5000 V, ion source temperature of 700 and medium collision gas. Multiple reaction monitoring (MRM) was used with two transitions monitored for each compound and one transition for the internal standards. The raw signal for each compound was normalized to the internal standard (fentanyl-d5), and concentration was calculated from the calibration curve. Only one internal standard was used because the method was qualitative.

LC–MS/MS assay validation

Validation of the final method included determining the lower limit of detection (LOD), linearity (around the cutoff), imprecision (coefficient of variation, % CV), matrix effect, recovery, carryover and interference for each analyte. Linearity was determined by fortifying known concentration of opioid standards into drug-free urine at 10, 25, 50, 100, 250, 500, 750, 1,000 and 2,000 ng/mL (1, 2.5, 5, 10, 25, 50, 75, 100 and 200 ng/mL for buprenorphine, fentanyl, norfentanyl and 6-MAM) and plotting the expected concentration against response. The LOD was defined as the lowest concentration at which the signal-to-noise ratio was greater than 10:1. Within-run % CV was estimated by analyzing five independent aliquots of each of the two QC samples within one run. Between-run % CV was estimated by analyzing two specimens of each level per day over 11 days. Matrix effects were determined by fortifying drug standards into water and five healthy and drug-free donor urine matrices at 500 ng/mL (50 ng/mL for buprenorphine, fentanyl, norfentanyl and 6-MAM) and was calculated using the following equation: $(B - A)/A \times 100\%$, where B is the mean signal intensity in donor urine matrix and A is the mean signal intensity in water. To calculate the percentage of extraction recovery, 500 ng/mL of opioids were fortified in three different aliquots of drug-free urine before extraction. Another three aliquots of drug-free urine were taken through the extraction and fortified with 500 ng/mL of compounds in the flow-through of the protein precipitation plate. The mean peak area of the samples fortified before extraction was divided by the mean peak area of the samples fortified after extraction. Interference studies were carried out by analyzing 140 compounds fortified in 10 drug-free urine samples. To determine an appropriate dilution scheme for patient samples that contained very high concentrations of opioids, samples were tested straight and at various dilutions based on the immunoassay value. Samples that had an opiate immunoassay value over 18,000 were subjected to a 1:20 dilution, and samples that had an immunoassay value between 15,000 and 18,000 were subjected to a 1:10 dilution. Samples that had an oxycodone immunoassay value over 300 were subjected to a 1:10 dilution. Blank samples were inserted after all diluted samples to determine any carryover. Sixty-two patient urine samples that were screened positive by the opioid immunoassay and were analyzed by GC–MS were analyzed by the LC–MS/MS method. In addition, 22 patient urine samples that tested positive for 6-MAM, buprenorphine/norbuprenorphine, methadone/EDDP and fentanyl/norfentanyl by an LC–MS/MS method from an outside reference laboratory were also analyzed by our LC–MS/MS method.

Data analysis

Analyst® and MultiQuant® software from ABSciex (Redwood city, CA, USA) were utilized for LC–MS/MS system control, data acquisition and data analysis. Calibration curves were generated using a weighted (1/×) linear regression curve. Analyte peaks were positively identified by a combination of retention time, two MRM transitions and an ion ratio. Because morphine was the first compound eluted from the chromatography column and was extremely sensitive to the pH of the mobile phase, the retention time of morphine was accurately determined from morphine-d6. For each compound, ion ratios were calculated by dividing the peak area of the qualifier ion by the peak area of the quantifier ion. Compounds identified in the patient samples were flagged if the ion ratio exceeded ±20% of the mean ion ratio of the calibrators. Analyte peaks were quantitated by normalizing the peak area of the quantifier ion of each compound to the internal standard, and concentration was calculated from the calibration curve. One-way ANOVA and Student's *t*-test were performed to determine the statistical difference between four different enzymes. A significant threshold of $P < 0.05$ was applied for all comparisons.

Results

Evaluation of four β-glucuronidase enzymes

The hydrolysis efficiencies of four β-glucuronidase enzymes for recovering morphine, codeine, hydromorphone, oxymorphone, buprenorphine and norbuprenorphine were evaluated. Under the optimal buffer conditions and temperature of each enzyme, hydrolysis efficiencies of two enzyme concentrations were compared: IMCSzyme 5 vs 10 KU/mL, BG100 10 vs 20 KU/mL, EBG 10.5 vs 17.5 KU/mL and *P. vulgata* 5 vs 10 KU/mL. It was determined that 10, 20, 10.5 and 10 KU/mL were the optimal doses for IMCSzyme, BG100, EBG and *P. vulgata*, respectively (data not shown). Hydrolysis efficiencies of the four β-glucuronidase enzymes at the optimal conditions for each enzyme are compared in Figure 1. As shown, IMCSzyme showed a significantly higher hydrolysis efficiency for C6G and H3G compared with all the other three enzymes. Red Abalone BG100 exhibited comparable results to IMCSzyme in hydrolyzing M3G, M6G, O3G and NBG, but

significantly lower for C6G, H3G and BG. EBG showed significantly lower efficiency for all glucuronide compounds compared with IMCSzyme and significantly lower efficiency for M3G, M6G, C6G, O3G and NBG compared with BG100. *P. vulgata* exhibited higher average efficiency in hydrolyzing BG and NBG compared with the other three enzymes, but the results were not significant due to a large standard deviation. The hydrolysis efficiency of *P. vulgata* for M3G was comparable to IMCSzyme, but its efficiency for M6G, C6G and H3G were significantly lower. Its efficiency for M6G and C6G were also significantly lower than BG100 and EBG.

To further understand the influence of substrate concentration on enzyme performance, three concentrations of the glucuronide conjugates, 0.1, 1 and 10 μg/mL, were incubated with the IMCSzyme enzyme for 1 h. Hydrolysis efficiency increased with the decrease in substrate concentration. At 0.1 μg/mL, almost 100% of M6G and H3G, 92% of M3G, 75% of O3G, BG and NBG and 63% of C6G were hydrolyzed. At 10 μg/mL, hydrolysis efficiency ranged from 40 to 60% for each glucuronide.

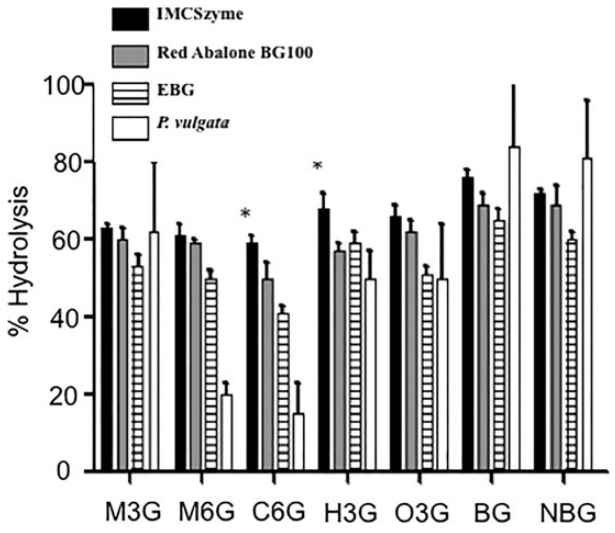

Figure 1. Evaluation of the hydrolysis efficiency of four β-glucuronidase enzymes for seven opioid glucuronide conjugates after 1-h incubation. Substrate concentrations were 1 μg/mL for each enzyme reaction. IMCSzyme exhibited a significantly higher efficiency in hydrolyzing codeine-6-β-D-glucuronide and hydromorphone-3-β-D-glucuronide compared with the other three enzymes. Results are reported as mean ± SD. *$P < 0.05$ compared with the other three groups.

Evaluation of three protein precipitation plates

The Supelco protein precipitation plate gave a higher overall compound recovery (88–103%) compared with the Phenomenex Impact plate (69–120%) and the Biotage Isolute PLD+ plate (72–81%), as shown in Table I. In addition, matrix effects using the Supelco plate (83–135%) were overall less significant than the Impact plate (68–122%) and the Isolute plate (62–137%).

Chromatography

A representative chromatogram for human urine fortified with 500 ng/mL of the 13 opioid compounds is shown in Figure 2a. The newly developed LC–MS/MS method provided a baseline separation of two pairs of isomers, morphine vs hydromorphone (Figure 2b) and codeine vs hydrocodone (Figure 2c). Oxycodone and 6-MAM (retention time, 2.5 min) were not completely separated in time, but spectrally they are distinguishable. They have different parent mass-to-charge ratios and do not share common product ions. Retention times and the two MRM transitions used for each analyte are listed in Table II. The separation was complete in 8 min.

Assay validation

A summary of the qualitative LC–MS/MS assay validation is shown in Table II. Calibration curves of each analyte exhibited consistent linearity and reproducibility in the range of 25–2,000 ng/mL (buprenorphine, 10–200 ng/mL, fentanyl, norfentanyl and 6-MAM, 2.5–200 ng/mL), with regression coefficients (r) ≥0.990 for all analytes. The LOD of each analyte was 10 ng/mL (1 ng/mL for buprenorphine, fentanyl, norfentanyl, and 6-MAM). The cutoffs for clinical reporting were selected to be 300 ng/mL for morphine, 10 ng/mL for buprenorphine, fentanyl, norfentanyl and 6-MAM and 100 ng/mL for all other analytes. No significant ion suppression was detected for any of the 13 analytes, with a few showing ion enhancement (83–135%).

Table I. Evaluation of the Extraction Recovery, Matrix Effects and Within-run Imprecision (% CV) at 80 and 120% of the Cutoff Concentrations for Three Commercially Available Protein Precipitation Plates

	Supelco protein precipitation filter plate (%)				Phenomenex impact protein precipitation plate (%)				Biotage Isolute PLD+ plate (%)			
	Recovery (%) ($n = 3$)	Matrix effect (%) ($n = 5$)	% CV ($n = 5$)		Recovery (%) ($n = 3$)	Matrix effect (%) ($n = 5$)	%CV ($n = 5$)		Recovery (%) ($n = 3$)	Matrix effect (%) ($n = 5$)	%CV ($n = 5$)	
			80%	120%			80%	120%			80%	120%
Morphine	98	90	4	7	74	77	18	16	78	70	7	4
Codeine	101	90	5	5	99	89	16	8	73	98	9	3
Hydromorphone	103	90	4	4	74	68	7	13	78	62	7	3
Hydrocodone	103	83	7	5	92	86	7	5	69	103	2	3
Oxymorphone	96	94	8	5	69	82	4	14	79	69	9	11
Oxycodone	98	113	6	3	89	86	13	6	80	81	7	6
6-MAM	102	97	7	5	97	92	10	7	81	79	16	6
Methadone	97	122	10	4	120	107	6	4	79	114	10	4
EDDP	96	113	6	3	114	93	3	2	74	137	6	3
Buprenorphine	88	135	7	6	119	122	4	2	79	81	10	1
Norbuprenorphine	100	115	12	7	104	85	10	6	78	99	15	8
Fentanyl	98	107	5	3	108	96	5	2	78	99	6	3
Norfentanyl	99	85	5	3	104	89	15	8	72	110	5	4

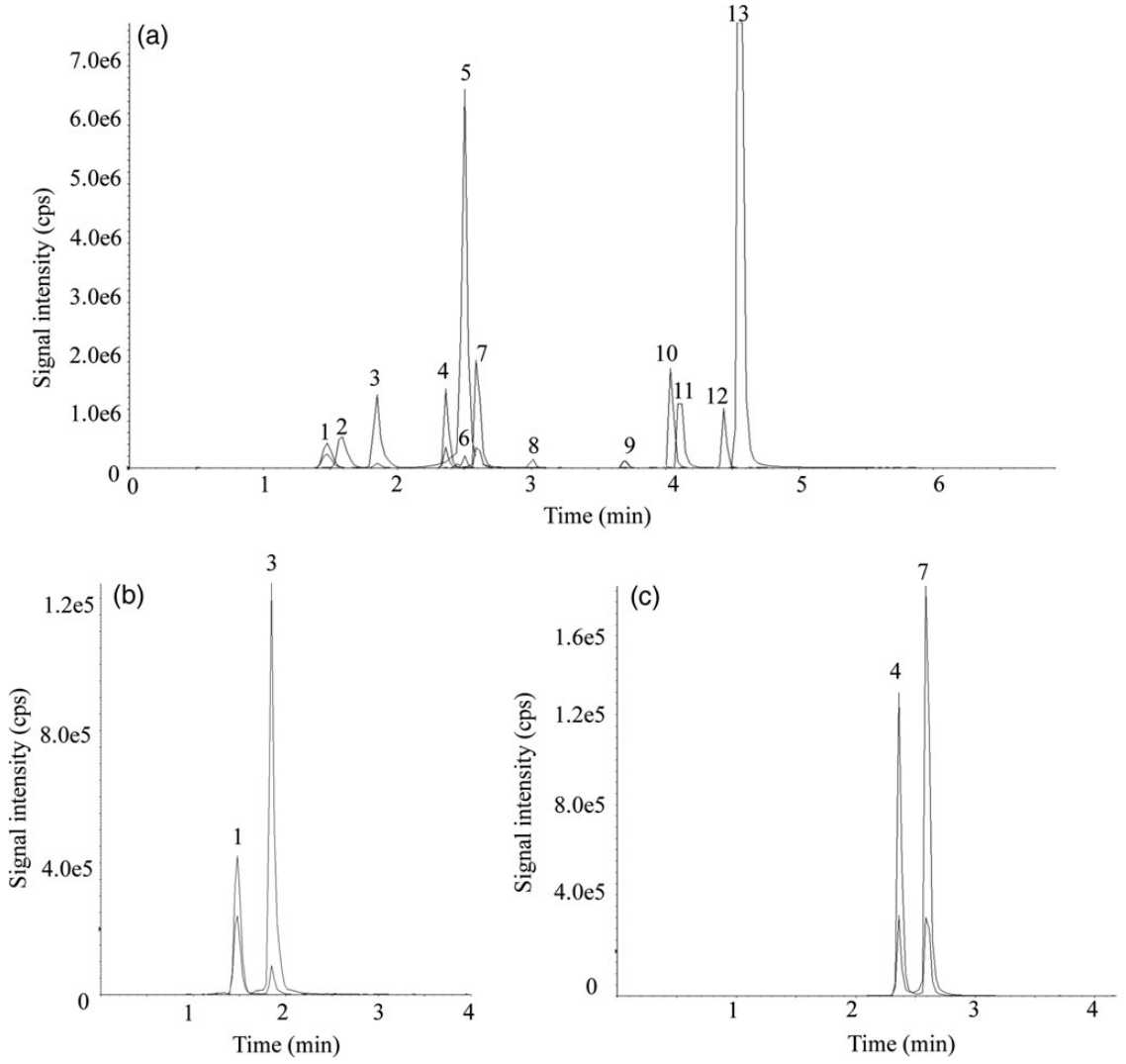

Figure 2. (a) A representative chromatogram for human urine fortified with 500 ng/mL of the 13 opioid compounds: morphine (1), oxymorphone (2), hydromorphone (3), codeine (4), oxycodone (5), 6-MAM (6), hydrocodone (7), norfentanyl (8), norbuprenorphine (9), fentanyl (10), EDDP (11), buprenorphine (12) and methadone (13). Signal intensity for methadone was 1.3e7 cps. The peak was truncated to better display other smaller peaks. (b) Baseline separation of morphine and hydromorphone. (c) Baseline separation of codeine and hydrocondone.

Extraction recovery using the Supelco protein precipitation plate ranged from 88 to 103%, with an average of 98%. Within-run coefficient of variation (% CV) at 80 and 120% of the cutoff values ranged from 4.0 to 11.7% (average 6.6%) and 3.0 to 7.2% (average 4.6%), respectively. Between-run % CV at 80 and 120% of the cutoff values ranged from 5.4 to 16.0% (average 10.5%) and 7.8 to 16.3% (average 12.1%), respectively. No interferences were found in the samples fortified with 1 µg/mL of 140 drugs that are common in our patient population. Samples were injected straight or diluted (1:10 or 1:20) based on the opiate and oxycodone immunoassay values. Carryover was less than the LOD in all blank samples injected after the samples that contained high concentrations of opioids.

Patient comparison

Sixty-two patient urine samples were analyzed by both the GC–MS and LC–MS/MS methods. A total of 40 morphine, 19 codeine, 13 hydrocodone, 22 hydromorphone, 26 oxycodone and 26 oxymorphone peaks were identified by GC–MS. Of these peaks, 96.6% were confirmed by the LC–MS/MS method. The LC–MS/MS assay detected an additional three patient samples that were positive for morphine, one for codeine, six for hydromorphone, five for hydrocodone, two for oxycodone and three for oxymorphone, which were reported as either negative or inconclusive by the GC–MS assay. These additional opioids were all confirmed by patient prescription information or were supported by the presence of metabolites and/or parent drugs in the same sample.

Twenty-two patient urine samples were tested positive for 6-MAM, methadone/EDDP, fentanyl/norfentanyl and buprenorphine/norbuprenorphine using a reference LC–MS/MS method from an outside laboratory. The reference method does not use a cutoff but rather calls a drug positive if a peak is identified and meets reporting criteria. The reference LC–MS/MS assay identified a total of five 6-MAM, nine methadone, eight EDDP, six fentanyl, six norfentanyl, five buprenorphine and six norbuprenorphine peaks. Of those peaks, 93.3% were confirmed by our LC–MS/MS method. Three peaks were called negative by our LC–MS/MS assay as they were below the cutoffs: one methadone (77 ng/mL), one buprenorphine (8.7 ng/mL) and one

Table II. Validation of the LC–MS/MS Assay

	Retention time (min)	Transitions	Linear range (ng/mL)	Matrix effect (%) ($n = 5$)	Recovery (%) ($n = 3$)	Within-run % CV ($n = 5$)		Between-run % CV ($n = 2$, over 11 days)		Cutoff (ng/mL)
						80% of the cutoff	120% of the cutoff	80% of the cutoff	120% of the cutoff	
Morphine	1.47	286.1–>165.1/152.2	25–2000	90	98	4	7	11	10	300
Codeine	2.40	300.2–>165.2/215.1	25–2000	90	101	5	5	14	13	100
Hydromorphone	1.90	286.1–>185.2/157.2	25–2000	90	103	4	4	10	12	100
Hydrocodone	2.62	300.2–>199.0/171.0	25–2000	83	103	7	5	11	13	100
Oxymorphone	1.66	302.1–>227.2/198.2	25–2000	94	96	8	5	12	14	100
Oxycodone	2.52	316.2–>298.2/241.2	25–2000	113	98	6	3	7	7	100
6-MAM	2.50	328.1–>165.1/211.1	2.5–200	97	102	7	5	11	11	10
Methadone	4.58	310.0–>105.2/77.1	25–2000	122	97	10	4	11	16	100
EDDP	4.12	278.2–>234.2/186.2	25–2000	113	96	6	3	6	10	100
Buprenorphine	4.45	468.3–>396.2/414.3	10–200	135	88	9	6	8	12	10
Norbuprenorphine	3.75	414.3–>83.1/101.3	25–2000	115	100	12	7	16	15	100
Fentanyl	4.06	337.3–>188.2/105.1	2.5–200	107	98	5	3	5	11	10
Norfentanyl	3.01	233.2–>150.1/84.1	2.5–200	85	100	5	3	14	13	10

norbuprenorphine peak (58 ng/mL). It also detected an additional buprenorphine peak (21 ng/mL). The buprenorphine peak was found in the presence of norbuprenorphine, and the reference method detected only norbuprenorphine.

Discussion

This study is the first to evaluate the hydrolysis efficiency of IMCSzyme recombinant β-glucuronidase for seven opioid glucuronide conjugates and to compare its performance with β-glucuronidase from abalone, *E. coli* and *P. vulgata*. IMCSzyme exhibited the best overall effectiveness in cleaving the glucuronide linkages in the opioid compounds tested. In particular, our data suggested that IMCSzyme was able to hydrolyze M6G and C6G significantly better than previous reports of other β-glucuronidase enzymes (11, 13). It has been proposed that the glucuronide group at carbon 6 is much less readily hydrolyzed than the one at carbon 3 of morphine. This is possibly due to the fact that the cleavage of a glucuronide bound at a phenolic position (M3G) requires less energy than a glucuronide bound to an alcoholic position (M6G, C6G) (17). It has been reported that the enzyme hydrolysis rate of M6G is ~25% that of M3G and requires 24 h for completion (14). A 2.5-h incubation with abalone, *E. coli* and *H. pomatia* glucuronidases hydrolyzed 81–91% of M3G but only 20–30% of M6G (14). Our data demonstrated that IMCSzyme hydrolyzed M6G at a similar rate to M3G and was able to achieve ~60% recovery after 1-h incubation. It is worth noting that BG100 β-glucuronidase from red abalone entrails, which has not been evaluated in other publications, was also fairly efficient for opioid hydrolysis, giving ~60% recovery for M3G and M6G and 50% for C6G. Its enzymatic activity for opioid conjugates was better than other generic abalone-sourced β-glucuronidase enzymes (13).

While a number of studies have characterized β-glucuronidase from *P. vulgata*, *H. pomatia*, *Helix aspersa*, *E. coli*, abalone entrails and bovine liver, no consensus has been reached on which enzyme is most effective for opioid hydrolysis. Combie *et al.* reported that *P. vulgata* was not only the most cost-effective β-glucuronidase compared with *H. aspersa*, *H. pomatia* and bovine liver but also the most thermal stable glucuronidase that can be used at a temperature (65°C) high enough to substantially shorten the incubation interval (12). However, the comparison was only based on the hydrolysis of morphine conjugates and cannot be extrapolated to many other opioid conjugates in clinical urine samples. Wang *et al.* also found that *P. vulgata* glucuronidase was able to recover 94% morphine from M3G after 2-h incubation but only 12% from M6G and 49% from H3G (11). The authors concluded that the performance of *P. vuglata* was better than *H. pomatia*, followed by *E. coli* β-glucuronidase (11). Romberg and Lee showed that abalone β-glucuronidase released 91% of morphine from M3G after 2.5-h incubation, which was slightly higher than *E. coli* and *H. pomatia* β-glucuronidase (80%) (14). In addition, Malik-Wolf *et al.* further demonstrated that abalone β-glucuronidase was able to completely hydrolyzed M3G but did not perform well for H3G and C6G (13). Here, our data shed light on the two novel β-glucuronidase enzymes, IMCSzyme and red abalone BG100, and suggest that they can be utilized as viable alternatives for opioid hydrolysis.

The only available publication on IMCSzyme was an investigation of its efficiency for benzodiazepine hydrolysis (18). In this study, samples were centrifuged after hydrolysis and then 10 µL was injected onto the instrument. The authors explained that the IMCSzyme appeared to provide a clean extract that may not adversely impact the analytical column. As such, sample cleanup was not necessary. However, a dilution procedure did not work well in our experiments and caused column

contamination with an increase in column backpressure and a decrease in column lifespan. We evaluated three different protein precipitation plates to remove the enzyme and to clean the urine samples. Among them, the Supelco protein precipitation filter plate exhibited the highest compound recovery and lowest matrix effects. The data presented here prove the combination of the IMCSzyme enzyme and the Supelco protein precipitation plate to be an efficient and convenient opioid hydrolysis and sample cleanup procedure.

As can be seen from the method comparison data, the newly developed qualitative LC–MS/MS method yielded a 96.6% agreement for 146 peaks that tested positive by GC–MS. The LC–MS/MS assay missed a total of five peaks in five specimens. Among them, it missed two oxymorphone peaks but was able to detect oxycodone in the specimens. Because oxymorphone is a metabolite of oxycodone, interpretation of these two samples remained the same by the two assays. In addition, it missed three hydromorphone peaks, two of which were close to the cutoff concentrations (110 and 120 ng/mL). This may be due to incomplete hydrolysis of hydromorphone glucuronide by IMCSzyme compared with acid hydrolysis used by GC–MS. In the third sample, hydrocodone was detected which is a parent drug of hydromorphone so the interpretation of the results was not changed. LC–MS/MS detected an additional 20 peaks in 17 specimens, and it was particularly more sensitive than GC–MS in detecting hydromorphone and hydrocodone peaks. Hydromorphone and hydrocodone are two analytes most affected by interfering substances in GC–MS, such as interference caused by a high oxycodone or oxymorphone concentration (7). Furthermore, for the GC–MS method, the presence of one compound at a very high concentration requires the sample to be diluted before running. Other compounds may be diluted out to concentrations below the LOD of the assay, resulting in inconclusive results.

Conclusion

This study performed an evaluation of four β-glucuronidase enzymes and three protein precipitation plates for opioid analysis. Our data suggested, for the first time, that the IMCSzyme recombinant β-glucuronidase is an effective option for opioid hydrolysis in urine. In addition, the Supelco protein precipitation plate provided a high compound recovery with low matrix effects. The new sample preparation procedure provides a significant reduction in time and cost compared with the previously used GC–MS method. Furthermore, we have developed and validated a qualitative LC–MS/MS assay for the detection of 13 opioid compounds in urine. The new assay exhibits higher sensitivities and fewer interferences leading to inconclusive results than the previous GC–MS assay used in our laboratory.

References

1. French, D. (2013) The challenges of LC-MS/MS analysis of opiates and opioids in urine. *Bioanalysis*, **5**, 2803–2820.
2. Fogger, S., McGuinness, T.M. (2014) Adolescents at risk: pain pills to heroin. Part I. *Journal of Psychosocial Nursing and Mental Health Services*, **52**, 17–20.
3. Jack, H., Masterson, A.R., Khoshnood, K. (2014) Violent conflict and opiate use in low and middle-income countries: a systematic review. *International Journal on Drug Policy*, **25**, 196–203.
4. Wendell, A.D. (2013) Overview and epidemiology of substance abuse in pregnancy. *Clinical Obstetrics and Gynecology*, **56**, 91–96.
5. Edinbora, L.E., Backer, R.C., Poklis, A. (2005) Direct analysis of opiates in urine by liquid chromatography–tandem mass spectrometry. *Journal of Analytical Toxicology*, **29**, 704–710.
6. Langman, L.J., Korman, E., Stauble, M.E., Boswell, M.V., Baumgartner, R. N., Jortani, S.A. (2013) Therapeutic monitoring of opioids: a sensitive LC-MS/MS method for quantitation of seven opioids including hydrocodone and its metabolites. *Therapeutic Drug Monitoring*, **35**, 352–359.
7. French, D., Wu, A.H., Lynch, K.L. (2011) Hydrophilic interaction LC-MS/MS analysis of opioids in urine: significance of glucuronide metabolites. *Bioanalysis*, **3**, 2603–2612.
8. Smith, H.S. (2009) Opioid metabolism. *Mayo Clinic Proceedings*, **84**, 613–624.
9. Dickerson, J.A., Laha, T.J., Pagano, M.B., O'Donnell, B.R., Hoofnagle, A.N. (2012) Improved detection of opioid use in chronic pain patients through monitoring of opioid glucuronides in urine. *Journal of Analytical Toxicology*, **36**, 541–547.
10. Zezulak, M., Snyder, J.J., Needleman, S.B. (1993) Simultaneous analysis of codeine, morphine, and heroin after B-glucuronidase hydrolysis. *Journal of Forensic Sciences*, **38**, 1275–1285.
11. Wang, P., Stone, J.A., Chen, K.H., Gross, S.F., Haller, C.A., Wu, A.H. (2006) Incomplete recovery of prescription opioid in urine using enzymatic hydrolysis of glucuronide metabolites. *Journal of Analytical Toxicology*, **30**, 570–575.
12. Combie, J., Blake, J.W., Nugent, T.E., Tobin, T. (1982) Morphine glucuronide hydrolysis: superiority of β-glucuronidase from *Patella vulgata*. *Clinical Chemistry*, **28**, 83–86.
13. Malik-Wolf, B., Vorce, S., Holler, J., Bosy, T. (2014) Evaluation of abalone β-glucuronidase substitution in current urine hydrolysis procedures. *Journal of Analytical Toxicology*, **38**, 171–176.
14. Romberg, R.W., Lee, L. (1995) Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and β-glucuronidase. *Journal of Analytical Toxicology*, **19**, 157–162.
15. Concheiro, M., Gray, T.R., Shakleya, D.M., Huestis, M.A. (2010) High-throughput simultaneous analysis of buprenorphine, methadone, cocaine, opiates, nicotine, and metabolites in oral fluid by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, **398**, 915–924.
16. Colby, J.M., Wu, A.H., Lynch, K.L. (2015) Analysis of codeine positivity in urine of pain management patients. *Journal of Analytical Toxicology*, **39**, 407–410.
17. Hackett, L.P., Dusci, L.J., Ilett, K.F., Chiswell, G.M. (2002) Optimizing the hydrolysis of codeine and morphine glucuronides in urine. *Therapeutic Drug Monitoring*, **24**, 652–657.
18. Morris, A.A., Chester, S.A., Strickland, E.C., McIntire, G.L. (2014) Rapid enzymatic hydrolysis using a novel recombinant β-glucuronidase in benzodiazepine urinalysis. *Journal of Analytical Toxicology*, **38**, 610–614.