Journal of Analytical Toxicology, 2018;1–7
doi: 10.1093/jat/bky082
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Comparison of Purified β-glucuronidases in Patient Urine Samples Indicates a Lack of Correlation Between Enzyme Activity and Drugs of Abuse Metabolite Hydrolysis Efficiencies Leading to Potential False Negatives

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Abstract

Pain management laboratories analyze biological fluids (urine, saliva or blood) from patients treated for chronic pain to ensure compliance and to detect undisclosed drug use. The quantitation of multi-panel drugs in urine and tissues utilizes β-glucuronidase to cleave the glucuronic acid and liberate the parent drug for mass spectrometry analysis. This work focuses on the comparison of three different, purified and commercially available β-glucuronidases across 83 patient urine samples. One enzyme is genetically modified, expressed in bacteria and the other two enzymes are purified from abalone. The results indicate that the source of β-glucuronidase plays an important role in substrate specificity which in turn dictates hydrolysis efficiency. Contaminants in the enzyme solutions also interfere with analyte detection. Altogether, these factors impact precision and accuracy of data interpretation, leading up to 13% positive/negative disagreement.

Introduction

Analytical chemistry techniques evolved rapidly in recent decades allowing simultaneous monitoring of multiple compounds in complex human biological matrices on mass spectrometry. Detecting prescribed or illicit drugs by liquid chromatography tandem mass spectrometry (LC–MS-MS) is a growing field and used by pain management and forensic toxicology laboratories to confirm results after immunoassay screens.

There are two main phases in human xenobiotic metabolism (1). Phase I (chemical modification) reactions are oxidation, reduction, hydrolysis, cyclization and decyclization. Phase II reactions involve conjugating parents of phase I products with highly polar species such as glucuronic acid, sulfate, glutathione or glycine. Phase II transforms xenobiotics into more polar, water-soluble species which is more suitable for excretion. Due to a limited number of commercially available glucuronide conjugated standards, a deconjugation prior to LC–MS-MS analysis is preferred. The deconjugation process also increases instrument sensitivity as the aglycones ionize better in MS.

Deconjugation of glucuronides can be achieved by acid, base or enzymatic hydrolysis. It has been previously documented that acid hydrolysis decomposes benzodiazepines into common benzophenones and renders unequivocal identification of target compounds impossible (2–4). Acid-catalyzed deconjugation can also lead to significant losses of some opiates (5, 6) and deacetylation of 6-monoacetylmorphine (6-MAM), a specific metabolite of heroin, to morphine (7). Base hydrolysis cleaved the ester linkage in 11-nor9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) glucuronide but reported ineffective on ether linkages found in Δ9 -tetrahydrocannabinol (THC) and 11-hydroxy-THC (11-OH-THC) glucuronides (8).

Enzymatic hydrolysis utilizing β-glucuronidase has been explored for hydrolyzing a wider range of glucuronidated substrates (9–12), especially with newer enzymes that reduce incubation times between 4 and 16 h to less than 1 h (13–15).

Commercially available β-glucuronidases come from different sources, giving each enzyme distinctive characteristics. Enzymes from mollusks—garden snail (*Helix pomatia*), red abalone (*Haliotis rufescens*) and limpet (*Patella vulgata*)—are most active at pH ≤ 4.5. β-Glucuronidase from red abalone has been reported as more effective than those from other sources (9). IMCSzyme, a genetically modified β-glucuronidase, has faster hydrolysis toward benzodiazepines in urinalysis compared to other enzymes, thereby reducing incubation time (13). IMCSzyme also has improved activity toward amitriptyline and cyclobenzaprine compared to mollusk enzymes (14). Hydrolysis efficiencies of four different β-glucuronidases against opioids were evaluated (15) and reported that IMCSzyme hydrolyzes most efficiently under the reported testing parameters for the seven glucuronidated opioids, particularly for codeine-6-β-*D*-glucuronide and hydromorphone-3-β-*D*-glucuronide.

In addition to the hydrolysis efficiency of β-glucuronidases, enzyme purity is important. Crude preparations of β-glucuronidase may contain other enzymes that modify target compounds. Impurities in three β-glucuronidases are reported to transform benzodiazepines (11). A loss of 3-hydroxy from the 1,4-benzodiazepine ring results in the conversion of the target analyte, resulting in conversion of temazepam to diazepam, lorazepam to delorazapam (10) and oxazepam to nordiazepam (11). Other impurities in crude preparations deacetylate 6-MAM to morphine, leading to inaccurate results (16). Such misleading artifacts increase the appeal of purified β-glucuronidases for clinical and forensic applications.

In this work, three commercially available purified β-glucuronidases are compared. One is a genetically modified bacterial enzyme; the other two are purified from abalone by two different manufacturers. Solid-phase extraction using dispersive pipette extraction technology was optimized for each enzyme to ensure the recovery of >80% of reported analytes. Experiments were performed on drug-free urine (DFU) as well as patient urine samples to represent realistic conditions in urine drug testing application. The study aims to demonstrate the importance of β-glucuronidase purity for assay accuracy.

Experimental

Materials

DFU was obtained from a healthy volunteer. Amitriptyline HCl (1 mg/mL), amitriptyline-*N*-β-*D*-glucuronide (100 µg/mL), amitriptyline-d_3 HCl (100 µg/mL), codeine (1 mg/mL), codeine-6-β-*D*-glucuronide (100 µg/mL), codeine-d_6 (1 mg/mL), morphine (1 mg/mL), morphine-3-β-*D*-glucuronide (100 µg/mL), morphine-d_3 (1 mg/mL), norbuprenorphine (1 mg/mL), norbuprenorphine glucuronide (100 µg/mL), norbuprenorphine-d_3 (100 µg/mL), temazepam (1 mg/mL), temazepam glucuronide lithium salt (100 µg/mL) and temazepam-d_5 (100 µg/mL) drug standards were purchased from Cerilliant Corporation (Round Rock, TX). Genetically modified β-glucuronidase, Enzyme A, and its proprietary rapid hydrolysis buffer (RHB) were provided by Integrated Micro-Chromatography Systems (Columbia, SC). Enzyme B and its accompanying buffer were purchased from United Chemical Technologies, and Enzyme C was purchased from Sigma-Aldrich. Disposable pipette extraction (DPX) WAX/RP tips were purchased from DPX Labs (Columbia, SC). Sodium acetate buffer (1 M, pH 4.5) was purchased from Teknova (Hollister, CA). Formic acid (99.5%) and all solvents were of LC–MS Optima grade purchased from Fisher Scientific (Waltham, MA).

Specimens

Eighty-three patient urine samples known positive for codeine, norbuprenorphine and buprenorphine were obtained from Pathway Diagnostics (Picayune, MS). These samples were previously confirmed positive by LC–MS-MS at Pathway Diagnostics and saved for this study. Reported cutoffs are in Supplementary Table S1.

Standards, calibrators and controls

Working solutions of drug analytes were prepared using certified reference material (CRM) purchased from Cerilliant. The CRM solutions were diluted into calibrator and quality control stock solutions in DFU at seven different concentrations to provide a seven-point calibration curve, ranging from the cut-off value to 50 times the cut-off value. Calibrator solutions contained a total of 73 analytes prepared in DFU. Calibration concentrations of the 10 reporting analytes are in Supplementary Table S2.

Quality control samples were 1.5 times the lowest calibrator and 0.9 times the highest calibrator. Deuterated internal standard (ISTD) CRM was used as an internal control in each sample run. All calibrations were linear with correlation coefficient, $R^2 \geq 0.99$, and quality control samples were within 20% accuracy of the target concentrations. All analytes were within 1% retention time of their respective internal standards.

Methods

Sample analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Enzymes were run on a 4–20% polyacrylamide gel (BioRad). One microgram of enzyme in Laemmli sample buffer was loaded per lane and electrophoresed for 30 min at 200 mV. Gels were stained with InstantBlue™ Protein Stain (Expedeon) and imaged using a GE Amersham Imager 600. Theoretical molecular weight for the monomeric unit of β-glucuronidase is between 68 and 72 kDa. Protein concentration was measured using Bradford Protein Assay and calculated based on a bovine serum albumin standard curve.

Enzyme activity

Talalay *et al.* introduced phenolphthalein glucuronide (PTGlcU) chromogenic assay to determine β-glucuronidase activity (17). Glucuronidase activity was monitored using protocols based on Fishman's PTGlcU chromogenic assay. The activity protocols performed were specific to each vendor using respective buffers, pH and temperature. Three activities, measured on three separate days, were averaged for each enzyme and compared to vendor-specified activity values.

Extraction method evaluation

Prior to urine drug confirmation by LC–MS-MS, the extraction method using DPX WAX/RP was assessed to evaluate any matrix effect.

Table I. Master mix composition for each enzyme hydrolysis per 80 µL urine sample[a]

Enzyme	β-Glucuronidase	Buffer (µL)	Water (µL)	Enzyme (µL)	ISTD (100% methanol) (µL)
A	IMCSzyme®	1X RHB pH 7.4 40	20	20	10
B	Purified abalone 1	1X vendor-provided buffer pH 4.5 60	–	20	10
C	Purified abalone 2	1 M sodium acetate pH 4.5 20	40	20	10

[a]Enzyme amount and ISTD were kept constant. Water was used to keep the total hydrolysis volume the same across different enzymes. Buffers for Enzymes A and B were used following manufacturer's recommendations.

Hydrolysis methods

Hydrolysis was measured every 15 min over 60 min. DFU was fortified with 500 ng/mL of amitriptyline glucuronide, 750 ng/mL of codeine-6-β-*D*-glucuronide, 750 ng/mL of morphine-3-β-*D*-glucuronide, 400 ng/mL of norbuprenorphine glucuronide and 500 ng/mL of temazepam glucuronide. Eighty microliter of fortified urine was incubated with 90 µL of each enzyme master mix containing enzyme, buffer, water and internal standards (ISTD) according to Table I. The enzyme amount per 80 µL urine sample was fixed at 20 µL. The incubation was performed at 58°C for 0, 15, 30, 45 and 60 min.

Patient samples were treated with the same enzyme master mix shown in Table I. Samples were incubated at 58°C for 30 min. Four representative patient samples were monitored frequently at 15-min intervals for 60 min.

LC–MS-MS analysis

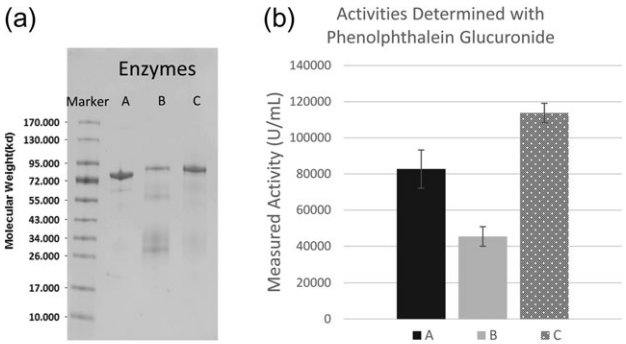
Ultraperformance liquid chromatography was performed on a Waters Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 µm). The column was heated to 40°C with a gradient elution with a flow rate of 0.6 mL/min. Mobile phase A consisted of 2.5 mM ammonium formate and 0.1% formic acid in ultrapure water and mobile phase B consisted of 2.5 mM ammonium formate and 0.1% formic acid in methanol. The system was equilibrated in 100% A and the gradient consisted of 10–16% B from 0.2 to 1.0 min, ramped to 30% B by 1.4 min, then to 60% B by 2.4 min. The final gradient steps consisted of 90% B by 3.2 min and 95% B by 3.4 min and re-equilibrated at initial conditions from 3.5 to 4.0 min. The separation of over 100 analytes was completed in 3.4 min. The liquid chromatography (LC) system was connected to Waters Xevo TQ-S tandem mass spectrometer with electrospray ionization source, operated in positive mode. Nitrogen was used as a nebulizer gas (7 bar), desolvation gas (1,000 L/h heated to 550°C) and cone gas (150 L/h). Argon was used as the collision-induced dissociation gas. Detection was performed by multiple-reaction monitoring analysis as in Supplementary Table S3.

Precision and accuracy

Precision and accuracy of the method were tested in replicates of five. The replicates were tested at both am and pm time intervals with a minimum of a 4-h time delay between tests, over a period of 5 days. Precision and accuracy were evaluated for inter- and intra-day. Results for inter-day tests are in Supplementary Table S4.

Results and Discussion

Three commercially available β-glucuronidases were compared. One microgrm of each enzyme was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Enzymes A and C display single intense bands of protein at ~72 kDa, while Enzyme B contains other lower molecular weight proteins apart from the major enzyme band (Figure 1a). The result suggests that Enzymes A and C are of higher purity than Enzyme B since there are fewer extraneous bands.



(c)

Enzyme	Activity (Fishman U/mL)	Std Dev	Vendor-specified enzyme activity (Fishman U/mL)	Temp (°C) of activity assay	pH in activity assay	Protein, mg/mL
A	82702	10557	> 50000	25	6.8	1.05
B	45416	5421	> 50000	37	4.5	0.89
C	113731	5367	≥ 100000	37	3.8	0.29

Figure 1. Characterization of three commercial β-glucuronidases. (a) SDS-PAGE showing 1 µg of each purified enzyme. (b) Enzyme activity of each enzyme on phenolphthalein glucuronide. (c) Table showing enzyme activities and their corresponding testing conditions for each enzyme.

By definition, one Fishman unit liberates 1 µg of phenolphthalein per hour at 37°C at pH 4.5. However, the PTGlcU assay conditions may be modified to suit the temperature and pH optima of a particular glucuronidase. For the three enzymes used in the experiment, vendor-recommended assay conditions were used for each enzyme (listed in Figure 1c). Enzyme activities are reported in Figure 1b and they are comparable to vendor-specified values in Figure 1c. Enzyme activities on PTGlcU: Enzyme C > Enzyme A > Enzyme B.

DPX WAX/RP extraction recover > 80% of codeine, dihydrocodeine and norbuprenorphine (Supplementary Figure S2) and was used to clean up hydrolyzed urine samples prior to LC–MS-MS analysis. Enzyme efficiency toward drug glucuronides was evaluated using fortified DFU by focusing on five drug metabolites. Drugs were selected from several classes, including tricyclic antidepressant (amitriptyline), opiate (morphine, codeine), opioid (norbuprenorphine) and benzodiazepine (temazepam). Fortified DFU samples were analyzed in triplicate. Each enzyme had its own set of calibrators and quality control samples. All samples comprised the same volume of urine, buffer/water mix, β-glucuronidase and internal

standard as outlined in Table I. The enzymes were dosed on a vendor-recommended ratio of 1:4 enzyme to urine volume.

Amitriptyline belongs to the tricyclic antidepressant class of drugs. During phase II metabolism, the tertiary amine of the analyte forms N-linked glucuronidation for excretion in urine. DFU was fortified with amitriptyline glucuronide at a concentration equivalent to 500 ng/mL of amitriptyline after complete hydrolysis. Enzyme efficiencies of the three different β-glucuronidases are drastically different as shown in Figure 2a. Enzyme A gives 100% recovery even without incubation, whereas both Enzymes B and C yield <40% recovery even after 60-min incubation. Similarly, Mastrianni *et al.* previously observed that Enzyme A hydrolyzes amitriptyline and cyclobenzaprine glucuronides faster than mollusk β-glucuronidases (14).

Based on the previous studies comparing β-glucuronidases, codeine-6-β-*D*-glucuronide is reportedly a difficult substrate to hydrolyze (18, 19). Enzyme A achieved a complete hydrolysis (>90%) after 30-min incubation, while both purified abalone β-glucuronidases showed poor hydrolysis efficiency and lower than 30% recovery after 60-min incubation (Figure 2b).

Morphine undergoes glucuronidation during phase II metabolism before urinary excretion. The majority of glucuronidated morphine is morphine-3-β-*D*-glucuronide (~60%), with traces amount of morphine-6-β-*D*-glucuronide (<10%) (20). Therefore, enzyme efficiency was compared on morphine-3-β-*D*-glucuronide in this study (Figure 2c), even though the 6-β-*D* linkage has been reported as a more difficult linkage to enzymatically cleave (9, 18, 19, 21). Depending on enzyme, morphine-3-β-*D*-glucuronide is often among the easiest glucuronidated opioids to hydrolyze enzymatically. Enzyme A achieved complete hydrolysis within 15 min; both purified abalone enzymes took longer. Enzyme C achieved complete hydrolysis after 30 min; Enzyme B only reached 80% hydrolysis, even after 60 min.

Norbuprenorphine glucuronide hydrolysis rate is usually similar to morphine-3-β-*D*-glucuronide, as both belong to the opioid classification. Enzyme A achieved 100% hydrolysis within 15 min of incubation (Figure 2d), as did one Enzyme B. The other Enzyme C, though it worked faster than Enzyme B on morphine-3-β-*D*-glucuronide, required 45 min to completely hydrolyze norbuprenorphine glucuronide. This demonstrates that while the abalone enzymes are from the same natural source, their hydrolysis efficiencies vary, probably as a function of their manufacturing processes.

Temazepam served as a representative benzodiazepine. Enzyme A hydrolyzed >80% of temazepam glucuronide without any incubation (Figure 2e). In contrast, purified enzymes from abalone required at least 15 min to achieve the same efficiency. Of all the drug glucuronides tested, temazepam glucuronide hydrolyzed most rapidly.

Four patient samples were selected for time course study over 60 min of incubation (Figure 3). Samples were extracted every 15 min, with 0 min referring to immediate extraction after the addition of the enzyme mixture. Two of the four patient samples were positive for codeine and the other two samples were positive for buprenorphine and its metabolite, norbuprenorphine. For both codeine-positive samples, Enzyme A achieved >80% recovery within 30 min, whereas purified abalone enzymes were <50% recovery after 60 min (Figure 3a). This result agrees with the data in Figure 2b that Enzyme A completes the hydrolysis of codeine-6-β-*D*-glucuronide in 30 min in both fortified DFU and patient urine.

In the case of buprenorphine-positive samples, both buprenorphine and norbuprenorphine were detected. Once absorbed into the body, buprenorphine is metabolized into norbuprenorphine via N-dealkylation (22). Both compounds then undergo glucuronidation during phase II metabolism. Buprenorphine was not included in the fortified DFU experiment because it has been reported to behave similarly to norbuprenorphine but is easier to hydrolyze than norbuprenorphine (5, 23). Two patient samples selected include low (< 500 ng/mL) and high (>1500 ng/mL) concentrations of norbuprenorphine. In a low-concentration scenario (patient 3), Enzyme A achieved a complete hydrolysis in 15 min, while purified abalone enzymes

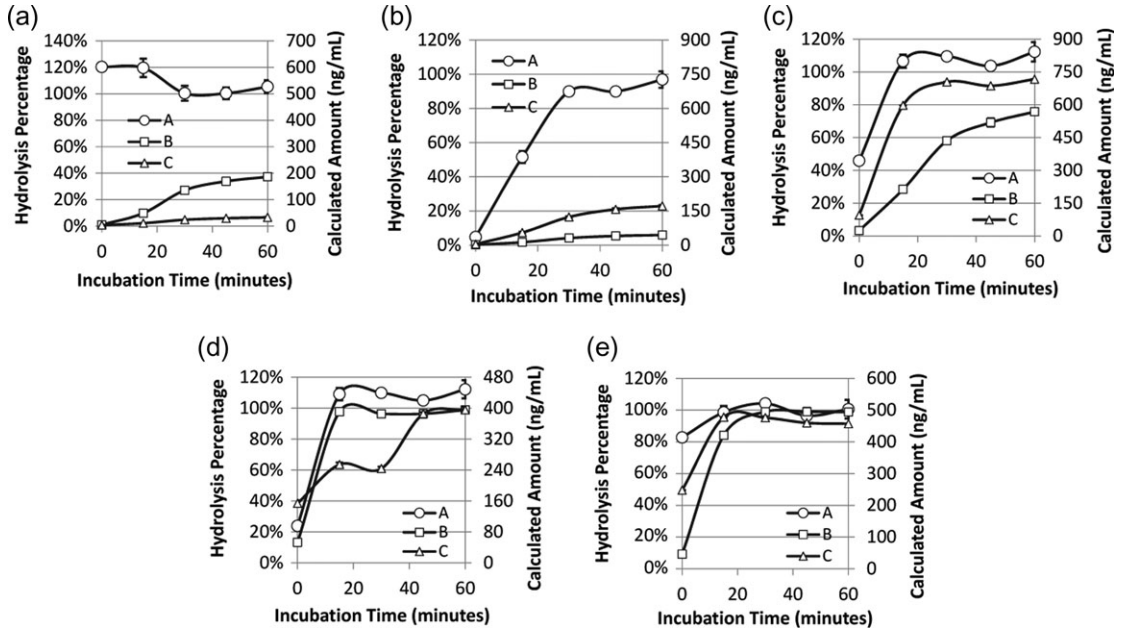


Figure 2. Recoveries of parent drug from (a) amitriptyline, (b) codeine, (c) morphine-3, (d) norbuprenorphine and (e) temazepam glucuronides with three different β-glucuronidases over 1-h incubation. Enzyme A: IMCSzyme; Enzyme B: purified abalone 1; Enzyme C: purified abalone 2. Abalone-derived enzymes (Enzymes B and C) gave lower recoveries of amitriptyline, codeine and morphine. Error bars show ± 1 SD.

reached only 75% recovery between 15 and 60 min (Figure 3b). The hydrolysis profiles differed more in the sample with a high concentration of norbuprenorphine (patient 4). IMCSzyme achieved >80% hydrolysis in 15 min, while purified abalone enzymes were unable to recover over 50% (Figure 3b). Though all three enzymes can achieve 100% hydrolysis within 15–45 min in DFU (Figure 2), data from patient urine demonstrates that patient samples are more complex and difficult matrices for some enzymes. Only Enzyme A completes norbuprenorphine hydrolysis in 15 min in both patient samples.

Buprenorphine was present in these two samples at lower concentrations compared to norbuprenorphine but shows a similar trend to norbuprenorphine (Figure 3). Enzyme A recovered >80% of buprenorphine without any incubation time (Figure 3c). Again, purified abalone enzymes only recovered 75% in patient 3 and barely recovered >50% in patient 4 over the 60-min incubation time (Figure 3c) in relative to the amounts recovered using Enzyme A.

Eighty-three patient samples were analyzed by LC–MS-MS to compare the results from three different enzyme treatments. The comparison was based on a 30-min incubation treatment with each enzyme. Purified abalone Enzymes B and C were compared to genetically modified β-glucuronidase Enzyme A. Ten compounds that each had more than 10 positives were selected to display regression slopes in Table II. A regression slope or coefficient <1 means that purified abalone enzymes (Enzyme B or C) have lower hydrolysis efficiency compared to genetically modified β-glucuronidase Enzyme A. These include alprazolam, buprenorphine, codeine, morphine, oxymorphone and temazepam. Low recovery could lead to false negatives, where the reported concentration falls below a cut-off due to incomplete

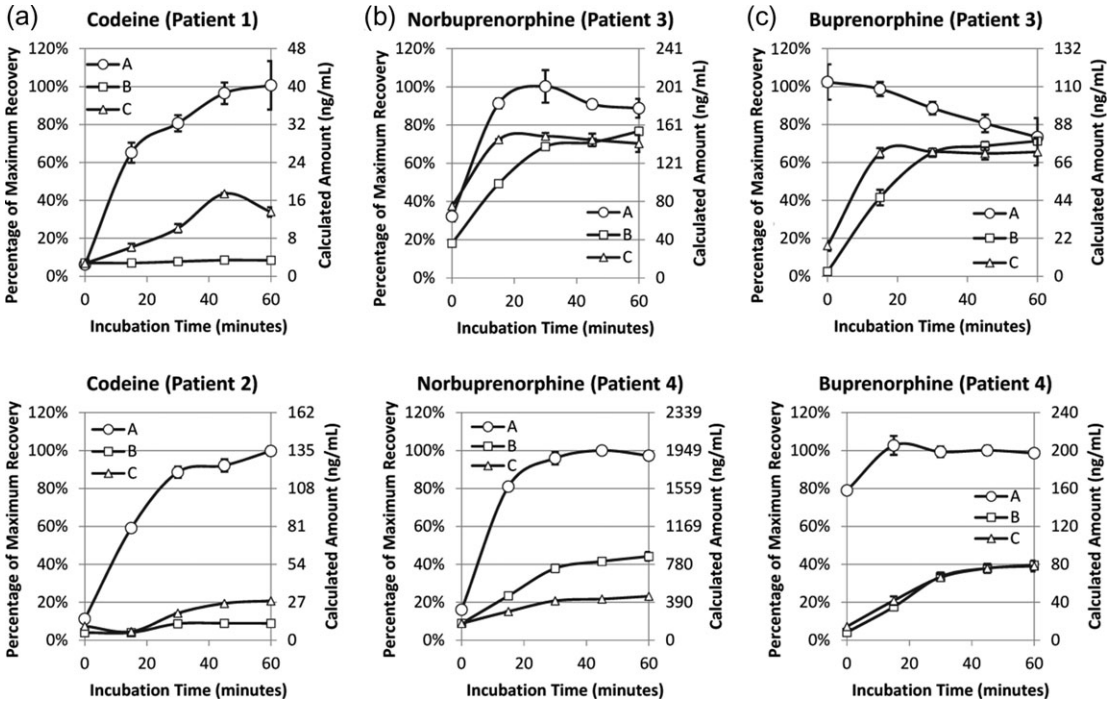


Figure 3. Recoveries of (a) codeine, (b) norbuprenorphine and (c) buprenorphine from four patient samples after treatment with three different β-glucuronidases over 60 min. A: IMCSzyme; B: purified abalone 1; C: purified abalone 2. Patients 1 and 2 are positive for codeine, while patients 3 and 4 are positive for norbuprenorphine and buprenorphine. Abalone-based enzymes (B, C) yield lower recoveries throughout the 60-min incubation. Error bars show ±1 SD.

Table II. Regression slopes of 10 glucuronidated drug recoveries comparing purified abalone enzymes (Enzymes B and C) to IMCSzyme (Enzyme A), including R^2 values and rates of agreement based on a pain management lab cut-off

	Enzyme B: Enzyme A ratio			Enzyme C: Enzyme A ratio		
	Slope	R^2	% Agreement (lab cut-off)	Slope	R^2	% Agreement (lab cut-off)
Alprazolam	0.89	0.90	91	0.63[a]	0.89	91
Buprenorphine	0.80[a]	0.99	100	0.44[a]	0.99	100
Codeine	0.57[a]	0.93	83	0.50[a]	0.96	87
Cotinine	0.95	0.95	98	0.99	0.93	98
Hydromorphone	0.88[a]	0.79	95	1.10	0.93	87
Morphine	0.64[a]	0.86	95	0.81[a]	0.91	100
Norbuprenorphine	1.10	0.99	100	0.41[a]	0.96	100
Oxazepam	1.22[b]	0.97	91	0.91	0.94	91
Oxymorphone	0.85[a]	0.97	90	0.90	0.98	90
Temazepam	0.75[a]	0.96	91	0.97	0.97	91

Annotations: [a] indicates slope < 0.88 and [b] indicates slope > 1.12. Highlight indicates R2 < 0.85 and % agreement < 90%.

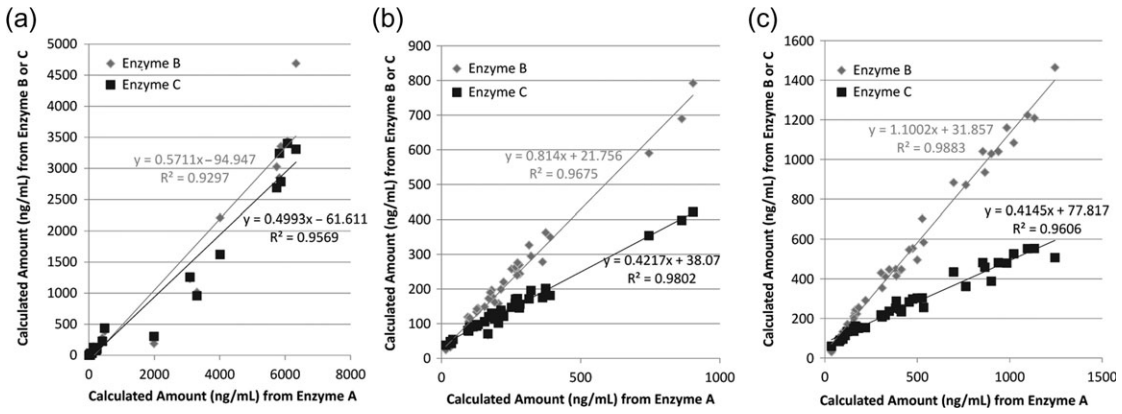


Figure 4. Regression plots of (a) codeine, (b) buprenorphine and (c) norbuprenorphine in patient urine samples recovered after 30-min incubation with three β-glucuronidases.

hydrolysis. The cut-off concentration used in this experiment and quadratic plot of agreements are shown in Supplementary Table S3. The percent agreement of all three enzymes is >83% for all analytes. This means that each compound has less than a 17% chance of being reported as a false negative or false positive depending on β-glucuronidase used.

The regression plots for codeine, buprenorphine and norbuprenorphine are displayed in Figure 4. Enzymes B and C exhibited similar regression slopes (0.57 and 0.5, respectively) against Enzyme A on codeine-6-β-*D*-glucuronide hydrolysis in patient samples (Figure 4a). For every 1 ng/mL of codeine recovered from Enzyme A hydrolysis, only 0.57 and 0.5 ng/mL of codeine were recovered from Enzymes B and C hydrolysis, respectively. Therefore, with a cut-off concentration of 20 ng/mL, Enzyme B yielded four false negatives and Enzyme C yielded three false negatives (Supplementary Table S3) compared to Enzyme A, due to their lower hydrolysis efficiencies. These false negatives result in 13% or 17% discrepancy between analyses, depending upon the choice of enzyme (Table II).

While both purified abalone enzymes behave similarly toward codeine, the purified abalone enzymes exhibit different hydrolysis efficiencies against buprenorphine and norbuprenorphine glucuronides. For these metabolites, the regression slopes of Enzyme B were higher than Enzyme C. This means that Enzyme B hydrolyzes these glucuronidated drugs more effectively than Enzyme C. For every 1 ng/mL of buprenorphine recovered from Enzyme A hydrolysis, only 0.81 and 0.42 ng/mL of buprenorphine is recovered from Enzymes B and C hydrolysis, respectively (Figure 4b). For every 1 ng/mL of norbuprenorphine recovered from Enzyme A hydrolysis, only 1.1 and 0.41 ng/mL of norbuprenorphine is recovered from Enzyme B and C hydrolysis, respectively (Figure 4c). This demonstrates that β-glucuronidases can have different hydrolysis efficiencies despite its source host as being identified as abalone.

Compared to the two purified abalone enzymes, genetically modified Enzyme A showed higher hydrolysis efficiencies toward all glucuronidated drugs tested except oxazepam (Table II). This could be contributed from matrix effect or benzodiazepine conversion as Figure 2e showed that all three purified enzymes can completely hydrolyze benzodiazepine (as represented with temazepam) within 15 min of incubation. Although Enzyme C had the highest enzyme activity as defined in Fishman units (Figure 1b), its activity hydrolysis efficiency toward various drug glucuronides in patient urine was the lowest (Table II). Furthermore, purified abalone enzymes show large differences in glucuronidated opioid hydrolysis efficiencies. Enzyme C with the highest activity has higher hydromorphone and morphine recoveries in patient samples, whereas Enzyme B demonstrates higher norbuprenorphine recovery. This lack of correlation between hydrolysis efficiencies and enzymatic activity reported using Fishman units is important to note. Furthermore, it should also be noted that despite stating the same source of enzyme, the differences in activities and its purity lead to large discrepancies when testing in patient urine samples.

Finally, during the automated data analysis phase, all calibrators and quality control samples prepared with Enzyme C were flagged. This required all chromatograms to be reinspected and replotted. An amphetamine interference was observed in Enzyme C which required a modification to the peak integration formula. DFU without enzyme showed no interference for amphetamine, however the interference peak at 1.43 min was observed when treated with Enzyme C (Supplementary Figure 4a). Extracted chromatograms from a calibration sample fortified with 400 ng/mL of amphetamine showed that the interference peak prevented baseline separation of the amphetamine peak, leading to faulty area integration and incorrect quantitation of amphetamine (Supplementary Figure 4b). DFU containing Enzyme B appeared to be without the amphetamine interference, but its recovery of amphetamine was lower than samples containing Enzymes A and C.

Conclusion

This work demonstrated that equal dosages of three different enzymes hydrolyze drug glucuronides with different efficiencies. Drugs were selected to represent a range of classes, such as tricyclic antidepressant (amitriptyline), opiate (codeine), opioid (morphine, norbuprenorphine) and benzodiazepine (temazepam). Overall, the genetically modified β-glucuronidase outperforms enzymes from abalone sources (Figure 2). A complete hydrolysis of amitriptyline glucuronide and temazepam glucuronide does not require an extensive incubation time with Enzyme A. All glucuronidated drugs are hydrolyzed with an incubation time of 30 min for Enzyme A but require over 60 min for abalone enzymes.

The regression slope correlating drug concentrations in 83 patient urine samples treated with different enzymes (Table II) reveals that Enzyme A has higher hydrolysis efficiency compared to abalone enzymes. The discrepancies in hydrolysis efficiency lead to up to 17% disagreement of positive or negative interpretation. One purified enzyme tested, Enzyme C, also showed an amphetamine

interference that prevented a baseline separation of the amphetamine peak. Therefore, the choice of β-glucuronidase directly affects patient result accuracy.

Supplementary Data

Supplementary material is available at *Journal of Analytical Toxicology* online.

Acknowledgments

The authors thank Pathway Diagnostics, LLC (Picayune, MS) for providing patient samples and for sample analyses on LC–MS-MS systems. The authors also thank John J. Tomashek for instructive discussions and careful editing of the manuscript.

Conflict of Interest

Pongkwan Sitasuwan, Cathleen Melendez and L. Andrew Lee are employees of Integrated Micro-Chromatography Systems, LLC.

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