Insufficient amount of glucuronidase in clinical samples can result in lowered recoveries

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INTRODUCTION

- Urine drug testing is a common practice for monitoring compliance of prescribed opioid medications.
- Screening and confirmatory assays, such as liquid chromatography coupled with mass spectrometry (LC-MS/MS), benefit from the use of β-glucuronidases (βGUS) that deconjugate glucuronidated analytes.
- In this study we compared two recombinant βGUS, IMCS's IMCSzyme RT and Kura Biotech's B-One in two ways:
  1. Ninety-six clinical samples were hydrolyzed with either IMCSzyme RT or B-One using 20 µg of protein in the reaction.
  2. Control and pooled clinical samples positive for morphine, oxymorphone, codeine and hydromorphone were hydrolyzed with multiple protein quantities ranging from 0 – 200 µg of protein in the reaction.

MATERIALS AND METHODS

Drug free urine control (UTAK) was fortified with glucuronide standards equivalent to 100 ng/mL of free base. Opioid-positive urine specimens were obtained from a national testing laboratory. Control and pooled clinical samples were hydrolyzed with either IMCSzyme RT (IMCS) or B-One (Kura Biotech) with multiple protein concentrations ranging from 0 – 200 µg (**Table 1**). Protein concentration for B-One was based on Bradford assay using BSA as standards, resulting in approximately 1 µg/µL. IMCSzyme RT is quantified by A280, resulting in 2 µg/µL. Hydrolyzed samples were diluted to 40% methanol with 5% formic acid in methanol elution solvent and filtered through a β-Gone plus plate to remove protein (Phenomenex). Samples were then diluted to 10% methanol with water prior to injection onto LC-MS/MS.

Table 1. IMCSzyme RT and B-One reaction set up.

IMCSzyme® RT					
Urine Sample (µL)	IMCSzyme® RT at 2 mg/mL (µL)	IMCSzyme® RT in reaction (µg)	Water (µL)*	Room Temperature Hydrolysis Buffer (µL)	Internal Standard (µL)
100	0	0	50	300	20
	2	4	48		
	4	8	46		
	6	12	44		
	8	16	42		
	10	20	40		
	30	60	20		
	50	100	0		

*water was added after hydrolysis so every sample had equivalent final volume

B-One®					
Urine Sample (µL)	B-One® at 1 mg/mL (µL)	B-One® in reaction (µg)	Water (µL)*	Hydrolysis Buffer (µL)	Internal Standard (µL)
100	0	0	200	B-One® comes all-in-one with hydrolysis buffer	20
	20	20	180		
	60	60	140		
	100	100	100		
	200	200	0		

*water was added after hydrolysis so every sample had equivalent final volume

10 µL of diluted sample was injected on a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Endura™ Triple Quadrupole Mass Spectrometer (**Table 2**). IMCSzyme RT and B-One had separate calibration curves using drug free urine as the matrix. All calibration curves had $r^2 \geq 0.992$ and calibration controls were within ± 20%. LLOQ is 10 ng/mL.

Table 2. HPLC and MS Parameters

Analysis Time	10 minutes
Column	Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å, 4.6 x 50 mm
Column Temperature	40°C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in acetonitrile
Electrospray voltage	1000 V
Sheath Gas	55 Arb
Auxiliary gas	11 Arb
Sweep gas	1 Arb
Ion Transfer Tube Temperature	300°C
Vaporizer Temperature	300°C

RESULTS

IMCSzyme RT and B-One hydrolysis of morphine, oxymorphone, codeine and hydromorphone in control or clinical samples at multiple protein concentrations.

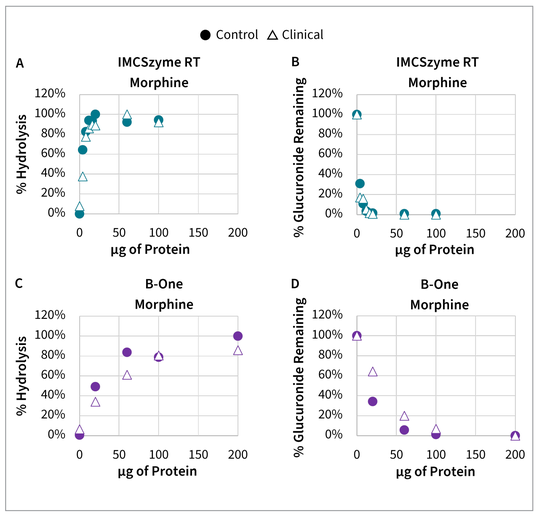

Figure 1. *(A) Recovery of morphine across different amounts of protein in the sample indicates that 8 µg of IMCSzyme RT was sufficient in control set to achieve > 80% recovery, in both the control ● and pooled clinical sample Δ. (B) The corresponding glucuronide peak diminishes as more protein is added, but < 20% glucuronide remains within the first sample of 4 µg of protein for both control ● and pooled clinical sample Δ. (C) Similar recovery plot with B-One as increasing amounts of protein is added indicates more than IMCSzyme RT is needed to achieve similar recoveries in the control ●, and greater amount of B-One is needed in the pooled clinical sample Δ. (D) The corresponding glucuronide remaining in the sample supports this need for more protein in pooled clinical sample Δ in comparison to control sample ●. B-One protein to volume conversion is 1 µg to 1 µL, and IMCSzyme RT conversion is 2 µg to 1 µL.*

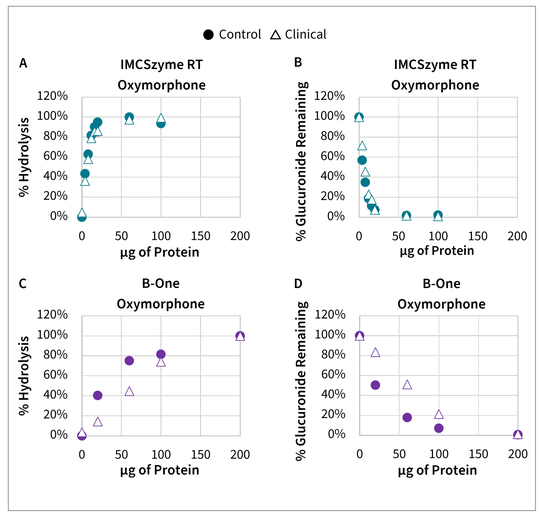

Figure 2. *Recovery of oxymorphone and corresponding decrease in glucuronidated metabolite across increasing amounts of protein. (A) Both control ● that was fortified with glucuronide standards equivalent of 100 ng/mL of free base and pooled clinical sample Δ performs in a comparable manner with consistent recoveries of oxymorphone, and (B) corresponding glucuronides diminish with increase amounts of protein in comparable manner for IMCSzyme RT in control ● and pooled clinical sample Δ. (C) Recovery of oxymorphone in control ● exhibits a classic plot whereas the pooled sample Δ shifts the recovery to the right, suggestive of sensitivity of the protein towards competitive inhibitors in the pooled sample, and (D) similar corresponding levels of glucuronide are detected in control ● and pooled clinical sample Δ. B-One protein to volume conversion is 1 µg to 1 µL, and IMCSzyme RT conversion is 2 µg to 1 µL.*

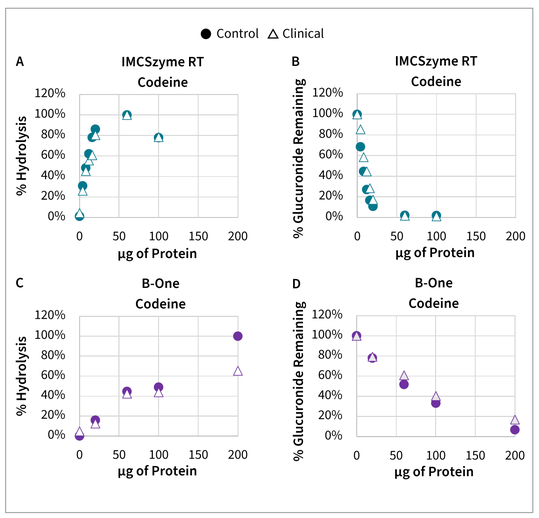

Figure 3. *Recovery of codeine and decreased detection of codeine-glucuronide across increasing amounts of protein. (A) Recovery of codeine with IMCSzyme RT for both control ● and pooled clinical sample Δ are closely aligned with over 80% recovery starting around 16 µg of protein. (B) Corresponding codeine glucuronide diminishes with increase in IMCSzyme RT in comparable manner for control ● and clinical samples Δ. (C) Codeine recovery is significantly challenged with B-One, showing 200 µg addition to achieve complete conversion in control sample ● but below 80% recovery threshold in clinical sample Δ. (D) Corresponding codeine glucuronide samples continue to be detected in both control ● and pooled clinical sample Δ, which suggests incomplete recovery of codeine. B-One protein to volume conversion is 1 µg to 1 µL, and IMCSzyme RT conversion is 2 µg to 1 µL.*

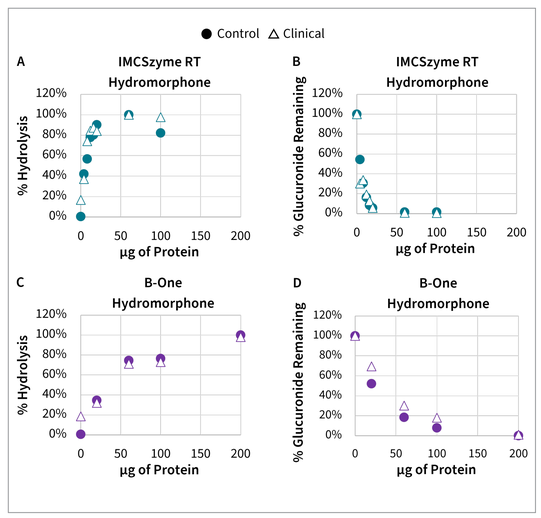

Figure 4. *Recovery of hydromorphone and corresponding glucuronide across increasing protein amounts. (A) Comparable recoveries are observed for control ● and pooled sample Δ, and (B) for glucuronide detection decreases as the corresponding free base increases in control ● and pooled clinical sample Δ. (C) While more protein is needed to achieve the similar recovery as IMCSzyme RT, this recoveries in control ● and pooled urine Δ are comparable, and (D) corresponding decrease in hydromorphone glucuronide decreases as greater amounts of protein is added to the sample. B-One protein to volume conversion is 1 µg to 1 µL, and IMCSzyme RT conversion is 2 µg to 1 µL.*

Due to limited supply of B-One, ninety-six clinical samples were hydrolyzed with either IMCSzyme RT or B-One using 20 µg of protein in the reaction containing 100 µL of sample, 300 µL of RTB for IMCSzyme RT or no buffer for B-One for fixed time of 15 minutes at room temperature (20-25 °C). All samples contained 20 µL internal standard. Each sample set had its own calibration standards. Samples are considered positive (+) with ≥ 25 ng/mL of drug and negative (-) with < 25 ng/mL of drug. Results for Oxymorphone and Codeine are shown in **Table 3** and **4**.

Table 3. Oxymorphone

IMCSzyme RT Hydrolysis \ B-One Hydrolysis	(+)	(-)
(+)	20	16
(-)	0	60

In Agreement:
- 20 samples as both positive
- 60 samples as both negative

In Disagreement:
- None of the samples that were positive by B-One were negative by IMCSzyme RT
- **16 samples disagreed where they were positive by IMCSzyme RT but negative by B-One**

Table 4. Codeine

IMCSzyme RT Hydrolysis \ B-One Hydrolysis	(+)	(-)
(+)	0	5
(-)	0	91

In Agreement:
- 91 samples as both negative

In Disagreement:
- None of the samples that were positive by B-One were negative by IMCSzyme RT
- **5 samples disagreed where they were positive by IMCSzyme RT but negative by B-One**

CONCLUSION

- Clinical samples contain endogenous urine chemicals that can affect hydrolysis efficiencies likely due to presence of denaturing chemicals like urea, and other competing glucuronidated metabolites.
- In specified testing parameters, both DFU and pooled urine samples required more B-One than IMCSzyme RT to achieve > 80% recovery for all four opiates.
- When using 20 µg of protein and 100 µL of urine with 15 minute room temperature incubation, there were more samples below the 25 ng/mL threshold for samples processed with B-One than samples processed with IMCSzyme RT.
- Using less βGUS product may result in higher number of false negatives.
- Future studies may entail comparing larger amount of B-One or modified buffer ratios to ensure appropriate pH conditions are achieved.

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