# Rapid Room Temperature Hydrolysis of Glucuronidated Drugs of Abuse using IMCSzyme® RT

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## ABSTRACT

- Only IMCSzyme® RT completely hydrolyzed 16 glucuronidated substrates in all three urines in 15 minutes at room temperature.
- Two other recombinant enzymes failed to complete hydrolysis within the same time frame at room temperature.
- Regardless of drug content, some human urine contains compounds that inhibit β-glucuronidase and reduce substrate recovery, though not all enzymes are equally affected.

## INTRODUCTION

β-glucuronidase is an enzyme commonly utilized by clinical and forensic laboratories to remove glucuronic acid conjugated to drug metabolites in biological fluids for improved detection and quantification by mass spectrometry. Hydrolysis efficiency of β-glucuronidase is highly dependent upon the source of the enzyme and the manufacturing process. Most commercially available β-glucuronidases have lower hydrolysis efficiencies against codeine-6-β-glucuronide (C6G) relative to the other glucuronidated substrates, requiring elevated incubation temperatures, longer incubation times, or higher enzyme dosages to achieve accurate recovery of codeine (1-3). Here we describe a new enzyme that completely hydrolyzes 16 glucuronidated drugs of abuse—including C6G—at room temperature in under 15 minutes **(Figure 1)**.

Three purified β-glucuronidases, IMCSzyme® RT, *Brachyspira pilosicoli* (BpGUS) and *Patella vulgata* (PvGUS), were compared in three different matrices — two synthetic urines and one certified drug-free urine. Synthetic urine is often used in clinical and forensic toxicology laboratories for the preparation of calibrators (4) because it mimics human urine without the variability present in biological samples. Human urine samples are highly heterogeneous with pH ranging from 4.6 to 8.0 (5) and specific gravity ranging from 1.002 to 1.030 (6). Personal health, disease or medication may further expand these ranges.

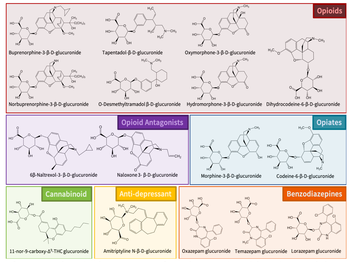


***Figure 1.*** *Chemical structures of 16 glucuronic acid conjugated drug substrates commonly quantified in urine by LC-MS/MS.*

## MATERIALS AND METHODS

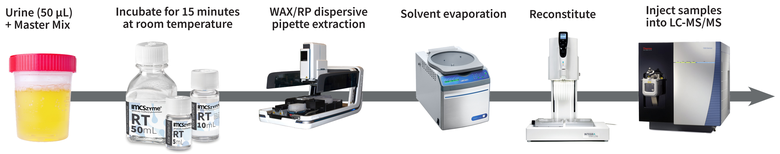


***Figure 2.*** *Schematic representation of hydrolysis and sample preparation procedures.*

Three different urine matrices (Surine™ from DTI part number 720-1, Synthetic Urine Solution from RICCA part number 8361 and Certified Drug-Free Urine from UTAK part number 88121-CDF(F)) were fortified with 16 glucuronide standards (Cerilliant) at equivalent to 5,000 ng/mL each when liberated. Substrates represent several different drug classes of interest: anti-depressant (amitriptyline), benzodiazepines (oxazepam, lorazepam, temazepam), opiates (morphine, codeine), opioids (hydromorphone, oxymorphone, dihydrocodeine, tapentadol, O-desmethyltramadol, norbuprenorphine, buprenorphine), opioid antagonists (6β-naltrexol, naloxone) and cannabinoid (11-nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol; cTHC). All 16 substrates were separated on a nine minute LC gradient **(Figure 3)**. A five-point calibration curve was created using a linear fit for each analyte. Quality control samples were within ± 20% deviation of the target values and correlation coefficients ($R^2$) were ≥ 0.98.

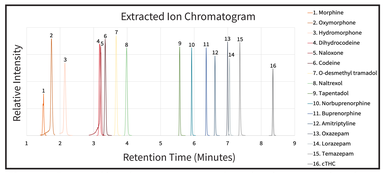


***Figure 3.*** *Extracted ion chromatogram overlays showing separation of 16 analytes.*

50 µL of fortified urine was mixed with 165 µL of master mix (refer to table) and incubated at room temperature (20.5 ± 1.0°C) for 15 minutes. The hydrolyzed urine samples were extracted using WAX/RP dispersive pipette extraction on a Hamilton Microlab® NIMBUS. Each sample was eluted with 400 µL of 1% formic acid in acetonitrile. The eluent was solvent evaporated and reconstituted with 50 µL of methanol and 400 µL of mobile phase A (0.1% formic acid in water). 5 µL of diluted sample was 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. Ion source voltage was set to 1000 V or 4500 V. Mobile phase B was 0.1% formic acid in acetonitrile.

***Table.*** *Internal standard comprised of all corresponding deuterated analytes. LC-MS/MS compatible buffers were prepared at an optimal pH for each enzyme. *15 µL of provided concentrated buffer mixed with 135 µL of water.*

| ***Reaction Composition*** | Master Mix | | | |
|---|---|---|---|---|
| **Urine (50 µL)** | **Purified Enzyme (5 µL)** | **Optimal Hydrolysis Buffer pH per Enzyme (150 µL)** | **Internal Standard in Methanol** | **Reaction Volume** |
| Surine Negative Control or Synthetic Urine Solution or Certified Drug-Free Urine | IMCSzyme® RT | pH 5.5* | 10 µL | 215 µL |
| | or BpGUS | pH 6.5 | | |
| | or PvGUS | pH 4.5 | | |

## RESULTS

Purified β-glucuronidases were characterized by phenolphthalein glucuronide (PTGlcU) activity assay **(Figure 4)**, based on the method of Fishman *et al.* (7). While IMCSzyme® RT and BpGUS have comparable protein concentration (data not shown), BpGUS exhibits higher PTGlcU activity **(Figure 4)**. Although PTGlcU activity in Fishman units is often used to compare β-glucuronidases for historic reasons, it correlates poorly with conjugated drug hydrolysis (8).

IMCSzyme® RT completely hydrolyzed all 16 substrates within 15 minutes at room temperature with less than 5% deviation between the three urine samples **(Figure 5a)**. BpGUS hydrolyzed less than 80% of several substrates and had up to 10% deviation between urine samples **(Figure 5b)**. PvGUS had the lowest PTGlcU activity **(Figure 4)**, and it hydrolyzed less than 80% within most drug classes **(Figure 5c)**. PvGUS performed similarly in the two synthetic urines, but it exhibited severe inhibition against some substrates in the certified drug-free urine which are a pool of multiple human urine samples **(Figure 5c)**. The degree of enzyme inhibition varied between substrates. Hydrolysis of hydromorphone and lorazepam glucuronides were mildly affected, while hydrolysis of other substrates decreased by more than half.

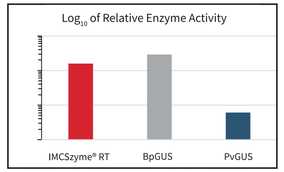


***Figure 4.*** *Relative enzyme activity in Fishman units/mL.*

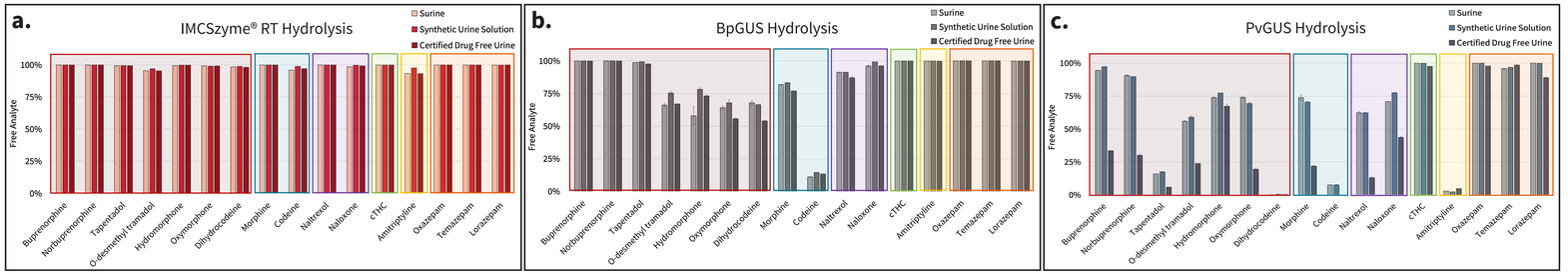


***Figure 5.*** *Enzyme hydrolysis in Surine, Synthetic Urine Solution and Certified Drug-Free Urine using a. IMCSzyme® RT, b. BpGUS and c. PvGUS. Colored boxes correspond to drug classes in Figure 1; red: opioids, blue: opiates, purple: opioid antagonists, green: cannabinoid, yellow: antidepressant and orange: benzodiazepines.*

Preliminary experiments to explore inhibition effects of drug-free urine on each enzyme utilized 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as the substrate because the fluorescent product is easy to detect and provides a linear signal across three orders of magnitude. Enzyme hydrolysis reactions utilized a range of both substrate and inhibitor and looked at the hydrolysis rate over one minute in 15-second increments. The optimal buffer for each enzyme was used. Hydrolysis rates were calculated, plotted as a function of substrate (in µM) and inhibitor (in percent drug-free urine) concentrations (shown for IMCSzyme® RT in **(Figure 6a)** and fit with a simple Michaelis-Menten competitive inhibition model (9) **(Figure 6b)** to quantify the effect of inhibition on enzyme activity. Using the parameters from the model fits, inhibition curves as a function of urine concentration were constructed **(Figure 6c)**.

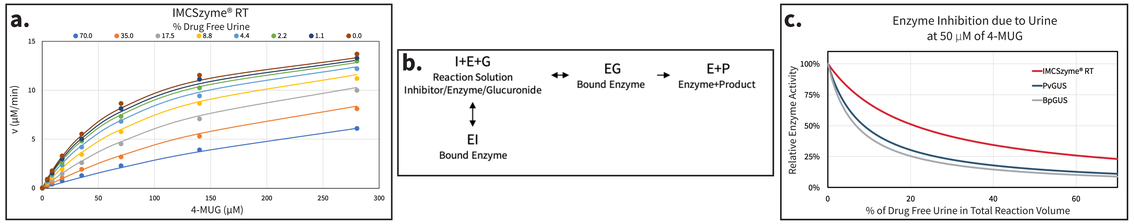


***Figure 6.*** *(a) IMCSzyme® RT velocity plot. (b) Simple Michaelis-Menten competitive inhibition model. (c) Enzyme activity inhibition as a function of urine concentration.*

Enzyme activity decreases at different rates depending on the specific affinities of an enzyme for both the substrate and the inhibitor(s) in the urine (as yet undefined). If an enzyme has a high affinity for an inhibitor, the reaction will slow down more significantly, even at low urine concentrations. IMCSzyme® RT is least affected by the inhibitor(s), retaining more than 50% of its activity at 20% urine content **(Figure 6a)**. In contrast, at 20% urine content BpGUS and PvGUS lose more than 70% of their activity. Inhibition becomes worse as substrate concentration decreases.

## REFERENCES

1. Lin Z, Lafolie P, and O Beck (1994) Evaluation of analytical procedures for urinary codeine and morphine measurements. *J Anal Toxicol*, **18**, 129-133.
2. Wang P, Stone JA, Chen KH, Gross SF, Haller CA, and AHB Wu (2006) Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites. *J Anal Toxicol*, **30**, 570-575.
3. Yang HS, Wu AHB, and KL Lynch (2016) Development and validation of a novel LC-MS/MS opioid confirmation assay: Evaluation of β-glucuronidase enzymes and sample cleanup methods. *J Anal Toxicol*, **40**, 323-329.
4. Kim VJ, Okano CK, Osborne CR, Frank DM, Meana CT, and MS Castaneto (2018) Can synthetic urine replace authentic urine to "beat" workplace drug testing? *Drug Test Anal*. DOI: 10.1002/dta.2497.
5. Martin LJ (2017) Urine pH test. Accessed February 25, 2019, from https://medlineplus.gov/ency/article/003583.htm
6. Stephens C (2018) Urine Specific Gravity Test. Accessed February 25, 2019, from https://www.healthline.com/health/urine-specific-gravity
7. Talalay P, Fishman WH, and C Huggins (1946) Chromogenic substrates; phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. *J Biol Chem*, **166**, 757–772.
8. Sitasuwan P, Melendez C, Marinova M, Spruill M, and LA Lee (2019) Comparison of purified β-glucuronidases in patient urine samples indicates a lack of correlation between enzyme activity and drugs of abuse metabolite hydrolysis efficiencies leading to potential false negatives. *J Anal Toxicol*, **43**, 221-227.
9. Segel I (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. New York, NY: John Wiley & Sons, Inc.

## CONCLUSION

- IMCSzyme® RT can rapidly hydrolyze samples for accurate detection of all glucuronidated drugs.
- IMCSzyme® RT better tolerates inhibitors found in human urine than other enzymes. Such inhibition could lead to false negatives and inaccurate results.
- Brief incubation at room temperature enables automated workflows, increasing throughput and efficiency for drug testing laboratories.

## DISCLOSURE

The authors are employees of Integrated Micro-Chromatography Systems, Inc.