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Variations in enzymatic hydrolysis efficiencies for amitriptyline and cyclobenzaprine in urine

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Abstract

A collaborative study was conducted to investigate discrepancies in recoveries of two commonly prescribed compounds, amitriptyline and cyclobenzaprine, in patient urine samples when hydrolyzed with different enzymes from different sources. A 2- to 10-fold increase in analyte recoveries was seen for patient samples hydrolyzed using a recombinant β-glucuronidase (IMCSzyme™) over samples hydrolyzed with β-glucuronidase from *Haliotis rufescens*. We report outcomes from four commercially available β-glucuronidase enzymes (IMCSzyme™, *Patella vulgata*, *Helix pomatia* and *H. rufescens*) on patient samples that tested positive for amitriptyline and cyclobenzaprine. Our results confirm reduced hydrolysis of glucuronides by β-glucuronidases isolated from mollusks, but near complete conversion when using the recombinant enzyme. Our premise is that systematic differences in hydrolysis efficiencies due to varying substrate affinity among enzyme subtypes potentially impacts accuracy and reliability of measurements.

Introduction

Amitriptyline and cyclobenzaprine are both tricyclic compounds with tertiary aliphatic amine groups that are subsequently metabolized to form quaternary ammonium-linked glucuronide (N^+-glucuronide). Amitriptyline is a tricyclic antidepressant that was first introduced in the 1950s (1). Amitriptyline is currently a widely used tricyclic antidepressant. Cyclobenzaprine is also a tricyclic compound, initially marketed under the name Flexeril™ in 1977 (2). The drug is a skeletal muscle relaxant commonly prescribed for acute neck and back pain as well as for fibromyalgia treatment (2). Owing to its high potential for abuse, cyclobenzaprine is monitored by many toxicology and pain management laboratories (3–8).

Cyclobenzaprine and amitriptyline are structurally related and hence metabolize similarly. Both can be demethylated, oxidated, hydroxylated at the aromatic carbons and glucuronidated (9–11). The glucuronides formed are quaternary ammonium-linked (Figure 1). Only trace levels of parent compound are found in urine due to the extensive metabolism. A pharmacokinetic study showed free unconjugated amitriptyline in urine at approximately 1% of the initial dose, while amitriptyline glucuronide accounts for 58% of the urinary metabolites at approximately 25% of the dose (9). Similarly, cyclobenzaprine N^+-glucuronide can be present in urine with concentrations up to 50% of the dose (10). Ideally, glucuronides could be targeted directly for monitoring amitriptyline or cyclobenzaprine use. Glucuronides typically require extended liquid chromatography run times, and exhibit poor recoveries with solid phase extractions and low ionization efficiencies with electronspray mass spectrometry. Hydrolysis of the glucuronides should lead to increased concentrations of the parent compounds, providing better sensitivity and accuracy for monitoring these drugs. Unfortunately, the quaternary ammonium-linked glucuronides are less common than primary or secondary glucuronides and have proven to be difficult to hydrolyze with β-glucuronidase enzymes isolated from mollusks (10, 12, 13).

After observing large discrepancies between samples hydrolyzed with *Haliotis rufescens* and IMCSzyme™, a more comprehensive study was conducted to monitor the hydrolysis of patient samples positive for cyclobenzaprine or amitriptyline with four different sources of β-glucuronidase enzyme: IMCSzyme™ (genetically engineered

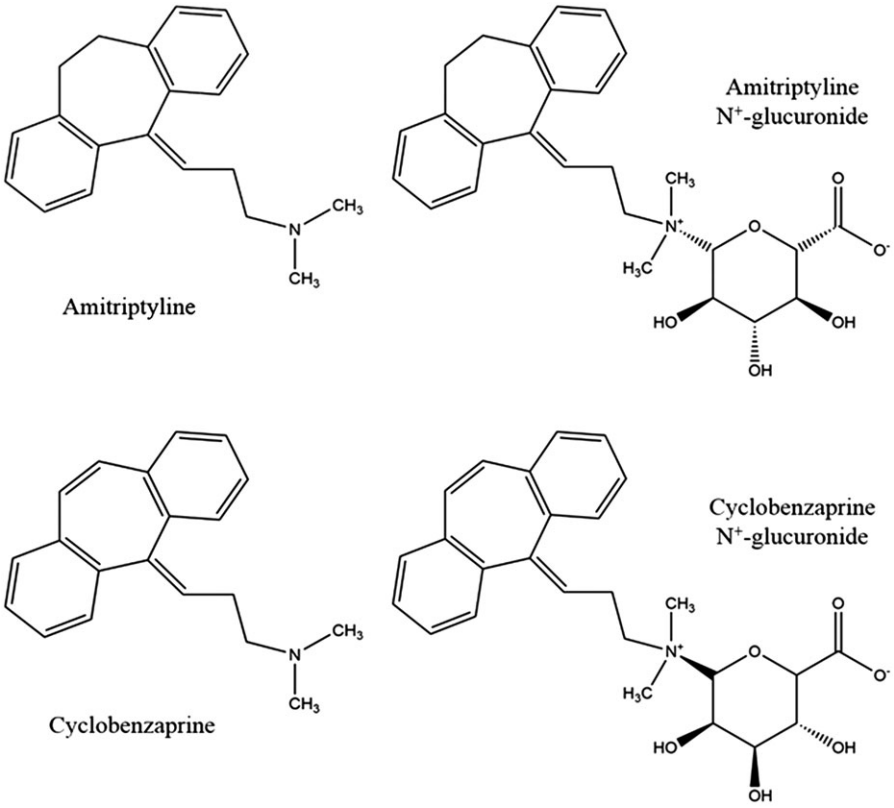

Figure 1. Structures of amitriptyline, cyclobenzaprine and their N^+-glucuronides.

Escherichia coli), *Patella vulgata* (limpet), *Helix pomatia* (Roman snail) and *H. rufescens* (red abalone). Patient samples were analyzed before and after hydrolysis with each of the enzymes and conversion of glucuronide conjugates being monitored by the increase in free (unconjugated) parent compound. The objective of this study was to evaluate the hydrolysis of quaternary ammonium-linked glucuronide metabolites with each of the named target enzymes.

Materials and Methods

Chemicals

Amitriptyline, amitriptyline-d_3 and cyclobenzaprine drug standards were purchased from Cerilliant Corporation (Round Rock, TX). The amitriptyline glucuronide standard, with a listed concentration of 1,000 ng/mL, was provided by TLC Pharmaceutical Standards (Ontario, Canada). Disposable pipette extraction (DPX) tips were provided by DPX Labs, LLC (Columbia, SC); the 1 mL RP-S tips contain styrene divinyl benzene sorbent with salt. IMCSzyme™ was obtained from Integrated MicroChromatography Systems, LLC (Columbia, SC). *Haliotis rufescens* β-glucuronidase was purchased from Campbell Science (Logan, UT). *Helix pomatia* and *P. vulgata* enzymes were purchased from Sigma Aldrich (St Louis, MO). All solvents were LC-MS Optima grade, from Fisher Scientific (Waltham, MA).

Specimens

Five urine samples positive for amitriptyline and five urine samples positive for cyclobenzaprine were obtained from PSO laboratory (Lansing, MI); all samples were anonymously coded. All 10 samples were analyzed in the initial method comparison study using *H. rufescens* (with results obtained by PSO Lab) and IMCSzyme™ (analyzed several days later). Further studies of the hydrolysis of spiked samples using other enzymes were conducted and presented herein. Patient samples, one positive for amitriptyline and one positive for cyclobenzaprine, were also analyzed using the various enzymes for direct comparison.

Sample Preparation

The following procedures were used for both the method comparison and the consequent enzyme hydrolysis comparison. Urine samples were prepared by aliquoting 200 µL into a 2 mL micro centrifuge tube (VWR, Radnor, PA). A master mix solution containing 10 µL of 1,000 ng/mL amitriptyline-d_3 in methanol, 50 µL of hydrolysis buffer and 40 µL of enzyme was added to the urine. Rapid hydrolysis buffer was provided by the vendor for IMCSzyme™ and 1 M sodium acetate at pH 4.5 was used for the other three enzymes. IMCSzyme™ had an activity of >50 kU/mL, while *H. pomatia, P. vulgata* and *H. rufescens* were >100 kU/mL. The following hydrolysis time and temperature were consistent with manufacturer specifications. Samples were heated and vortexed at 60 °C and 550 RPM for 30 min with IMCSzyme™ and 1 h for the other enzymes using a Labnet VorTemp™ 56 Benchtop Incubator/Shaker (Woodbridge, NJ).

Subsequent to hydrolysis, 500 µL acetonitrile was added to each sample, and disposable pipette extractions were performed by aspirating and dispensing the sample solutions in and out of the DPX (RP-S) tips. The extraction produces two distinct layers, aqueous solution containing salts on the bottom, and acetonitrile containing analytes of interest on the top. After transferring a 300 µL portion

of the top acetonitrile layer into a vial, and adding 700 µL water, the samples were ready for analysis.

Calibrators and Controls

All calibrators and controls were prepared in drug-free urine, obtained from healthy volunteers. The calibration design used for the method comparison study included eight concentration points at 0, 50, 100, 250, 500, 1,000, and 2,000 ng/mL of amitriptyline and cyclobenzaprine. Calibrations were done in duplicate to account for the difference in buffer pH needed for IMCSzyme™ (pH ~7.4) and *H. rufescens*, *P. vulgata* and *H. pomatia* (pH ~4.5).

Instrumental Analysis

Analyses were performed using a Thermo TSQ Vantage™ triple quadrupole mass spectrometer (Milwaukee, WI) coupled to an Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent Poroshell EC-C18 column (3.0 × 50 mm, 2.7 µm). Sample injections of 20 µL were made using a 6 port (0.25 mm) Cheminert C2V injection valve (Houston, TX) incorporated on a dual rail GERSTEL MPS autosampler (Linthicum, MD).

The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient started at 70% A for 0.5 min, ramped to 5% A at 3 min, held at 5% A at 3.5 min, and then changed back to 70% A. The total run time was 6 min. Eluent was diverted to waste during the intervals of 0–0.75 and 4.5–6 min after the injection. The column flow rate was 0.4 mL/min. Mass spectrometer parameters were set to the following values: electrospray voltage, 4,000 V; auxiliary gas pressure, 60 psi; sheath gas pressure, 47 psi; vaporizer temperature, 400°C; auxiliary gas temperature, 24°C; capillary temperature, 350°C. The selected ion pairs for multiple reaction monitoring (MRM) for each analyte can be found in Table I.

Results and Discussion

Comparative Study with 10 Patient Samples

Researchers at **the collaborating** PSO laboratory recognized a discrepancy between hydrolysis results of *H. rufescens* and IMCSzyme™ in a number of patient samples, and donated samples to the University of South Carolina for further investigation. Patient sample results comparing IMCSzyme™ and *H. rufescens* are summarized in Table II, showing the concentrations of the unhydrolyzed parent compound (amitriptyline/ cyclobenzaprine) and concentrations of the parent compound after hydrolysis (hydrolyzed). The amount of glucuronide conjugates can be calculated from hydrolyzed versus unhydrolyzed concentration of the parent compound. IMCSzyme™ and *H. rufescens* unhydrolyzed sample concentration should be the same. Differences, however, can potentially be attributed to hydrolysis in the two month storage time between analyses.

Table I. Ions selected for multiple reaction monitoring

Analyte	Ions	
	Precursor	Product
Amitriptyline	278	91.1, 233.0
Amitriptyline d_3	281	190.9, 233.0
Amitriptyline glucuronide	454.2	91.1, 233.0
Cyclobenzaprine	275.9	215.9, 231.0
Cyclobenzaprine glucuronide	452.2	215.9, 231.1

IMCSzyme™ and *H. rufescens* produced different hydrolyzed parent compound concentrations, ranging over almost an order of magnitude. For example, in patient sample 42,756, the hydrolyzed concentration of cyclobenzaprine using IMCSzyme™ was 4,153 ng/mL; the hydrolyzed concentration using *H. rufescens* was only 450 ng/mL. The amount of glucuronide conjugates hydrolyzed by the *H. rufescens* was consistently lower in all samples compared to that produced by IMCSzyme™. Thus, enzyme choice (*H. rufescens* for PSO, and IMCSzyme™ for this method) significantly impacted the amount of glucuronide converted. Consequently, further experiments were performed evaluate the ability of four commercially available β-glucuronidase enzymes to hydrolyze this unique class of glucuronides.

Glucuronides were monitored using the same product ions as the parent precursors shown in Table I. These measurements enabled qualitative assessment of the glucuronide decrease as a result of hydrolysis, and to monitor consistency of the relationship between the parent compound peak area increase and glucuronide peak area decrease as a result of hydrolysis. The ratio (Δ[Parent]/Δ[Glucuronide]), shown in Table III, can be employed to ascertain the likelihood that the increase in parent compound is a direct result of glucuronide decrease. If the ratio remains consistent throughout the patient samples, then it is likely that the parent compound peak area increase is a direct result of the glucuronide peak area decrease. However, if the ratios are highly variable, then it is likely that there is a confounding factor causing the parent compound increase. Ratios for IMCSzyme™ data were consistent (approximately 6 for cyclobenzaprine and 18 for amitriptyline) except when the glucuronide peak areas for unhydrolyzed compounds were low (~30,000 area counts). This occurred for patient samples 48,675 and 42,746, which were most likely outside the linear dynamic range. We therefore concluded that the observed parent compound peak intensity increases were a direct result of glucuronide hydrolysis.

Hydrolysis of Amitriptyline Glucuronide Standard in Drug Free Urine

Drug-free urine was spiked with the acquired amitriptyline glucuronide standard. However, the concentration of amitriptyline glucuronide standard that was provided was inaccurate for two reasons.

Table II. Comparison of patient sample cyclobenzaprine and amitriptyline concentrations (ng/mL) prior to hydrolysis (unhydrolyzed), and after hydrolysis (hydrolyzed) in after use of IMCSzyme™ and *Haliotis rufescens (red abalone)* (NF = not found)

Patient sample	*IMCSzyme™*		*Haliotis rufescens*	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
Cyclobenzaprine				
42,756	1,407	4,153	29	450
48,675	3,618	3,918	214	661
48,678	NF	NF	NF	87
48,735	298	2,985	81	170
49,187	167	2,715	33	182
Amitriptyline				
42,746	7	95	6	11
42,748	117	2,253	67	85
42,753	47	799	26	76
48,663	587	4,175	312	496
48,665	35	1,542	20	191

Table III. Comparison of peak areas for parent (cyclobenzaprine/amitriptyline) and glucuronide conjugates before and after hydrolysis with IMCSzyme™ as described in sample preparation. NF = not found, N/A = not applicable

Patient Sample	Cyclobenzaprine glucuronide		Cyclobenzaprine		Δ[Parent]/Δ[Glucuronide]
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed	
42,756	820,983	1182	2,904,217.4	7,440,808.8	5.5
48,675	31,197	NF	10,290,532	11,237,703	30.4
48,678	NF	NF	930.5	2,799.9	N/A
48,735	1,305,618	912	665,501.5	9,123,170.9	6.5
49,187	735,546	929	380,286	6,597,721.7	8.5
	Amitriptyline glucuronide		Amitriptyline		
42,746	31,269	NF	5,114	308,288	9.7
42,748	372,287	4,938	480,017	6,824,697	17.3
42,753	162,822	1,823	199,265	3,072,325	17.9
48,663	696,569	11,806	2,158,599	15,531,667	19.5
48,665	247,575	233	133,743	4,702,824	18.5

After "complete" hydrolysis (where no glucuronide peak was detected), the yield of amitriptyline did not match the manufacturer's specified amitriptyline glucuronide concentration. Further, amitriptyline was also present in the glucuronide standard solution, thus suggesting degradation had occurred. Therefore, calibration for product amounts was not conducted, and the peak areas of both compounds were monitored before and after hydrolysis.

The four sources of β-glucuronidase chosen here include a genetically engineered β-glucuronidase and three different types of sea snail sources. The goal of this work was not to find the optimum hydrolysis conditions for different enzymes, but rather, to compare their relative hydrolysis efficiencies. For that reason, the manufacturer recommendations for hydrolysis conditions were followed as described in the Experimental section.

Average peak areas for amitriptyline and amitriptyline glucuronide before and after hydrolysis with each of the enzymes are shown in Table IV. Percent hydrolysis was calculated as

$$\% \text{ hydrolysis} = (1 - A/B) \times 100\%$$

where A is the average ratio ($n = 3$) of amitriptyline glucuronide peak area to internal standard peak area for post-hydrolysis, and B is the average ratio ($n = 3$) of amitriptyline glucuronide peak area to internal standard peak area for pre-hydrolysis.

IMCSzyme™ achieved 99.3% hydrolysis in 30 min of incubation. The three mollusk enzymes (*H. pomatia*, *H. rufescens* and *P. vulgata*) produced 6.3%, 23.2% and 31.4% hydrolysis, respectively, under 1 h of incubation. The incubation times employed are based on manufacturer recommendations and common laboratory procedures for the respective enzymes. Amitriptyline glucuronide peak area decrease and amitriptyline peak area increase, when comparing pre- and post-hydrolysis chromatograms for each enzyme, as shown in Table IV.

Table IV. Average peak areas ($n = 3$) before and after hydrolysis with *Helix pomatia, Haliotis rufescens, Patella vulgata* and IMCSzyme™ and the subsequent calculated percent hydrolysis.

	Average peak area			% Hydrolysis
	Am. Gluc.	*Amitriptyline*	*Am. Gluc./I.S.*	
Helix pomatia				
Pre-hydrolysis	126,093.1	11,987.6	0.293	**6.3**
Post-hydrolysis	123,873.1	34,786.1	0.274	
Haliotis rufescens				
Pre-hydrolysis	116,697.4	13,315.4	0.255	**23.2**
Post-hydrolysis	92,136.2	96,844.6	0.196	
Patella vulgata				
Pre-hydrolysis	112,592.8	12,715	0.244	**31.4**
Post-hydrolysis	76,658.2	137,821.6	0.168	
IMCSzyme™				
Pre-hydrolysis	107,857.2	16,285.1	0.194	**99.3**
Post-hydrolysis	500.3	356,913.9	0.001	

Hydrolysis of Patient Samples by Four Different Enzymes

Further experiments to estimate the percent glucuronidation in actual patient samples were performed for the same four enzymes. In this case, however, the percent glucuronidation was calculated based on monitoring changes in the concentration of the parent compound (amitriptyline or cyclobenzaprine). The calculated percent glucuronidation for each enzyme can be compared to average ratios reported in the literature for parent and glucuronide metabolites in human urine. We assume that, if calculated glucuronidation percentages are similar to reported averages, then the enzyme has likely converted close to all of the available glucuronide.

The patient samples chosen for comparison of hydrolysis capability were patient sample 49,187 (positive for cyclobenzaprine) and 42,748 (positive for amitriptyline). Samples ($n = 2$) were analyzed for amitriptyline or cyclobenzaprine before hydrolysis (unhydrolyzed) and after hydrolysis (hydrolyzed) for each of the four enzymes. The N^+-glucuronide metabolites of cyclobenzaprine and amitriptyline were resistant to hydrolysis with *H. pomatia, P. vulgata* and *H. rufescens*, but not with IMCSzyme™.

Table V shows total parent compound (amitriptyline/cyclobenzaprine) concentration (ng/mL) prior to hydrolysis ("unhydrolyzed") and after hydrolysis ("hydrolyzed") with each of the four β-glucuronidase enzymes. Based on these values, the percent glucuronidation is calculated as the percentage of glucuronide metabolite compared to the amount of total parent compound after hydrolysis, and represents the relative amount of hydrolyzed glucuronide produced by each enzyme. Hence, the percent glucuronidation increases with the increase in hydrolysis of the glucuronide metabolites.

Table V. Amitriptyline and cyclobenzaprine concentrations (mean ± standard deviation, ng/mL) ($n = 2$) post-hydrolysis for each enzyme and the calculated percent glucuronidation based on concentrations of parent drug pre-hydrolysis (N/A = not applicable).

	Total parent concentration	% Glucuronidation
Amitriptyline		
Unhydrolyzed	88.4 +/– 2.1	N/A
IMCSzyme™	1,892.6 +/– 21.4	95.3
Helix pomatia	209.3 (one rep)	57.8
Patella vulgata	245.8 +/– 2.8	64.0
Haliotis rufescens	271.9 +/– 3.1	67.5
Cyclobenzaprine		
Unhydrolyzed	144.4 +/– 10.9	N/A
IMCSzyme™	2,322.6 +/– 16.1	93.8
Helix pomatia	300.9 (one rep)	52.0
Patella vulgata	523.7 +/– 3.6	72.4
Haliotis rufescens	532.8 +/– 3.7	72.9

Of the four enzymes tested, IMCSzyme™ converted the highest amount of amitriptyline N^+-glucuronide during hydrolysis (95.3%). The other enzymes exhibited glucuronidation percentages ranging from 57.8 to 67.5%. The glucuronidation percentage with IMCSzyme™ hydrolysis is consistent with reported amitriptyline and amitriptyline glucuronide concentrations from metabolism studies in the literature. Using a hot alkali treatment (2 M sodium hydroxide at 100°C for 15 min), Dahl-Puustinen *et al.* (14) found free amitriptyline concentration in urine to be 0.45 ± 0.46% of total dose, while amitriptyline glucuronide was found to be 8.2 ± 3.2% of total dose. These values represent an average of 94.8% glucuronidation. Other studies report that excreted amitriptyline N^+-glucuronide is 5–10% of the administered dose (10), and that free amitriptyline constitutes 0.2–0.4% of the dose (10). These results imply 92–98% binding of glucuronide conjugates. Lastly, Rana *et al.* (1) reported an average of 90% glucuronidation for amitriptyline in urine using K12 β-glucuronidase from *E. coli* at 52°C for 1 h.

Our results show that the concentration of cyclobenzaprine post-hydrolysis with IMCSzyme™ was much higher than the post-hydrolysis cyclobenzaprine concentrations for the other enzymes. The IMCSzyme™ post-hydrolysis concentration of cyclobenzaprine was 2,322.6 ± 16 ng/mL, while the concentration of free cyclobenzaprine in the unhydrolyzed sample was 144.4 ± 11 ng/mL, which correlates to a glucuronidation percentage of 93.8%. The other three enzymes had percentages that ranged from 52.0 to72.9%. The IMCSzyme™ glucuronidation percentage is comparable to the free cyclobenzaprine and cyclobenzaprine glucuronide ratios found in the literature. Hucker *et al.* (11) reported 1.7% of metabolites in urine as free cyclobenzaprine and 34.0% was attributed to the N^+-glucuronide during the first 24 h after administration—an average glucuronidation percentage of 95.2%. Between 24 and 48 h 1.1 ± 0.6% of metabolites in urine was free cyclobenzaprine, and 24.1 ± 6.9% was cyclobenzaprine glucuronide, corresponding to an average glucuronidation percentage of 95.6%. Hucker's study was carried out over 5 days. Cyclobenzaprine was present for the first 48 h while cyclobenzaprine glucuronide was present for all 5 days. High hydrolysis efficiency from IMCSzyme™ for cyclobenzaprine glucuronide increases sensitivity and provides a longer window of detection for the parent compound.

We employed drug-free urine spiked with amitriptyline glucuronide standard to provide known reference samples for testing hydrolysis efficiency for the four different enzymes. Percent hydrolysis ranged from 6.3% with *H. pomatia* in 1 h to 99.3% with IMCSzyme™ in 30 min. This amitriptyline glucuronide standard hydrolysis study was performed at a relatively low level (~1,000 ng/mL) of amitriptyline glucuronide at which the enzymes perform optimally. However, patient samples often have a much greater glucuronide concentration. Thus, when comparing the post-hydrolysis glucuronide amounts in both the patient sample study and the glucuronide standard study, the three snail sourced enzymes performed more poorly. For example, *P. vulgata* hydrolyzed approximately 30% of the amount that IMCSzyme™ did in the low glucuronide spiked urine. However, in patient samples, *P. vulgata* only hydrolyzed approximately 13% of that hydrolyzed by IMCSzyme™ (based on post-hydrolysis amitriptyline concentrations). Use of IMCSzyme™ provided ratios of parent compound to glucuronide most consistent with the literature reports of average human excretion patterns (6, 10, 11). The other three enzymes convert less amitriptyline and cyclobenzaprine glucuronide, even under more rigorous conditions (i.e. longer hydrolysis time and higher activity). This variation of hydrolysis based on enzyme source for N^+-glucuronides is consistent with previous literature. Kowalczyk *et al.* compared the ability of three different enzymes (bovine liver, *E. coli* and *H. pomatia*) to hydrolyze two cyclic and two acyclic quaternary ammonium-linked glucuronide metabolites. This work showed that the enzymes hydrolyzed the compounds to different degrees and adjustment of buffer pH and amount of enzyme did not achieve complete hydrolysis (13).

Conclusion

Although variation in hydrolysis of these quaternary ammonium-linked glucuronides with enzyme source is evident, the high degree of hydrolysis with IMCSzyme™ provides a reliable β-glucuronidase source for amitriptyline and cyclobenzaprine N^+-glucuronide hydrolysis. Hydrolysis efficiencies may vary due to many factors, including impurities in the enzyme solution, and/or differing binding affinities for this specific class of glucuronides. Large discrepancies in the hydrolysis efficiency of different enzymes is of great practical significance due to the potential for incomplete hydrolysis of samples. Insufficient hydrolysis results in limited sensitivity and positive samples may fall below limits of detection and/or limits of quantitation. Results presented here demonstrate that enzyme choice and/or optimal hydrolysis conditions should be evaluated specifically for N^+-glucuronides. Other combinations of N^+-glucuronide metabolites of different drugs (30+ known) and sources of β-glucuronidase may produce different hydrolysis results.

For both amitriptyline and cyclobenzaprine, *H. pomatia, P. vulgata* and *H. rufescens* produced lower hydrolysis efficiencies for amitriptyline N^+-glucuronide and produce glucuronide percentages in patient samples well below literature reported human metabolism ratios of these drugs. IMCSzyme™ hydrolysis resulted in calculated glucuronidation percentages closer to the literature averages in half the hydrolysis time. Based on the data reported, we conclude that IMCSzyme™ is more efficient at hydrolyzing these N^+-glucuronide metabolites (at the conditions tested) and could improve the sensitivity of the analysis of these compounds.

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