Reduced Hydrolysis Due to Natural Chemicals Inhibiting Glucuronidase Activity Resulting in Poor Liberation of Aglycones

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INTRODUCTION

- Small molecules progress through phase I and phase II metabolism.
- Phase II metabolism involves conjugation reactions such as glucuronidation.
- β-Glucuronidases are used to liberate glucuronide conjugates to make drugs of abuse analysis easier.
- We present data to show that β-glucuronidase can exhibit high sensitivity to urea in urine, and all the enzymes exhibit different inhibition profiles in urine.

MATERIALS AND METHODS

IMCSzyme RT was provided by Integrated Micro-Chromatography Systems (IMCS). BpGUS is a recombinant enzyme expressed in *E. coli* with gene sequence sourced from *Brachyspira pilosicoli.* The enzyme contained poly-his tag for immobilized metal affinity chromatography (IMAC) followed by buffer exchange. The concentration of both enzymes was approximately 2 mg/mL.

Inhibition Models:
Enzyme activities were measured using a substrate-metabolite cross titration assay where substrate and metabolite concentrations varied while enzyme concentration remained constant. 4-Methylumbelliferyl glucuronide (4-MUG) was utilized as the substrate with 4-methylumbelliferyl (4-MU) product formation measured by fluorescence. Substrate and metabolites were mixed, and hydrolysis was initiated with IMCSzyme RT or BpGUS addition to substrate-metabolite mixture. Reactions were quenched in 20 second intervals for 120 seconds total.

Patient Specimens:
Opioid-positive urine samples were obtained from a national testing laboratory. Negative controls and patient samples were buffered and hydrolyzed with IMCSzyme RT or BpGUS. Hydrolyzed urine samples were cleaned with solid phase extraction, solvent evaporated, reconstituted in methanol and diluted with mobile phase A (0.1% formic acid in water). 10 µL of sample was injected 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 column. Mobile phase B was 0.1% formic acid in acetonitrile.

INHIBITION

Hydrolysis rates from each metabolite and substrate concentration were used to calculate kinetic values using a Michaelis-Menten model equation for mixed-type partial inhibition[1]. The inhibitor constant (Ki) values from each metabolite for IMCSzyme RT or BpGUS are shown in **Table 1.** Ki indicates the affinity an enzyme has for a metabolite where smaller numbers indicate higher affinity and, ultimately, stronger inhibition. D-Saccharic acid 1,4-lactone monohydrate is a well known β-glucuronidase inhibitor and displayed the lowest Ki of all metabolites analyzed.

Table 1. *IMCSzyme RT and BpGUS Ki values for each metabolite.*

	Ki (mM)	
Metabolite	**IMCSzyme RT**	**BpGUS**
D-Saccharic acid	0.003	0.001
Urea	102.6	97.8
Caffeine	9.0E+04	1.1E+10
Glucuronic acid	1.4E+05	81.6
L-Ascorbic acid	5.4E+07	109.9

Kinetic values were used to model relative enzyme activity at a fixed substrate concentration as metabolite concentration increased (**Figure 1**).

INHIBITION

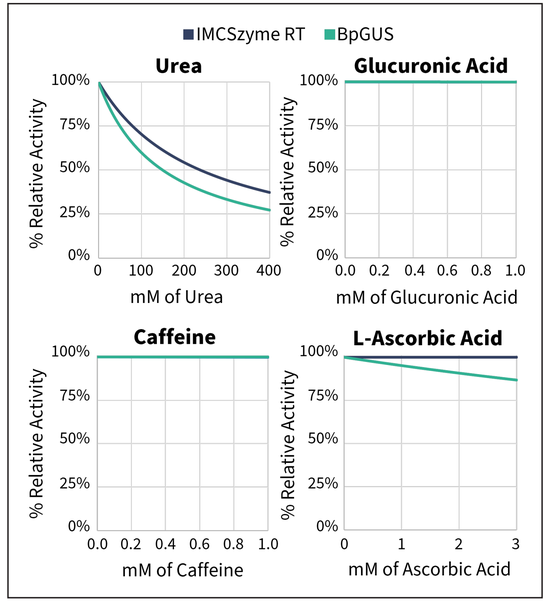

Figure 1. *Relative activity was calculated as metabolite concentration in the reaction increased. Urea, a urine component excreted between 155-338 mM[2], decreased enzyme activity up to 50-75% in this range. IMCSzyme RT activity decreased slower than BpGUS in the presence of urea. Glucuronic acid, the byproduct of β-glucuronidase drug of abuse hydrolysis, had no measurable effect on enzyme hydrolysis. Caffeine, a common component in many foods and drinks, also had no measurable effect on enzyme hydrolysis. L-Ascorbic acid is excreted in urine after consumption (>2.7 is highly positive[3]) and had no measurable effect on IMCSzyme RT but decreased BpGUS activity up to 13%.*

REFERENCES

1. Segal I. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. 1975;New York, NY: John Wiley & Sons, Inc.
2. Rose C, *et al.* The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. *Crit Rev Environ Sci Technol* 2015;**45**:1827-79.
3. Gabaj N, *et al.* Ascorbic Acid in Urine Still Compromises Urinalysis Results. *Ann Clin Biochem* 2019;**57**:64-68.

PATIENT SAMPLES

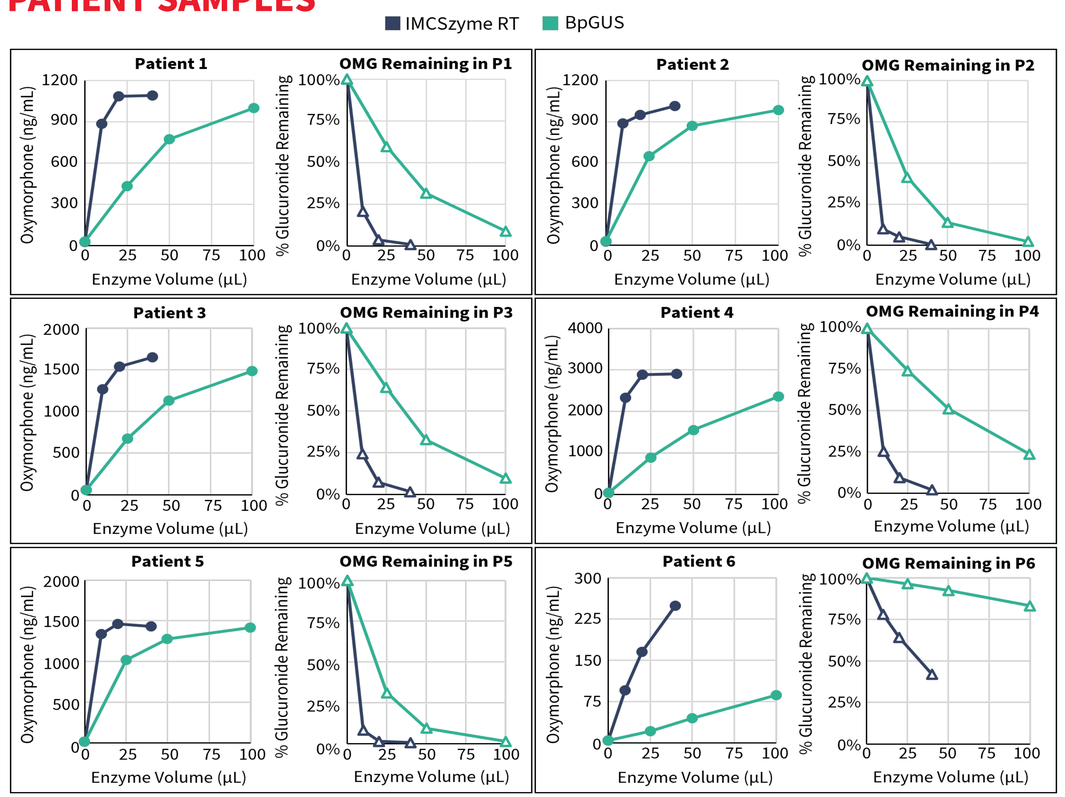

Figure 2. *Oxymorphone hydrolysis from six patient samples hydrolyzed with IMCSzyme RT or BpGUS (both enzymes are 2 mg/mL). Oxymorphone recovery is represented on the left graphs and oxymorphone glucuronide remaining (OMG) is represented on the right graphs of each patient (P). P1, P2, P3 and P5 exhibited minimal inhibition on IMCSzyme RT where 10 µL completed hydrolysis (< 20 % glucuronide remaining) while BpGUS required 50 or 100 µL to complete hydrolysis. P4 exhibited mild inhibition where 20 µL of IMCSzyme RT was required to complete hydrolysis and 100 µL of BpGUS could not complete hydrolysis (23% glucuronide remaining). P6 exhibited drastic inhibition on both enzymes where neither enzyme was able to complete hydrolysis which was confirmed with 40% or 80% of oxymorphone glucuronide remaining.*

CONCLUSION

- Some metabolites in urine are able to differentially inhibit β-glucuronidases.
- Urine samples could be diluted to avoid the natural complexities of endogenous metabolites.

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