The Importance of pH in β-Glucuronidase Hydrolysis Efficiency

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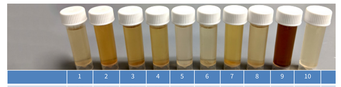
ABSTRACT

- Different enzymes have different optimal hydrolysis pH
- pH optima are substrate dependent
- Urine samples have a range of pH 4.0-9.0 and require buffers to adjust pH for the most efficient hydrolysis
- Comparison of two β-glucuronidase hydrolysis efficiencies at their optimal pH in both fortified synthetic urine and authentic patient urine samples

INTRODUCTION

β-glucuronidase is an enzyme commonly utilized in clinical and forensic labs to convert drug metabolites present in biological fluids into their parent compounds for better detection and quantification. In the case of urine drug testing, urine samples are highly heterogeneous and range from pH 4.6 to pH 8.0 (1), though pH may be outside this range due to the effects of disease or medication. Authentic patient urine samples in ***Figure 1*** represent two sets of pH, acidic (pH < 5.0) and basic (pH > 8.5). Urine chemical properties cannot be predicted by visual inspection.



	1	2	3	4	5	6	7	8	9	10
pH	4.2	4.2	4.5	4.5	4.9	8.7	8.7	8.8	8.9	9
Creatinine	45	110	64	82	34	112	116	61	<5	25
Specific Gravity	1.012	1.01	1.013	1.022	1.015	1.018	1.02	1.013	1.015	1.009

Figure 1. *Heterogeneity of urine samples. Physical characteristics do not correlate to any chemical properties.*

Different enzymes have different requirements for hydrolysis, such as incubation temperature, incubation time, hydrolysis buffer pH, etc. This poster shows the importance of pH adjustment in the glucuronide hydrolysis step. Two β-glucuronidases, IMCSzyme® and purified from abalone, are used to demonstrate distinct hydrolysis pH requirements.

METHODS

β-glucuronidase activity was measured colorimetrically using Fishman's phenolphthalein-glucuronide assay (2). The buffer system used to test pH effect was made from acetic acid, MES, and Tris; pH was adjusted with either HCl or NaOH. The pH profile of each enzyme was determined by testing enzyme activity at room temperature over a pH range from 4.5 to 8.0 in 0.5 increments.

To measure drug glucuronide hydrolysis efficiency, synthetic urine was fortified with 500 ng/mL of nine drug metabolites, including opiates, benzodiazepines, and tricyclic antidepressants. A master mix was prepared for each glucuronidase containing 30 µL of enzyme, 100 µL of buffer, and 10 µL of internal standards in methanol. The hydrolysis buffer was prepared from acetic acid, MES, and Tris. IMCSzyme was tested at pH 6.0, 7.0 and 8.0, while purified abalone was tested at pH 4.5, 5.5, and 6.5. 100 µL of spiked urine was incubated with each master mix in triplicate at room temperature for 60 minutes. Hydrolyzed samples were then extracted and eluted using DPX WAX/RP tips and analyzed on a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Endura™ Triple Quadrupole Mass Spectrometer using a Phenomenex Kinetex® 2.6 µm Phenyl-Hexyl 100 Å, 50 x 4.6 mm LC column. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.

To compare enzyme hydrolysis efficiency in fortified urine and authentic urine at the optimal pH, the protocol above was repeated, but incubation was carried out at 55°C instead. The hydrolysis buffer was changed to the vendor-recommended buffer system for each enzyme, which is Rapid Hydrolysis Buffer for IMCSzyme and sodium acetate buffer pH 4.5 for purified abalone.

RESULTS

Chromogenic assay using phenolphthalein glucuronide showed different pH optima for IMCSzyme and purified abalone. The >50% activity range for IMCSzyme falls from pH 5.5 to 8.0, while purified abalone has <50% activity at pH above 4.5 (***Figure 2***).

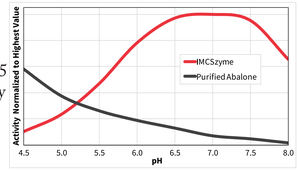


Figure 2. *Activity measured by the hydrolysis of phenolphthalein glucuronide by each β-glucuronidase at pH ranging from 4.5 to 8.0. The relative activity is normalized to each enzyme's highest activity.*

We extended our study to pH effects on the hydrolysis of drug-glucuronides. The chemical structures of nine glucuronidated drugs are shown in ***Figure 3***. In order to observe kinetics of hydrolysis, some experiments were performed at room temperature incubation and at lower enzyme concentration than typically used in the clinical setting.

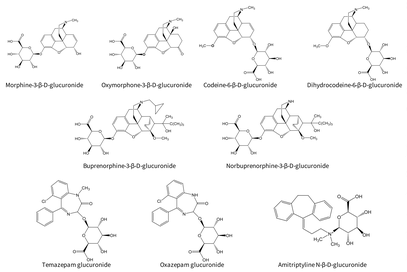


Figure 3. *Chemical structures of 9 glucuronidated drugs used to observe pH-dependent urine hydrolysis.*

Optima determined for IMCSzyme and purified abalone were pH 7.0 and 4.5, respectively, so the hydrolysis experiment tested short ranges with the optima in the middle. However, purified abalone has low pH optima at 4.5 and it is difficult to pH urine below pH 4.0, the pH range tested was +1.0 and +2.0 pH units from the optimal value. **IMCSzyme maintains activity at pH 8.0 with only 5% decrease in hydrolysis efficiency, whereas purified abalone significantly decreases over 30% if pH increases from 4.5 to 5.5.**

Enzyme activity towards phenolphthalein glucuronide does not correlate strongly to urine drug hydrolysis, and the correlation varies drug to drug. Each substrate is affected by pH differently, and this is due in part to their chemical structures (***Figure 3***).

IMCSzyme showed a mixed trend while purified abalone has optimal hydrolysis efficiency on most of the glucuronidated drugs at pH 4.5 (***Figure 4***). However, IMCSzyme has a broader pH active range since the ± 1.0 pH unit shift only caused 5-10% loss in overall activity, compared to >33% loss in purified abalone. The exception to the low pH trending of purified abalone is amitriptyline N-β-D glucuronide, which hydrolyzes faster at higher pH. The same trend for amitriptyline is observed when using IMCSzyme. The protonation of nitrogen at the N-linkage may facilitate glucuronide bond cleavage.

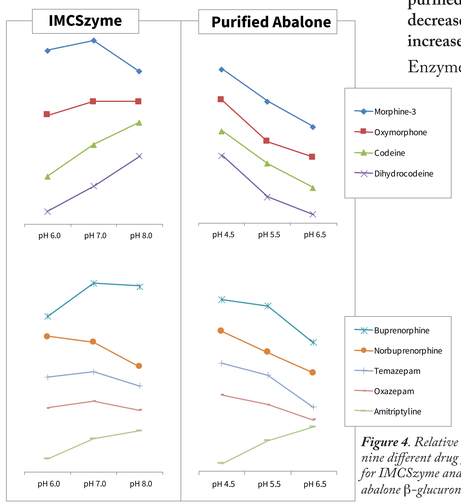


Figure 4. *Relative hydrolysis efficiencies towards nine different drug glucuronides at pH 6.0 – 8.0 for IMCSzyme and at pH 4.5 – 6.5 for purified abalone β-glucuronidase.*

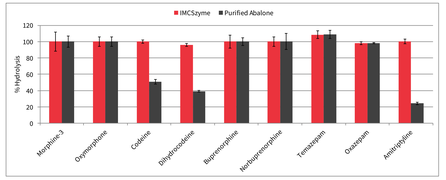


Figure 5. *Percent hydrolysis efficiencies of IMCSzyme and purified abalone at their respective pH optima. 30 µL of each enzyme was added to 100 µL of urine and incubated at 55°C for 30 minutes.*

The two enzymes were compared side by side using their optimal pH conditions at 55°C instead of room temperature. Hydrolysis in synthetic urine was performed using Rapid Hydrolysis Buffer, pH 7.4 (RHB) for IMCSzyme and 0.2 M acetate buffer, pH 4.5 for purified abalone. Using the same volume of enzyme, IMCSzyme yielded higher hydrolysis recovery for codeine, dihydrocodeine, and amitriptyline (***Figure 5***).

Ten representative patient urine samples shown in ***Figure 1*** were buffered to an acceptable range ± 0.5 pH unit of optimal pH. Since IMCSzyme has optimal activity at both pH 7.0 and 8.0 with less than 5% difference, the acceptable range is pH 6.5 to 8.5 and this is achieved by the addition of RHB. Purified abalone has an optimal activity at pH 4.5 so the acceptable range is pH 4.0 to 5.0. pH adjustment was achieved by the addition of 0.2 M sodium acetate pH 4.5. ***Figure 6*** illustrates that it takes 3 times the volume of urine to reach an acceptable pH range for IMCSzyme, whereas up to 5 times the volume to reach an acceptable pH range for purified abalone.

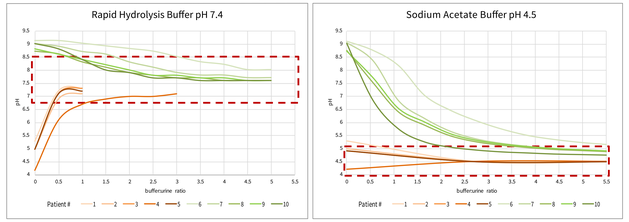


Figure 6. *pH adjustment of ten patient urine samples shown in **Figure 1**, using Rapid Hydrolysis Buffer (RHB) for IMCSzyme or 0.2 M sodium acetate pH 4.5 for purified abalone. Red box shows pH ranges for optimal hydrolysis efficiency. Since IMCSzyme shows comparable overall efficiency at pH 7.0 and 8.0, the optimal pH range is 6.5 to 8.5. However, purified abalone shows higher hydrolysis efficiency at pH 4.5, the optimal pH range is 4.0 to 5.0.*

Because urine samples are highly heterogeneous, we studied hydrolysis recovery over time in eighty four authentic patient urine samples known to be positive for codeine and buprenorphine. Using the same volume of enzyme and their respective optimal buffers, IMCSzyme showed higher hydrolysis efficiency in all patient urine samples. ***Figure 7*** shows calculated drug amounts plotted in a log scale recovered from hydrolysis with IMCSzyme or purified abalone. Average efficiency of purified β-glucuronidase from abalone was 50% of IMCSzyme for codeine and 42% for buprenorphine. Three patients were also positive for amitriptyline and purified abalone only recovered an average of 25% compared to IMCSzyme. Although each enzyme is used at their optimal pH, different enzymes demonstrate different hydrolysis efficiencies towards glucuronidated drugs.

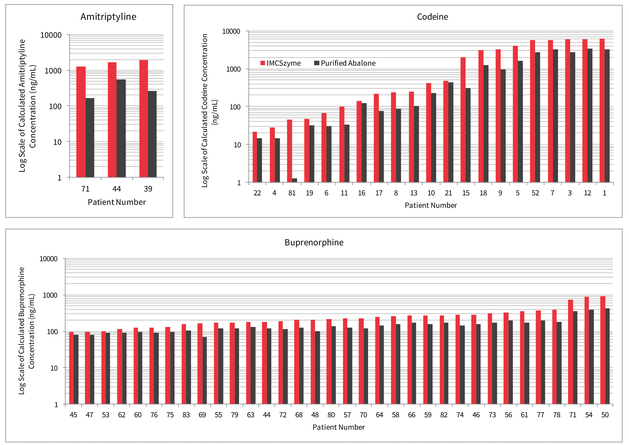


Figure 7. *Eighty four patient urine hydrolysis at each enzyme's optimal pH, incubated for 30 minutes at 58°C. Calculated drug concentration is expressed in log scale.*

CONCLUSIONS

- Choosing the right β-glucuronidase and buffer is an important step for complete glucuronide hydrolysis and accurate results.
- Every enzyme has a different pH optimum and range. Because urine samples are highly heterogeneous, buffering to an optimal pH is essential for highest enzyme activity.
- pH adjustment to neutral is easier than to acidic pH at the end of pH spectrum.
- IMCSzyme maintains activity at pH 8.0 with only 5% decrease in hydrolysis efficiency, whereas purified abalone significantly decreases over 30% if pH increases from 4.5 to 5.5.
- Despite using the optimal pH, each enzyme has different catalytic activity towards different glucuronidated substrates. Data from 84 patient urine samples shows that compared to IMCSzyme, purified abalone β-glucuronidase recovered an average of 50% for codeine, 42% for norbuprenorphine and 25% for amitriptyline.

REFERENCES

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(2)Talalay, P.; Fishman, W. H.; Huggins, C. (1946) Chromogenic substrates; phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. The Journal of biological chemistry, 166 (2), 757-72.

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