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Technical Note

Evaluation of Abalone β-Glucuronidase Substitution in Current Urine Hydrolysis Procedures

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This study examined the potential of abalone β-glucuronidase as a viable and cost effective alternative to current hydrolysis procedures using acid, *Helix pomatia* β-glucuronidase and *Escherichia coli* β-glucuronidase. Abalone β-glucuronidase successfully hydrolyzed oxazepam-glucuronide and lorazepam-glucuronide within 5% of the spiked control concentration. Benzodiazepines present in authentic urine specimens were within 20% of the concentrations obtained with the current hydrolysis procedure using *H. pomatia* β-glucuronidase. JWH 018 *N*-(5-hydroxypentyl) β-D-glucuronide was hydrolyzed within 10% of the control concentration. Authentic urine specimens showed improved glucuronide cleavage using abalone β-glucuronidase with up to an 85% increase of drug concentration, compared with the results obtained using *E. coli* β-glucuronidase. The JWH 018 and JWH 073 carboxylic acid metabolites also showed increased drug concentrations of up to 24%. Abalone β-glucuronidase was able to completely hydrolyze a morphine-3-glucuronide control, but only 82% of total morphine was hydrolyzed in authentic urine specimens compared with acid hydrolysis results. Hydrolysis of codeine and hydromorphone varied between specimens, suggesting that abalone β-glucuronidase may not be as efficient in hydrolyzing the glucuronide linkages in opioid compounds compared with acid hydrolysis. Abalone β-glucuronidase demonstrates effectiveness as a low cost option for enzyme hydrolysis of benzodiazepines and synthetic cannabinoids.

Introduction

The body utilizes conjugation reactions to increase the hydrophilicity and polarity of xenobiotics to facilitate excretion via bile and urine. Two commonly prescribed drug classes that undergo Phase II metabolism and are routinely encountered in forensic analysis are benzodiazepines and opioids (1–4). Metabolism studies of synthetic cannabinoids have shown that JWH 018 5-hydroxypentyl also reacts with uridine diphosphate-glucuronosyltransferase to form glucuronide conjugates (5–7). It is common practice to include a hydrolysis reaction to cleave the glucuronide linkage when extracting conjugated analytes from urine to measure the total drug concentration.

Hydrolysis reactions can be performed using an enzyme, acid or base though consideration must be given to efficiency and the effect it may have on drug structure. Acid hydrolysis is not recommended for benzodiazepines due to the formation of benzophenones. Although acid offers a fast method of hydrolysis for opioids that is not afforded by enzyme hydrolysis, it will convert 6-acetylmorphine to morphine thus precluding its use in workplace drug testing programs (1, 3, 8). Various sources of β-glucuronidase exist such as *Helix pomatia* (*H. pomatia*), *Escherichia coli* (*E. coli*), bovine liver, *Patella vulgatta* and abalone, with each exhibiting different hydrolysis efficiencies and rates. Abalone β-glucuronidase is an economic option for enzymatic hydrolysis, but is less often cited in scientific literature than other sources of β-glucuronidase. This study examined the potential of abalone β-glucuronidase to replace current hydrolysis methods as a more cost-effective option in sample extraction.

Experimental

Specimens

The urine specimens used in this study were previously submitted to the Armed Forces Medical Examiner System (AFMES) Division of Forensic Toxicology for research purposes. The specimens contained multiple analytes of interest including oxazepam, lorazepam, temazepam, hydromorphone, oxymorphone, codeine and morphine. A small number of specimens also contained JWH 018 5-hydroxypentyl metabolite (JWH 018-OH), JWH 018 5-pentanoic acid metabolite (JWH 018-COOH) and JWH 073 4-butanoic acid metabolite (JWH 073-COOH). Specimens were extracted using the laboratory's current procedures to ensure the analytes of interest had not degraded since the initial analysis, and to provide a reference for comparison of the experimental hydrolysis results. Specimens containing opioids were also extracted using *H. pomatia* β-glucuronidase to allow for an enzymatic hydrolysis comparison. Hydrolysis was performed with abalone β-glucuronidase using parameters derived from manufacturer's documents, existing literature and in-house examination of various pH levels, incubation times and incubation temperatures (9–11).

Reagents and materials

High-performance liquid chromatography (HPLC) grade solvents, concentrated hydrochloric acid (HCl) and sodium acetate trihydrate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Glacial acetic acid, concentrated ammonium hydroxide (NH_4OH), hydroxylamine HCl, concentrated potassium hydroxide (KOH), ammonium formate, formic acid, sodium phosphate monobasic and dibasic, bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), β-glucuronidase *H. pomatia* HP-2 aqueous solution >100,000 units/mL and β-glucuronidase *E. coli* IX-A lyophilized powder 1MU [reconstituted in 20 mL deionized water (DI H_2O)] were purchased from Sigma-Aldrich (St Louis, MO, USA). Abalone β-glucuronidase lyophilized powder 2MU (reconstituted in 20 mL of DI H_2O) was purchased from Campbell Science (Rockford, IL, USA). Liquichek™ Urine Toxicology Control Level C3 #433 was purchased from Bio-Rad (Irvine, CA, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) w/1% *tert*-butyldimethylchlorosilane (TBDMCS) and mixed-mode solid-phase extraction (SPE) columns (ZCDAU020) were purchased from United Chemical Technologies (UCT, Bristol, PA, USA). Strong cation mixed-mode SPE columns (Strata-X-C) were purchased from Phenomenex (Torrance, CA,

Table I
Analyte conjugation of authentic urine samples and hydrolysis recovery

Compound	Sample number	Concentration range (ng/mL)	Observed % conjugation range	Observed % conjugation (average ± SD)	Abalone		*H. pomatia*
					Concentration range (ng/mL)	Relative average % hydrolysis (average ± SD)	Relative average % hydrolysis (average ± SD)
Benzodiazepines							
Lorazepam	2	229–728	92.3–99.5	95.9 ± 5.1	224–733	99.5 ± 5.1	
Oxazepam	5	58.2–878	94.7–99.1	96.3 ± 1.6	55.9–835	96.3 ± 1.6	
Temazepam	5	34.8–24673	62.4–97.1	83.2 ± 15	31.9–27723	103 ± 7.4	
α-Hydroxyalprazolam	3	147–1031	14.2–98.5	46 ± 45	149–830	96 ± 13	
Alprazolam	3	44.7–678	2.5–59.8	32.1 ± 28	44.3–546	90.1 ± 9.2	
Nordiazepam	2	41.0–307	1.2–84.4	42.8 ± 58	48.2–320	110 ± 9.4	
Opioids							
Hydromorphone	6	112–2256	90.1–95.1	93.2 ± 1.8	95.4–1831	62.3 ± 19	69.9 ± 21
Oymorphone	2	222–715	97.7–98.3	98 ± 0.4	198–594	80.7 ± 3.2	82.6 ± 3.4
Codeine	4	224–16599	87.5–93.8	91.2 ± 3.3	52.3–14397	75.7 ± 35	60.1 ± 26
Morphine	4	420–2464	94.0–97.2	95.9 ± 1.4	372–2128	78.8 ± 7.8	83.7 ± 11
Hydrocodone	6	213–1515	8.3–15.4	11.3 ± 2.8	215–1437	99.1 ± 4.7	96.8 ± 3.6
Oxycodone	3	253–343	12.3–16.3	14.3 ± 2.8	255–337	98.6 ± 1.9	93.5 ± 0.9
Synthetic cannabinoids[a]							
JWH 018-OH	9	4.09–10.8	3.92–97.6	64.4 ± 36	6.12–14.9	140 ± 36	
JWH 018-COOH	9	6.06–15.1	4.78–34.4	20.4 ± 9.0	7.7–19.4	124 ± 11	
JWH 073-COOH	8	0.26–1.7	1.04–28.2	16.9 ± 8.6	0.39–1.9	115 ± 6.2	

[a]The concentration ranges for the synthetic cannabinoids are from analysis using *E. coli* β-glucuronidase for a 30-min incubation time, while observed percent conjugation and abalone data are from incubation for 60 min at 70°C using abalone β-glucuronidase.

USA). Drug standards and internal standards were purchased from Cerilliant (Round Rock, TX, USA) and Cayman Chemical (Ann Arbor, MI, USA). Table I lists the analytes of interest.

Standards, calibrators and controls

Benzodiazepine calibrator working solutions were created using 13 commercial standards. The working solutions were used to prepare calibrators in blood at concentrations of 0.025, 0.050, 0.10, 0.25, 0.50 and 1.0 mg/L. Two controls that contained alprazolam and diazepam were prepared in blood at concentrations of 0.20 and 0.75 mg/L. A urine hydrolysis control containing oxazepam-glucuronide and lorazepam-glucuronide with total drug concentrations of 0.49 and 0.51 mg/L, respectively, was included to monitor the hydrolysis reaction. An internal standard containing five deuterated benzodiazepines was prepared at a concentration of 10 mg/L.

Opioid calibrator working solutions were created using six commercial standards. The working solutions were used to prepare calibrators in blood at concentrations of 0.050, 0.10, 0.20, 0.50, 1.5 and 3.0 mg/L. Two positive controls containing codeine, morphine and oxycodone were prepared in blood at concentrations of 0.25 and 2.5 mg/L. A Liquichek™ C3 control containing morphine-3-β-D-glucuronide was extracted with each batch to monitor the hydrolysis reaction. An additional hydrolysis control containing hydromorphone-3-β-D-glucuronide and oxymorphone-3-β-D-glucuronide prepared at a 0.25 mg/L (total-drug) concentration was included to evaluate the hydrolysis of additional commercial glucuronide standards. An internal standard solution containing six deuterated opioids was prepared at a concentration of 10 mg/L.

Synthetic cannabinoid calibrator working solutions were prepared using nine commercial standards. The working solutions were used to prepare calibrators in urine at concentrations of 0.050, 0.10, 0.50, 1.0, 5.0 and 10 ng/mL. A control containing the nine analytes was prepared in urine at a concentration of 0.75 ng/mL. A hydrolysis control was included containing JWH 018 *N*-(5-hydroxypentyl) β-D-glucuronide (JWH 018-OH-glucuronide) in urine at a 6.6 ng/mL total drug concentration. An internal standard solution containing two compounds was prepared at a concentration of 0.10 mg/L.

Benzodiazepine extraction

Specimens were extracted using the existing validated SPE procedure (12). A 1-mL aliquot of each specimen was prepared with 20 μL of internal standard solution and 2 mL of 0.1 M sodium acetate buffer (pH 4.5). Urine specimens were hydrolyzed with 50 μL of *H. pomatia* β-glucuronidase (>5,000 units/mL of sample) followed by incubation at 70°C for 30 min. Blood specimens were sonicated for 15 min, and all specimens were centrifuged at 3000 rpm for 10 min. Strata-XC SPE columns were conditioned with sequential additions of 1 mL of 2% NH_4OH in ethyl acetate (elution solvent), 3 mL of methanol, 3 mL of DI H_2O and 1 mL of 0.1 M sodium acetate buffer (pH 4.5). Specimens were added to the columns and allowed to elute by gravity. The columns were sequentially washed with 2 mL of DI H_2O and 2 mL of 20% acetonitrile in 0.1 M sodium acetate buffer (pH 4.5), dried for 20 min under vacuum, treated with 2 mL of hexane wash and dried under vacuum again for 30 min. Analytes were eluted with 3 mL of elution solvent and dried under nitrogen at 40°C. Samples were reconstituted with 40 μL of a 4 : 1 acetonitrile/MTBSTFA w/1% TBDMCS derivatizing solution and transferred to autosampler vials with conical inserts. *tert*-Butyldimethyl-silyl derivatives were formed by flash derivatization in the injection port.

Opioid extraction

Specimens were extracted using the existing validated SPE quantitation procedure (12). A 2-mL aliquot of each specimen was prepared with 100 μL of internal standard solution. Blood specimens were precipitated with 3 mL of acetonitrile, vortexed, allowed to equilibrate for 5 min and centrifuged at 3000 rpm for

5 min. The supernatant was transferred to a clean tube, concentrated to ~1 mL under nitrogen at 55°C, then diluted with 2 mL of DI H_2O and 2 mL of 0.3 M phosphate buffer (pH 6.0). Urine specimens were hydrolyzed with 1 mL of HCl in an autoclave at 15 psi for 45 min, and then allowed to cool. The pH was adjusted to 6.0 ± 0.2 using KOH and 2 mL of 0.3 M phosphate buffer (pH 6.0). All blood and urine specimens were reacted with 250 μL of 10% hydroxylamine and incubated at 70°C for 15 min to form oxime derivatives of the keto opiates. Specimens were centrifuged at 3000 rpm for 5 min before addition to the SPE column. SPE columns (ZCDAU020) were conditioned with the sequential addition of 3 mL of methanol, 2 mL of DI H_2O and 2 mL of 0.3 M phosphate buffer (pH 6.0). Specimens were added to the columns and allowed to elute by gravity. Columns were sequentially washed with 2 mL of DI H_2O and 2 mL of 0.1 M acetic acid, dried for 2 min under vacuum, washed with 3 mL of methanol and dried again under vacuum for 10 min. Analytes were eluted with 3 mL of 2% NH_4OH in ethyl acetate : methanol (2 : 1) and dried under nitrogen at 50°C. Trimethylsilyl derivatives were formed using 50 μL of acetonitrile and 25 μL of BSTFA w/1% TMCS followed by incubation at 70°C for 35 min before being transferred to autosampler vials with conical inserts.

Synthetic cannabinoid extraction

Specimens were extracted using an existing validated liquid–liquid procedure. A 2-mL aliquot of each specimen was prepared with 50 μL of internal standard solution followed by hydrolysis using 1 mL of 0.5 M phosphate buffer (pH 6.8), 50 μL of *E. coli* β-glucuronidase (>1,250 units/mL of sample) and a 30-min incubation at 55°C. Samples were cooled to room temperature, 200 μL of HCl and 5 mL of chlorobutane were added, and the mixture rotated for 20 min and centrifuged at 3000 rpm for 5 min. The organic layer was transferred to a clean tube and evaporated under nitrogen at 55°C. Extracts were reconstituted in 50 μL of (50 : 50) mobile phase (10 mM ammonium formate : 20% methanol in acetonitrile with 0.1% formic acid) and transferred to polypropylene vials.

Instrumental analysis

Benzodiazepines were quantitated using an Agilent 6890 GC coupled with a 5975 MS detector (Palo Alto, CA, USA). Separation occurred using a Restek (Bellefonte, PA, USA) RTX-200 capillary column (30 m × 0.25 mm × 0.25 μm) with a 1-mL/min constant flow of helium. The injection port was operated at 295°C with a 2-μL injection and a 2 : 1 pulsed split (40 psi for 2.0 min). The oven parameters were as follows: 150°C (1.5 min), increased by 15°C per min to 265°C (held 1.2 min), increased 4°C per min to 280°C and increased 30°C per min to 320°C (held 4 min) for a 19.45 total run time. The detector was operated in the selected ion monitoring (SIM) mode with a 7.5-min solvent delay. The following ions were monitored for the analytes of interest and the respective internal standard: oxazepam (m/z 457*, 513, 459), lorazepam (m/z 491*, 513, 493), temazepam (m/z 357*, 283, 255) and oxazepam-d_5 (m/z 462*, 519); alprazolam (m/z 279*, 308, 204), nordiazepam (m/z 327*, 329, 328) and diazepam-d_5 (m/z 261*, 288); α-hydroxyalprazolam (m/z 381*, 383, 382) and α-hydroxyalprazolam-d_5 (m/z 386*, 388). The quantitation ions are indicated by asterisks.

Opioids were quantitated using an Agilent 6890 GC coupled with a 5973 MS detector. Separation occurred using an Agilent DB1-MS capillary column (30 m × 0.25 mm × 0.25 μm) with a 1-mL/min constant flow of helium. The injection port was operated at 250°C with a 2-μL injection and a 10 : 1 pulsed split (40 psi for 2.0 min). The oven parameters were as follows: 100°C increased by 18°C per min to 250°C, and increased 10°C per min to 300°C (held 5 min) for an 18.33-min run time. The detector was operated in the SIM mode with a 10.5-min solvent delay. The following ions were monitored for each drug and the respective internal standard: codeine (m/z 371*, 343, 372) and codeine-d_3 (m/z 374*, 346); morphine (m/z 429*, 430, 401) and morphine-d_3 (m/z 432*, 417); hydrocodone (m/z 386*, 297, 387) and hydrocodone-d_3 (m/z 389*, 374); hydromorphone (m/z 444*, 355, 429) and hydromorphone-d_3 (m/z 447*, 358); oxycodone (m/z 474*, 475, 459) and oxycodone-d_3 (m/z 477*, 462); oxymorphone (m/z 532*, 517, 533) and oxymorphone-d_3 (m/z 535*, 520). The quantitation ions are indicated by asterisks.

Synthetic cannabinoids were quantitated using an Agilent 1200 LC coupled with a 3200 ABSciex Q Trap MS-MS detector (Framingham, MA, USA) operated in positive electrospray ionization in the multiple reaction monitoring (MRM) mode. Separation occurred using a Phenomenex Gemini-C18 analytical column (4.6 mm × 150 mm ID × 3.0 μm). The mobile phases were 10 mM ammonium formate pH 4.5 (A) and 20% methanol in acetonitrile with 0.1% formic acid (B). A 10-μl injection with a 0.8-mL/min constant mobile phase flow rate was utilized with the following gradient: initial conditions of 25 : 75 (A : B), increased to 5 : 95 (A : B) over 5.0 min, held until 7.5 min and then transitioned back to 25 : 75 (A : B) by 7.75 min with a total run time of 8.0 min. The following MRM transitions were monitored: JWH 018-COOH (372.1/155.2, 372.1/127.2), JWH 073-COOH (358.1/155.2, 358.1/127.2), JWH 018-OH (358.2/155.2, 358.2/127.2) and JWH 073-COOH-d_5 (363.2/155.2, 363.2/127.2). Quantitations were performed using the first MRM transition.

Hydrolysis conditions

All specimens were extracted with and without the hydrolysis reaction to measure the total drug and free drug concentrations. While it is not possible to determine the exact level of conjugation using this method, it allowed for the calculation of an observed percent conjugation for each individual sample. The appropriate buffers were added with the omission of enzyme or acid and incubation. The opioid extraction was also performed with enzyme hydrolysis using *H. pomatia* β-glucuronidase to provide an enzyme hydrolysis comparison (10, 13–18). See Table II for hydrolysis conditions.

Hydrolysis conditions for abalone β-glucuronidase were evaluated using glucuronide controls and authentic urine specimens. A series of experiments were performed to analyze the pH, incubation time and incubation temperature to determine the optimum hydrolysis conditions. The sample pH in the benzodiazepine extraction was varied by half-step increments from pH 4.0 to 6.0. Hydrolysis at pH 4.5 yielded optimum results for all the analytes and was consistent with the existing literature utilizing pH 4.0 and 5.0 (9, 10, 19). The incubation temperature was evaluated at 40, 55 and 70°C. Both 55 and 70°C produced

Table II
Hydrolysis conditions

Assay	Reference hydrolysis	Evaluation hydrolysis 1	Evaluation hydrolysis 2
Benzodiazepines	1 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 50 µL of β-glucuronidase *H. pomatia* (>5,000 units) 30 min incubation at 70°C	1 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 50 µL β-glucuronidase abalone (>5,000 units) 30 min incubation at 70°C	
Opioids	2 mL specimen 1 mL concentrated HCl Autoclaved at 15 psi for 45 min	2 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 50 µL β-glucuronidase abalone (>2,500 units) 16 h incubation at 60°C	2 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 50 µL β-glucuronidase *H. pomatia* (>2,500 units) 16 h incubation at 60°C
Synthetic cannabinoids	2 mL specimen 2 mL 0.5 M phosphate buffer pH 6.8 50 µL β-glucuronidase *E. coli* (>1,250 units) 30 min incubation at 55°C	2 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 Sample pH adjusted to 4.5 ± 0.2 50 µL β-glucuronidase abalone (>2,500 units) 30 min incubation at 70°C	2 mL specimen 2 mL 0.1 M sodium acetate buffer pH 4.5 Sample pH adjusted to 4.5 ± 0.2 50 µL β-glucuronidase abalone (>2,500 units) 60 min incubation at 70°C

acceptable results, which are similar to other published incubation temperatures of 60 and 65°C (10, 11). Two incubation times, 30 and 60 min, were evaluated for both temperatures, and all the results were within 5% of both the target concentration and the *H. pomatia* β-glucuronidase hydrolysis result. The optimum benzodiazepine hydrolysis conditions were determined to be pH 4.5 at 70°C for 30 min with >5,000 units of enzyme per mL of sample. The pH was kept consistent for opioid analysis, but the incubation parameters were changed to 60°C for 16 h due to the literature reporting poor hydrolysis above 60°C for morphine-3-glucuronide (10). The hydrolysis conditions were re-examined for JWH 018 OH-glucuronide. It was found that 55 and 70°C produced similar results, with a slight improvement at 70°C. Urine specimens were initially incubated for 30 min at 70°C, but complete hydrolysis was not achieved. Further experimentation indicated that an hour incubation time was required for complete hydrolysis. Specimens were re-analyzed with incubation for 60 min and yielded increased hydrolyzed drug concentrations relative to concentrations obtained by the current hydrolysis method.

Results and discussion

Benzodiazepines

When analyzing the free and total analyte concentrations, it was identified that hydrolyzed samples containing alprazolam exhibited falsely elevated concentrations when compared with unhydrolyzed results. The increase in alprazolam concentration was only seen in specimens that contained α-hydroxyalprazolam and could not be replicated using unconjugated drug standards. Fu *et al.* (8) published results documenting the transformation of oxazepam to nordiazepam under various hydrolysis conditions. Owing to their study, additional urine specimens were examined in the full scan mode to investigate the potential of analyte transformation. Analysis showed the presence of additional ions in the hydrolyzed samples, so it is more probable that the production of an interfering compound is the source of the increased alprazolam concentration and not the transformation of α-hydroxyalprazolam to alprazolam. This elevation of the drug concentration needs to be investigated further for the source of the ion production.

Table I includes a summary of the data obtained in this study for each analyte of interest including concentration ranges, observed percent conjugation and relative percent hydrolysis. The benzodiazepine hydrolysis control was within 4% of the target concentration for oxazepam and lorazepam using abalone β-glucuronidase. Urine specimens containing lorazepam, oxazepam and temazepam with high levels of observed conjugation were successfully hydrolyzed using abalone β-glucuronidase as quantitative results were within 20% of the concentration obtained when using *H. pomatia* β-glucuronidase. One specimen with an α-hydroxyalprazolam concentration of 1,031 ng/mL and 98.5% observed conjugation was hydrolyzed within 20% for both alprazolam and α-hydroxyalprazolam. This indicates that β-glucuronidase derived from abalone is capable of hydrolyzing samples with high analyte concentrations that exhibit a high percentage of observed conjugation. Nordiazepam exhibited varying degrees of observed conjugation, 84.4 and 1.2%, but analyses of both specimens using abalone β-glucuronidase were within 20% of the current hydrolysis result. Nordiazepam conjugation variations may be explained by the presence of an amine glucuronide linkage that does not exhibit the stability observed in oxygen-linked glucuronides (3).

Opioids

Enzymatic hydrolysis of morphine-3-glucuronide present in the hydrolysis control was found to be similar for both *H. pomatia* and abalone β-glucuronidases and was within 5% of the result obtained using acid hydrolysis. These results are consistent with Romberg and Lee (10) who reported >90% hydrolysis of morphine-3-glucuronide (3 h, 60°C, 2 mL sample, 1100–1500 units of enzyme per mL) for both *H. pomatia* and abalone β-glucuronidases. Hydrolysis of hydromorphone-3-glucuronide was within the acceptable range (± 20% of the target concentration), whereas that of oxymorphone-3-glucuronide was −23.9% using *H. pomatia* β-glucuronidase and −20.6% using abalone β-glucuronidase.

Hydrolysis controls indicate that both enzymes are capable of cleaving the morphine-3-glucuronide linkage. Morphine-6-glucuronide has been identified to hydrolyze at a slower rate than morphine-3-glucuronide, but was not directly analyzed in this study (10, 13, 15, 17). It has been proposed that the cleavage of a glucuronide bound at a phenolic position, such as morphine-3-glucuronide, would more readily be cleaved and stabilized by resonance than a glucuronide bound to an alcoholic position as seen in morphine-6-glucuronide (10). Given this, the likely presence of morphine-6-glucuronide in the urine specimens could result in

reduced recoveries and therefore the high standard deviations seen in this study. The hydrolysis results for specimens containing codeine and hydromorphone also exhibited high standard deviations as summarized in Table I. Wang *et al.* (13) examined a commercial hydromorphone-3-glucuronide control in their study and found that *H. pomatia* β-glucuronidase was able to only hydrolyze 20% of the concentration when incubated at 60°C for 16 h with 5,000 units of enzyme per mL of specimen. Lin *et al.* (17) reported that enzymatic hydrolysis of codeine resulted in variable results (average 69.8%; 63.7–88.8%) when incubated at 55°C for 18 h with 5,000 units of *H. pomatia* β-glucuronidase. The results in the current study in combination with the previous findings suggest that the variability associated with enzyme hydrolysis using abalone and *H. pomatia* β-glucuronidases do not provide the accuracy necessary for forensic analysis (13, 17).

Synthetic cannabinoids

Upon initial analysis with *E. coli* β-glucuronidase, JWH 018-OH showed an average observed conjugation of 70.5 ± 24.1% and a range of 31.8–97.3% with the carboxylic acid metabolites not exhibiting conjugation. Observed conjugation was re-examined when it was determined that abalone β-glucuronidase hydrolysis displayed increased hydrolysis efficiency. The highest levels of conjugation observed when using abalone β-glucuronidase were 28.2% for JWH 073-COOH, 34.4% for JWH 018-COOH and 97.6% for JWH 018-OH. The levels of observed conjugation exhibited variation within each analyte as summarized in Table I. Sobolevsky *et al.* (5) and Chimalakonda *et al.* (6) suggest that JWH-018 5-hydroxypentyl metabolite and JWH-018 5-pentanoic acid metabolite would exhibit conjugation when excreted in urine. The increased hydrolysis efficiency of abalone β-glucuronidase permitted the identification of conjugation on the carboxylic acid metabolites not previously identified in this study using *E. coli* β-glucuronidase. Wohlfarth *et al.* (19) did not observe conjugation of the JWH 018 5-pentanoic acid metabolite and suggested that deconjugation may have occurred prior to analysis due to the instability of the ester glucuronide linkage. The level of variation in the current study may be due to the instability of the glucuronide linkage as previously reported (19).

The samples were hydrolyzed for 30 min and incomplete hydrolysis was observed for JWH 018-OH with 79.3 ± 12.1% hydrolysis recovery compared with result obtained using *E. coli* β-glucuronidase. JWH 018-COOH and JWH 073-COOH were hydrolyzed within 97.5 and 95.5% of the current procedures, respectively. Further analysis of the glucuronide control indicated that a longer incubation time was necessary to obtain complete hydrolysis using abalone β-glucuronidase. All specimens were hydrolyzed with a 60-min incubation time and resulted in increased hydrolysis for all analytes. The abalone hydrolysis results from the 60-min incubation time have been included in Table I. The extended incubation period produced improved hydrolysis results; JWH 018 *N*-(5-hydroxypentyl) β-D-glucuronide control was within 10% of the target concentration, the carboxylic acid metabolites showed increased hydrolysis of up to 35% and JWH 018-OH showed hydrolysis percentages ranging from 84.6 to 185%. The increased hydrolysis results indicated the need to re-examine the *E. coli* β-glucuronidase parameters. The hydrolysis control was readily hydrolyzed using *E. coli* β-glucuronidase, but the specimen that exhibited 185% recovery did not show maximum hydrolysis recovery until 2 h. Hydrolysis was not examined past 2 h due to the increased extraction time, so it cannot be determined if more analyte would have been cleaved past that point. The highest concentration obtained for the previously mentioned urine specimen using *E. coli* β-glucuronidase was 8.2 ng/mL, while abalone β-glucuronidase was able to hydrolyze 14.2 ng/mL of JWH 018-OH in 1 h. Wohlfarth *et al.* (19) found that 2,000 units of abalone β-glucuronidase incubated for 2 h at 55°C provided optimum hydrolysis of JWH 018 *N*-(5-hydroxypentyl) β-D-glucuronide. This equates to 20 units per μL of specimen. The current study found that 1-h incubation at 70°C with only 2.5 units per μL of specimen was able to achieve optimum hydrolysis.

Conclusion

The purpose of this study was to explore the efficiency of abalone β-glucuronidase and to compare it to current hydrolysis procedures. Abalone β-glucuronidase was able to sufficiently hydrolyze benzodiazepines of interest in specimens and controls within ± 20% of expected concentrations. Hydrolysis of commonly encountered opioids generated similar results when compared with hydrolysis with *H. pomatia* β-glucuronidase. Abalone β-glucuronidase was able to completely hydrolyze the morphine-3-glucuronide hydrolysis control, but did not perform as well for the two additional commercial glucuronide standards examined in this study. Abalone β-glucuronidase also provided poor hydrolysis results when used to hydrolyze hydromorphone and codeine in authentic urine specimens. Hydrolysis of select synthetic cannabinoids showed improved results over the current method. JWH 018 *N*-(5-hydroxypentyl) β-D-glucuronide was hydrolyzed within 10% of the target concentration. Analysis of authentic urine samples showed improved hydrolysis for all analytes examined resulting in increased analyte concentrations over current laboratory procedures. Abalone β-glucuronidase appears to be a viable low cost option for enzyme hydrolysis of benzodiazepines and synthetic cannabinoids.

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