Overcoming Heterogeneity in Urine Specimens: Avoiding False Negatives caused by Endogenous Inhibitors

Amanda C. McGee[1], P. Nikki Sitasuwan[1†], John J. Tomashek[1], Ana Celia Muñoz-Muñoz[2], Lawrence Andrade[2], and L. Andrew Lee[1]*

[1]Integrated Micro-Chromatography Systems, Inc, Irmo, SC 29063, USA; [2]Dominion Diagnostics, LLC, North Kingstown, RI 02852, USA. [†]Current affiliation: 3M Company, St. Paul, MN 55144, USA. *Presenting author: john.tomashek@imcstips.com

IMCS

DOMINION DIAGNOSTICS

Introduction

There are three critical parameters to know when working with enzymes:

1. Temperature and pH optima;
2. Substrate profile;
3. Matrix (urine) tolerance.

These parameters are unique for each enzyme; all are dictated by the structure of the enzyme and the chemistry of the analyte. Therefore, a single substrate, pH, and temperature cannot be used to compare different enzymes or easily predict performance across a drug panel [1]. More importantly, **compounds in clinical samples—absent from synthetic matrices—compromise hydrolysis performance both in an enzyme- and analyte-specific manner**. Though the variability of urine is well known to testing practitioners, the effects on hydrolysis efficiency have not yet been characterized in detail.

In this study, two new enzyme products—IMCSzyme RT and Enzyme B, both designed for room temperature hydrolysis—are compared. Enzyme activities are determined at the optimum pH of each enzyme, and a new method for quantifying promiscuous enzyme activity is proposed. Finally, sensitivity of different enzymes to authentic urine specimens is compared across 90 patient samples. **Different enzymes have widely divergent sensitivity to the natural variability of clinical samples.**

Results

Enzymes have Unique pH and Substrate Profiles, Dependent on Enzyme and Substrate

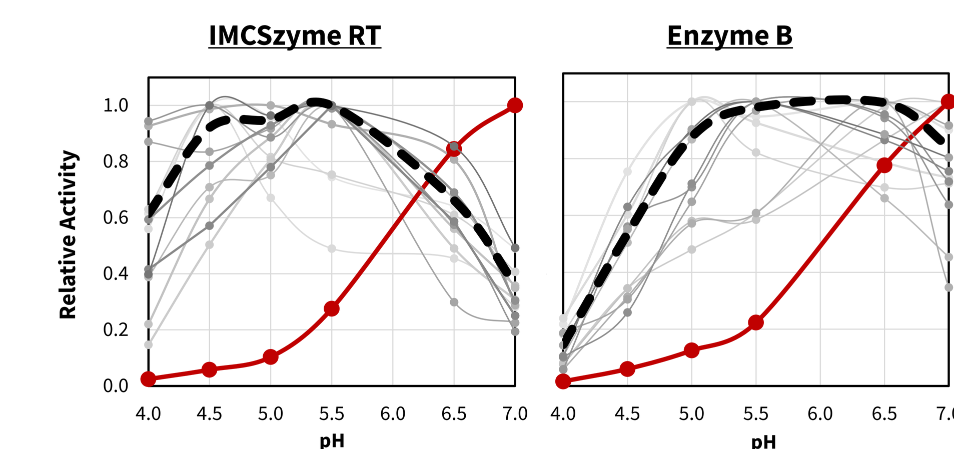


Figure 1. Each enzyme and substrate pair has a unique pH profile. Thirteen drug metabolites hydrolyzed with either IMCSzyme RT or Enzyme B at pH from 4.0 to 7.0. Activity has been normalized to the maximum activity for each profile. The average activity (excluding amitriptyline) is indicated by the ***black dashed line***. **Amitriptyline**, indicated in **red**, has an N-linked glucuronide and prefers a more neutral pH in both enzymes.

Table 1. Enzyme activities at their optimal pH for 13 substrates (in pmol/min/mg).

Substrate	IMCSzyme RT	Enzyme B	Enzyme E
Amitriptyline	7,600	17,000	9,400
Buprenorphine	8,900	8,000	2,000
Codeine	900	60	1,600
Dihydrocodeine	940	380	2,000
Hydromorphone	1,100	280	570
Lorazepam	39,000	22,000	19,000
Morphine	2,100	630	1,900
Norbuprenorphine	30,000	2,600	1,300
O-desmethyltramadol	1,100	340	2,600
Oxymorphone	790	270	250
Oxazepam	4,700	6,000	9,000
Tapentadol	2,900	1,400	5,700
Temazepam	4,600	6,000	9,000
Average	**8,048**	**4,997**	**4,948**
SD	12,168	7,035	5,390
Avg/SD	**0.66**	**0.71**	**0.92**
Root Sum	**3,410**	**1,466**	**2,737**

Two enzymes, IMCSzyme RT and Enzyme B, were evaluated against a panel of thirteen common forensic analytes from pH 4 to 7 (**Figure 1**). Most substrates are *O*-glucuronidated and have similar pH optima (***grey lines***) for a given enzyme. However, *N*-glucuronidated substrates (amitriptyline; ***red lines***) have pH optimum shifted to higher pH regardless of enzyme. For each enzyme, an average pH curve was plotted (***heavy black dashed lines***) for all substrates excluding amitriptyline. IMCSzyme RT pH_{opt} = 5.5; Enzyme B pH_{opt} = 6.5.

Enzyme activity (pmol analyte per minute per mg enzyme) was determined at pH_{opt} for all thirteen analytes (**Table 1**). Another enzyme—Enzyme E (pH_{opt} = 5.5)—is included for comparison. It is impossible to assess enzyme performance from a single substrate [2]. Reliance on the Fishman unit, which is based on activity against phenolphthalein-β-glucuronide, is the classic—and calamitous—example. An alternative criterion for comparison is needed.

$$\text{Average} = \frac{\sum_{1}^{n} x_i}{n} \quad \text{Equation 1} \qquad \text{Root Sum} = \sqrt[n]{\prod_{1}^{n} x_i} \quad \text{Equation 2}$$

Two important qualities should be considered: average activity across a range of substrates (higher the better) and variability of activity between substrates (lower the better). These might be determined from the average activity (**Equation 1**) and standard deviation (SD), which can be combined into a single parameter (average/SD). We propose an alternative, the Root Sum (**Equation 2**), which is the nth root of the product of activities on n analytes. This captures both variability and average activity. It also correlates most positively across all analytes (R_{av} = 0.38), compared to either the average (R_{av} = 0.19) or the average/SD (R_{av} = 0.15).

Enzymes are Sensitive to Substances in the Matrix (Urine)

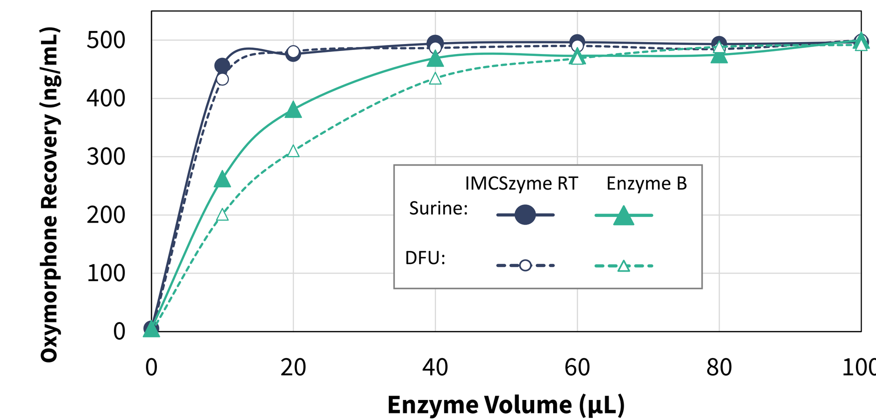


Figure 2. ***IMCSzyme RT tolerates urine better than Enzyme B.*** *Synthetic urine (Surine) and certified drug-free urine (DFU) were fortified with oxymorphone glucuronide to yield 500 ng/mL of oxymorphone when liberated.*

Real urine contains numerous compounds that that can hurt enzyme efficiency, and these would not be present in synthetic urine controls. Hydrolysis differences between IMCSzyme RT and Enzyme B in Surine and in Drug Free Urine (DFU), a mixture of clean urine from 11 anonymous donors, demonstrates this effect (**Figure 2**).

For clarity, we followed a single analyte, oxymorphone-3-β-D-glucuronide (OMG), which converts to oxymorphone (OM) upon hydrolysis. Several features become obvious.

1. IMCSzyme RT has higher activity and releases more OM per unit of enzyme than Enzyme B, regardless of matrix (**Table 1**; **Figure 2**), with ~3x better activity in Surine.
2. DFU shows a modest but measurable negative effect on Enzyme B, relative to Surine, that is not observed for IMCSzyme RT.
3. Because DFU is collected from multiple donors, it means that either:
   a. The offending compound is very potent;
   b. The offending compound is common to most of the population;
   c. There are multiple compounds, and Enzyme B is sensitive to more of them.

Therefore, IMCSzyme RT should be more tolerant to a majority of forensic and clinical samples.

Some Enzymes are Less Sensitive to Inhibitors

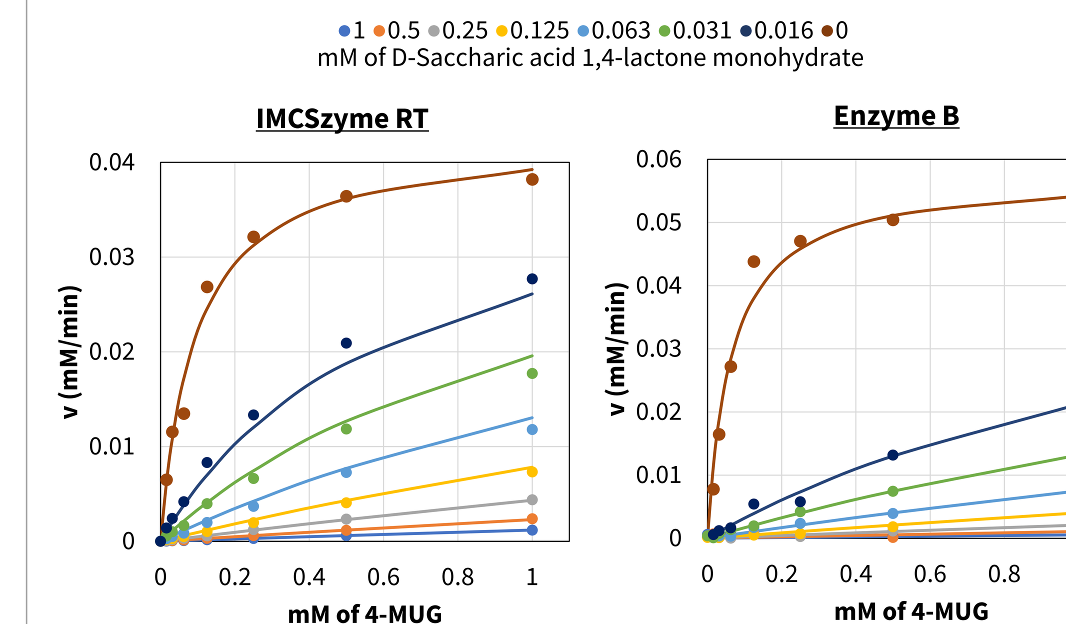


Figure 4. IMCSzyme RT tolerates specific inhibitor better than Enzyme B. Michaelis plots of IMCSzyme RT and Enzyme B with D-saccharic acid 1,4-lactone (DSAL), a known β-glucuronidase inhibitor, and 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as substrate. Data (circles) were fit with a Michalis-Menten competitive inhibition model (lines). Kinetic constants are in **Table 3**.

Table 3. Kinetic constants for D-saccharic acid 1,4 lactone inhibition.

	IMCSzyme RT	Enzyme B
k_{cat} (min^{-1})	9256	13518
K_{4MUG} (µM)	93.6	65.0
K_{DSAL} (µM)	2.7	0.6

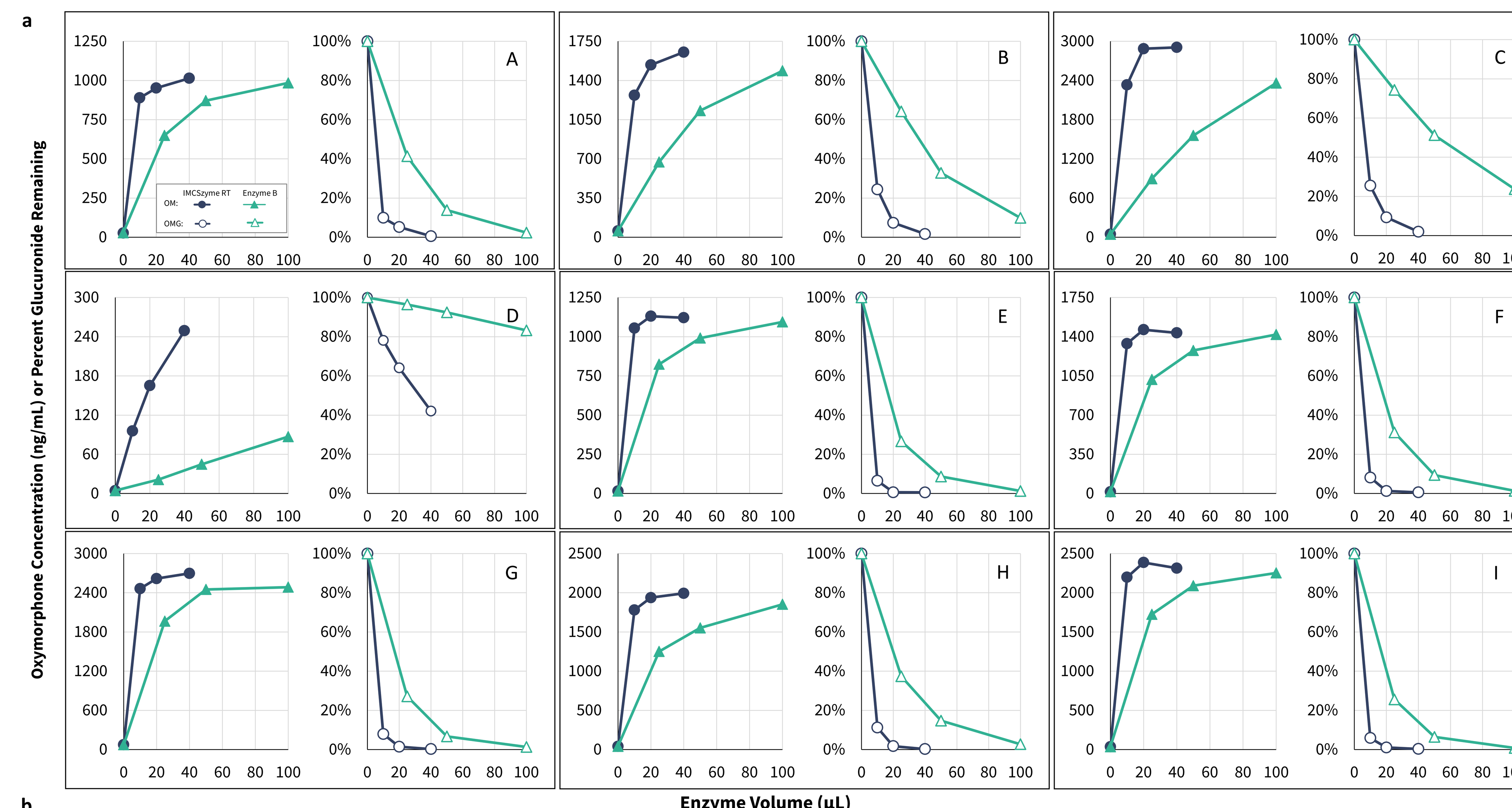
We have begun a detailed investigation to identify inhibitors in urine samples and their mechanism of action. Here we present some preliminary results.

A well characterized general inhibitor of β-glucuronidases is D-saccharic acid-1,4-lactone (DSAL), which typically has an inhibition constant (K_i) of 1-10 µM [JJT, unpublished data].

We performed assays using 4-methylumbelliferyl-β-D-glucuronide (4MUG) as the substrate and determined the Michaelis constants for substrate and inhibitor (**Figure 4**; **Table 3**). In the absence of inhibitor, IMCSzyme RT has about two-thirds the activity (k_{cat}) of Enzyme B, and similar affinity (K_{4MUG}). However, Enzyme B is about 5x more inhibited by DSAL (K_{DSAL} = 0.6 µM) relative to IMCSzyme RT (K_{DSAL} = 3.0 µM). A competitive model of inhibition fit the data best. Other inhibitors may have more complex mechanisms.

Some Enzymes Perform More Evenly Sample-to-Sample

We treated nine anonymous patient samples that contain varying amounts of endogenous oxymorphone glucuronide with either IMCSzyme RT or Enzyme B at five different enzyme loads (**Figure 3a**); concentrations (mg/mL) were the same for both. The analyte (OM) and its glucuronide (OMG) were monitored to confirm mass balance. Activity loss is significantly greater for Enzyme B than for IMCSzyme RT. IMCSzyme RT is ~4x more active than Enzyme B toward oxymorphone in most samples. In some patients, Enzyme B is severely inhibited, *e.g.* Patient C, with an 8x difference, and Patient D—where both enzymes underperform—IMCSzyme RT is still 10x more potent.



b

Fortified Patient Hydrolysis

IMCSzyme RT ■ Enzyme B

Oxymorphone Concentration (ng/mL)

Patient Samples

Figure 3. IMCSzyme RT outperforms Enzyme B in actual Clinical Samples. **(a)** Patient specimens (A-I; unfortified) with endogenous oxymorphone glucuronide were hydrolyzed at room temperature (22°C) at five different volumes of each enzyme. Starting enzyme concentrations were nearly identical. Therefore, volumetric additions from 10 to 100 µL of enzyme is proportional. For each patient, the right graph represents hydrolyzed oxymorphone; the left graph represents percent oxymorphone glucuronide remaining. **(b)** Patient specimens (1-19) were fortified with oxymorphone glucuronide to yield 500 ng/mL of oxymorphone after hydrolysis (10 µL of enzyme; 100 µL of urine; 300 µL of buffer; 20 µL of internal standard; 15-minute incubation). Fortified analyte recovery was calculated by subtracting the unfortified analyte from fortified analyte.

Nineteen patient samples were fortified with OMG to yield an expected 500 ng/mL of OM (**Figure 3b**). Recovery of fortified analyte was determined by subtracting recovery without fortification. IMCSzyme RT fully recovered analyte in all samples; Enzyme B in only 1 out of 19 (~5%).

Ninety patient samples (unfortified) were screened for oxymorphone using enzyme at a single dose in a standard protocol (10 µL of enzyme; 100 µL of urine, 300 µL of buffer; 20 µL of internal standard; 15-minute incubation). Setting the positive/negative cutoff at 100 ng/mL, 26 samples tested positive with both IMCSzyme RT and Enzyme B and 31 samples tested negative. Zero samples were negative with IMCSzyme RT but positive with Enzyme B, whereas 33 samples tested positive with IMCSzyme RT but negative with Enzyme B. This suggests Enzyme B may have given 33 false negatives (37%) (**Table 2**).

Table 2. Ninety specimens being screened for oxymorphone were processed with either IMCSzyme RT or Enzyme B for 15 minutes.

Green = positive
Orange = negative
Blue = enzymes agree
Red = false negatives from Enzyme B
Gray = false negatives from IMCSzyme RT

		Samples processed with Enzyme B	
		> 100 ng/mL	< 100 ng/mL
Samples processed with IMCSzyme RT	> 100 ng/mL	26	33
	< 100 ng/mL	0	31

Conclusions

Two commercially available enzymes were compared for pH profiles and activities on 13 high-value analytes. Based on the data:

- An improved approach for evaluating and comparing enzymes is proposed.
- Matrix contaminants affect enzymes differently and can seriously compromise results.
- **IMCSzyme RT** shows excellent tolerance of inhibitors found in clinical samples.

References

1. Taylor LL *et al.* J Anal Toxicol 2017;41:407–11.
2. Sitasuwan P *et al.* J Anal Toxicol 2019;43:221–7.

Disclosure

ACM, JJT, LAL are employees of Integrated Micro-Chromatography Systems, Inc. (IMCS). IMCS supports the research presented in this poster and has paid for the attendance of JJT. PNS is a former employee of IMCS. ACMM and LA are employees of Dominion Diagnostics.