Enzyme Activities (Fishman units) Correlate Poorly with Hydrolysis Efficiencies

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ABSTRACT

- Activity of five different β-glucuronidases was tested via a phenolphthalein-glucuronide chromogenic assay.
- Hydrolysis efficiency of ten drugs was monitored for all five β-glucuronidases at five time points.
- Activity level measured with phenolphthalein-glucuronide did not correlate well with drug hydrolysis efficiency.

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. Since Fishman et al. introduced a quick and simple way to determine β-glucuronidase activity in the 1940s, the phenolphthalein-glucuronide chromogenic assay has been universally accepted and performed to indicate β-glucuronidase activity. By definition, one Fishman unit of activity liberates 1 µg of phenolphthalein per hour at 38°C at pH 4.5.[1] However, the phenolphthalein-glucuronide assay temperature and pH conditions may be modified to suit a particular glucuronidase whose characteristics may differ due to its source and/or genetic modification. Commercial β-glucuronidase vendors test and report activity in Fishman units per milliliter. It is generally accepted that glucuronidases with higher measured activity will perform drug hydrolysis more efficiently. To challenge this hypothesis, four commercially available β-glucuronidases and one non-commercially available β-glucuronidase construct were compared in this study.

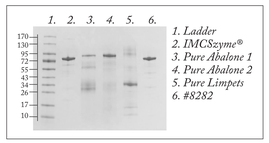


***Figure 1**. One microgram of each β-glucuronidase was loaded onto a gel for SDS-PAGE.*

METHODS

Activity of each glucuronidase was monitored over three nonconsecutive days using protocols based on Fishman's phenolphthalein-glucuronide chromogenic assay. The activity protocols performed were specific to each vendor using respective buffers, pH, and temperature. The three activities for each enzyme were averaged and compared to vendor-specified activity values. The effects of ethanol percentage on calculation of activity was evaluated, and the pH profile of each enzyme was determined by testing each enzyme's activity at 37°C over a range of buffers with pH values from 3.8 to 8.8.

For the hydrolysis efficiency test, drug-free synthetic urine was spiked with 1000 ng/mL of ten drug metabolites, including opiates, benzodiazepines, and tricyclic antidepressants. A master mix was prepared for each glucuronidase containing 20 µL of enzyme, 40 µL of buffer, 20 µL of water and 10 µL of internal standards in methanol. 80 µL of spiked urine was incubated with each master mix in triplicate at 58°C for time periods of 0, 15, 30, 45 and 60 minutes. 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. The extracted samples were run on two separate methods.

Enzyme	Source	Protein (mg/mL)	Vendor-specified enzyme activity (U/mL)
IMCSzyme	Recombinant/Genetically modified *Escherichia coli*	0.90	> 50,000
Pure Abalone 1	Purified *Haliotis rufescens (Abalone)*	0.77	> 50,000
Pure Abalone 2	Purified *Haliotis rufescens (Abalone)*	0.24	≥ 100,000
Pure Limpets	Purified *Patella vulgata (Limpets)*	0.58	100,000 – 200,000
#8282	Construct #8282 expressed in *Escherichia coli*	1.05	Not commercially available

Table 1. The five different β-glucuronidases analyzed in this study

Method A	Method B[2]
Mobile Phase A: 0.1% formic acid in water	Mobile Phase A: 10 mM ammonium bicarbonate with 5% methanol in water, pH 10
Mobile Phase B: 0.1% formic acid in acetonitrile	Mobile Phase B: Methanol
Gradient:	Gradient:

Min.	%A	%B
0	95	5
0.5	95	5
3	5	95
3.8	5	95
4	95	5
6	95	5

Min.	%A	%B
0	60	40
2	60	40
10	10	90
12	10	90
12.1	60	40
14.0	60	40

Table 2. LC methods used to analyze the hydrolyzed samples

RESULTS

***Figure 2**. Graph of average activities collected over three days*

Enzyme	Activity (U/mL)	Std Dev	Vendor-specified enzyme activity (U/mL)	Temp (°C) of activity assay	pH in activity assay
IMCSzyme	82702	10557	> 50000	25	6.8
Pure Abalone 1	45416	5421	> 50000	37	4.5
Pure Abalone 2	113731	5367	≥ 100000	37	3.8
Pure Limpets	88484	534	100000 – 200000	37	3.8
#8282	398290	24643	N/A	25	5.2

Table 3. Results and conditions of each enzyme assay

As seen in Table 3, the average activities of Pure Abalone 1 and Pure Limpets were slightly below the vendor-specified range. The #8282 glucuronidase had the highest measured activity at 4- to 8-fold greater than the other enzymes, while Pure Abalone 1 had the lowest. Pure Abalone 1, 2 and Pure Limpets were tested at 37°C while IMCSzyme and #8282 were tested at 25°C according to vendor protocols.

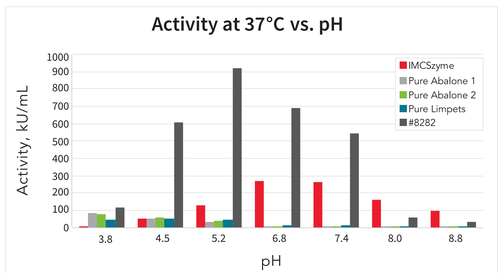


***Figure 3**. pH profiles of each enzyme tested at 37°C*

Figure 3 demonstrates the optimal pH values for each enzyme. pH is a critical factor to consider when performing urine drug hydrolysis with a β-glucuronidase since patient urine samples are found to be within a wide range of pH values, typically from approximately pH 4.6 to 8.0, and may vary outside this range due to the effects of disease or medication.[3] As seen in ***Figure 3***, Pure Abalone 1, 2 and Pure Limpets are virtually inactive at pH values over 5.2. IMCSzyme is completely inactive at pH 3.8, while this pH is optimal for enzymes purified from abalone and limpets.

Calculating enzyme activity requires a phenolphthalein (PT) standard curve in order to convert the absorbance values into concentrations of product. A colorimetric product such as PT typically contains aromatic rings and addition of an alcohol facilitates its solubility in solution. Although the addition of the co-solvent like ethanol or methanol helps solubilize PT, depending on the percentage of the co-solvent, the slope of the PT curve will vary, as seen in ***Figure 4***. When two PT curves were prepared in either 1% or 10% ethanol, the slopes varied such that the calculated enzyme activity would differ by 25% depending on the concentration of ethanol present in the PT standards.

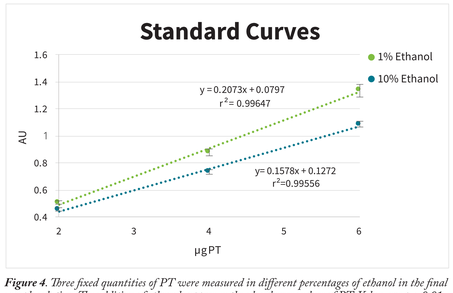


***Figure 4**. Three fixed quantities of PT were measured in different percentages of ethanol in the final sample solution. The addition of ethanol suppresses the absorbance values of PT. Values are $p < 0.01$ using a two-tailed T-test.*

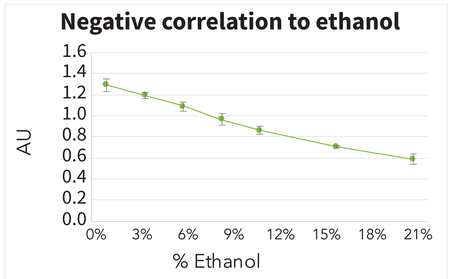
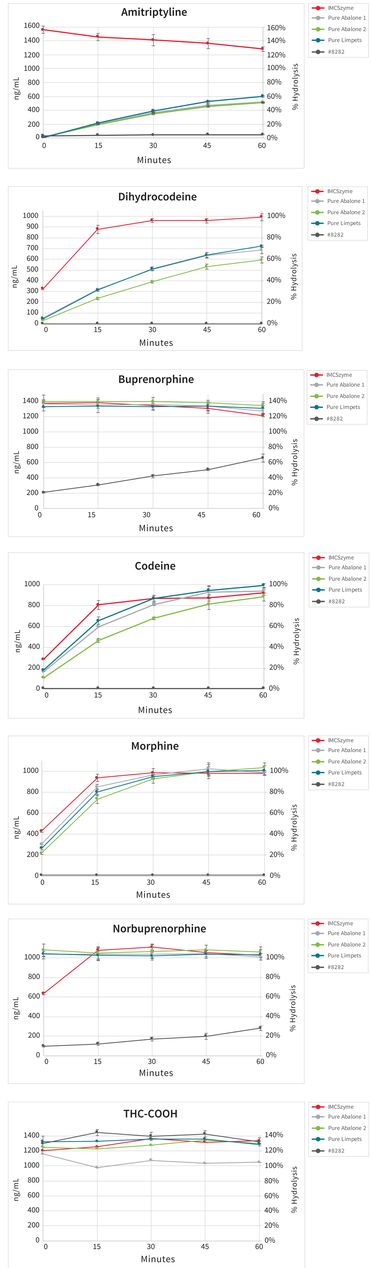
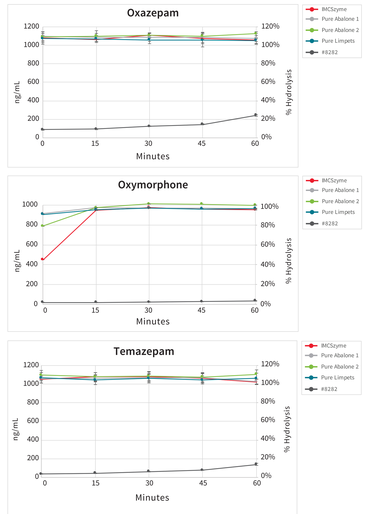


***Figure 5**. Graph demonstrating the linear decrease in phenolphthalein absorbance with increasing percentage of ethanol*

Multiple factors will always impact the enzyme and its hydrolysis efficiency and it is important to establish a standardized unit of measure. The conditions at which enzyme activity is determined are often not clearly defined, as indicated with β-glucuronidase and its standardized Fishman unit. And more often, the conditions that affect the enzyme have a contrasting effect on the substrate, such as with phenolphthalein in the presence of ethanol. β-glucuronidases can hydrolyze more effectively in 10% ethanol, as indicated by higher absorbance values from phenolphthalein-glucuronide measurements. However, the absorbance intensities are decreased by increasing amount of ethanol for phenolphthalein (***Figure 5***). This suggests that a correction factor should be applied to the enzyme activities when using different percentages of organic solvents in order for a more accurate comparison of enzyme activities.





***Figures 6–15**. Graphs comparing drug hydrolysis efficiency of the five β-glucuronidases*

Though the #8282 enzyme had the highest measured phenolphthalein-glucuronide activity by far, it had the lowest rate of hydrolysis, with little to no hydrolysis of amitriptyline, codeine, dihydrocodeine, morphine, oxymorphone and temazepam glucuronides. IMCSzyme showed a significantly higher rate of dihydrocodeine glucuronide hydrolysis and instant hydrolysis of amitriptyline glucuronide. Four drug metabolites, buprenorphine, oxazepam, temazepam and THC-COOH glucuronides, were hydrolyzed instantly by IMCSzyme, Pure Abalone 1 and 2 and Pure Limpets regardless of measured phenolphthalein-glucuronide activity.

CONCLUSIONS

- Activity measured with the chromogenic substrate phenolphthalein-glucuronide is not a good indicator of drug hydrolysis due to substrate specificity.
- Consumers should be aware when choosing a β-glucuronidase that higher activity in Fishman units will not necessarily correlate with drug hydrolysis efficiency.
- Laboratories should validate β-glucuronidase hydrolysis in-house to ensure effectiveness with drugs being monitored and compatibility with patient samples.

REFERENCES

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