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**Article**

# Factors Compromising Glucuronidase Performance in Urine Drug Testing Potentially Resulting in False Negatives

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## Abstract

Next generation β-glucuronidases can effectively cleave glucuronides in urine at room temperature. However, during the discovery studies, additional challenges were identified for urine drug testing across biologically relevant pH extremes and patient urine specimens. Different enzymes were evaluated across clinical urine specimens and commercially available urine control matrices. Each enzyme shows distinct substrate preferences, pH optima, and variability across clinical specimens. These results demonstrate how reliance on a single glucuronidated substrate as the internal hydrolysis control cannot ensure performance across a broader panel of analytes. Moreover, sample specific urine properties compromise β-glucuronidases to varying levels, more pronounced for some enzymes, and thereby lower the recovery of some drug analytes in an enzyme-specific manner. A minimum of 3-fold dilution of urine with buffer yields measurable improvements in achieving target pH and reducing the impact of endogenous compounds on enzyme performance. After subjecting the enzymes to pH extremes and compromising chemicals, one particular β-glucuronidase was identified that addressed many of these challenges and greatly lower the risk of failed hydrolyses. In summary, we present strategies to evaluate glucuronidases that aid in higher accuracy urine drug tests with lower potential for false negatives.

## Introduction

Urine drug testing is one of the most common practices for monitoring the use of prescribed opioid medications. Testing is typically performed by a preliminary screening assay, such as immunoassay, followed by a confirmatory assay, such as liquid chromatography coupled with mass spectrometry (LC–MS-MS). Screening and confirmatory assays benefit from the use of enzymes that hydrolyze, or deconjugate, glucuronidated analytes. These enzymes improve assay sensitivity because glucuronic acid can mask target analytes in immunoassays or suppress ionization in mass spectrometry (1–4). Particularly in LC–MS-MS, analysis of de-conjugated (free base) drugs is preferred over the analysis of intact glucuronide molecules because of multiple LC–MS-MS analytical challenges presented by glucuronide drugs, for example, ionization suppression, in-source fragmentation, poor analyte retention and irregular peak shape on reverse phase chromatography (5–8).

Effective hydrolysis of glucuronides is indispensable for accurate drug detection (9–11). The choice of hydrolysis method, either chemical or enzymatic, can affect the testing outcome and thereby influence treatment decisions, since each may reveal different urine drug profiles for prescription compliance and illegal substance use (12–16). While recombinant enzymes have proved to be more efficient than either chemical hydrolysis or crude enzyme extracts (17, 18), the next generation of recombinant enzymes has high activity at room temperature and requires shorter incubation times (5 to 15 minutes), thereby increasing test throughput and simplicity by eliminating heating or prolonged incubation steps (17, 18).

Enzymes have substrate profiles—biases for or against specific substrates—and pH optima, all dictated by their structure and the environment in which they evolved. Glucuronidated analytes also contribute to the pH optimum and velocity of a reaction. Therefore, a single glucuronide substrate cannot be used to compare the performance of different enzymes or to predict performance across a drug panel (19). Furthermore, the results presented here demonstrate that compounds and metabolites in clinical samples—absent from synthetic samples—compromise the performance of hydrolyses in both an enzyme- and analyte-specific manner. Additionally, biological and chemical compositions of urine are highly variable, and their effects on glucuronide hydrolysis efficiency are not well understood.

In this study, we demonstrate expanded approaches to evaluate new enzymes. These approaches were used during discovery and selection of enzymes designed for room temperature hydrolysis. Previously characterized and reported enzymes, older generation recombinant enzymes, and naturally sourced enzymes (13, 14, 20, 21) were not used in this

study. We focused instead in new β-glucuronidase preparations that have not been previously reported and are designed to perform hydrolysis at room temperature. For comparison purposes with the ones designed at IMCS, we also designed and cloned in-house a similar construct to one that is commercially available that performs hydrolysis at room temperature, which here is identified as *Bp*. Different enzymes are tested from pH 4 to 7 at 0.5 increments with 13 glucuronidated analytes to determine their substrate profiles. The enzymes are further evaluated by comparing hydrolysis efficiencies in over 100 authentic urine specimens. The results demonstrate that one enzyme shows consistent performance across a range of diverse urine specimen samples and highlights the importance of challenging enzyme hydrolysis with clinical specimens during enzyme selection.

## Materials and Methods

Certified reference materials were purchased from Cerilliant Corporation (Round Rock, TX) that include Amitriptyline HCl (1 mg/mL), amitriptyline-*N*-β-D glucuronide (100 μg/mL), amitriptyline-$d_3$ HCl (100 μg/mL), buprenorphine (100 μg/mL), buprenorphine-3-β-D glucuronide (100 μg/mL), buprenorphine-$d_3$ (100 μg/mL), codeine (1 mg/mL), codeine-6-β-D glucuronide (100 μg/mL), codeine-$d_6$ (1 mg/mL), dihydrocodeine (1 mg/mL), dihydrocodeine-6-β-D glucuronide (100 μg/mL), dihydrocodeine-$d_6$ (1 mg/mL), hydromorphone (1 mg/mL), hydromorphone-3-β-D glucuronide (100 μg/mL), hydromorphone-$d_3$ (100 μg/mL), lorazepam (1 mg/mL), lorazepam glucuronide (100 μg/mL), lorazepam-$d_4$ (100 μg/mL), morphine (1 mg/mL), morphine-3-β-D glucuronide (100 μg/mL), morphine-$d_3$ (1 mg/mL), norbuprenorphine (1 mg/mL), norbuprenorphine glucuronide (100 μg/mL), norbuprenorphine-$d_3$ (100 μg/mL), O-desmethyltramadol (1 mg/mL), O-desmethyltramadol β-D glucuronide (1 mg/mL), O-desmethyltramadol-$d_6$ (100 μg/mL), oxazepam (1 mg/mL), oxazepam glucuronide (100 μg/mL), oxazepam-$d_5$ (100 μg/mL), oxymorphone (1 mg/mL), oxymorphone-3-β-D glucuronide (100 μg/mL), oxymorphone-$d_3$ (100 μg/mL), tapentadol (1 mg/mL), tapentadol-β-D glucuronide (100 μg/mL), tapentadol-$d_3$ (100 μg/mL), temazepam (1 mg/mL), temazepam glucuronide lithium salt (100 μg/mL) and temazepam-$d_5$ (100 μg/mL).

Synthetic urine, Surine™, was purchased from DTI (Lenexa, KS). Certified drug-free urine (DFU) was purchased from UTAK (Valencia, CA). Certified reference materials were used to prepare calibrator and quality control stock solutions in Surine™ and DFU at five different concentrations to provide a five-point calibration curve in duplicates per plate. Two quality control solutions were prepared in duplicates per plate between the lowest and highest calibration concentration. All calibrations were linear with correlation coefficient, $R^2 \geq 0.99$, and quality control solutions were within ±20% accuracy of the nominal concentrations. Deuterated internal standard was prepared in methanol with all analytes of interest and was used as an internal control in each sample. All analytes were within 1% retention time of their respective internal standards.

A genetically modified β-glucuronidase, IMCSzyme *RT* (annotated as *RT*), and sodium acetate buffer, pH 5.5, was from Integrated Micro-Chromatography Systems (Irmo, SC). GusA genes from *Bp* (*Brachyspira pilosocoli*) and *Ee* (*Eubacterium eligens)* were cloned based on published sequences in GenBank and expressed in *Escherichia coli* as recombinant versions of the two enzymes. The two enzymes were tagged with polyhistidine for IMAC purification after lysis with high-pressure homogenizer. Buffers were prepared in-house and the two recombinant enzymes were purified using vendor-specified protocols (Cytiva). Mixed mode solid phase resin tips were purchased from DPX Technologies (Columbia, SC). All reagents were LC grade or better and were purchased from Fisher Scientific (Waltham, MA).

### Methods

#### Enzyme catalytic activity

Surine was fortified with 13 glucuronide standards, each standard equivalent to 500 ng/mL of free base. *RT, Bp* and *Ee* were diluted with water and enzyme concentrations ranging from 0.001 to 2.2 mg/mL. The impact of pH on enzyme activities was tested on all drugs by preparing acetate buffer with pH ranging between 4.0 and 7.0. Fortified Surine (50 μL) samples were processed with 20 μL of diluted enzymes, 150 μL of hydrolysis buffer (pH 4 to 7) and 10 μL of internal standard. Samples were hydrolyzed for 15 minutes at room temperature (20–25°C). Each sample treatment was prepared and analyzed in duplicate. Activity is measured as picomole of free analyte per minute of reaction per milligram of enzyme at room temperature (21°C).

#### Urine titration with buffers

We titrated the amount of buffer needed to adjust the pH of 10 urine specimens: five samples with pH below 5.5 and five samples with pH above 8.5. Samples were adjusted to two pH targets, either acidic or neutral. To this end, 0.2 M acetate buffer was used to adjust the 10 samples to pH 4.5 and 0.2 M phosphate buffer was used to adjust the 10 samples to pH 7.4.

#### Urine hydrolysis and post-hydrolysis preparation

Surine and DFU were fortified with oxymorphone glucuronide equivalent to 500 ng/mL of free base. Surine or DFU samples (100 μL) were treated with 0 to 100 μL of stock *RT* or *Bp* at concentration of 2 mg/mL, 300 μL of hydrolysis buffer and 20 μL of internal standard. Samples were hydrolyzed for 15 minutes at room temperature. Each sample treatment was prepared and analyzed in duplicate.

Enzyme hydrolysis performance was also evaluated on clinical urine specimens. These urine specimens were a subset of those samples submitted for drug screening to Dominion Diagnostics (North Kingstown, RI). Nineteen of those urine specimens with extreme pH were selected for initial drug screening and quantitation. These specimens were subsequently fortified with additional oxymorphone glucuronide equivalent to 500 ng/mL of free base. Fortified urine specimens (100 μL) were treated with 10 μL of *RT* or *Bp*, 300 μL of hydrolysis buffer and 20 μL of internal standard for 15 minutes at room temperature. All 19 specimens were processed identically: same buffer amount (3-fold volume ratio) and same volume of enzyme fixed at 2 mg/mL concentration. Another set of 90 urine specimens—which were not fortified with oxymorphone glucuronide—were mixed with 10 μL of *RT* or *Bp,* both having stock concentration of 2 mg/mL, 300 μL of hydrolysis buffer and 20 μL of internal standard for 15 minutes at room temperature.

The pH of the hydrolyzed samples was verified to make sure that proper pH levels were achieved. These samples were then extracted using weak anion exchange and reverse phase mixed mode solid phase extraction. Samples were eluted with 400 µL of 1% formic acid in acetonitrile. The eluent was evaporated, reconstituted with 50 µL of methanol and diluted with 400 µL of 0.1% formic acid in water. Ten microliters of diluted sample was analyzed by LC–MS-MS. All samples were analyzed in duplicate.

### LC–MS-MS

Ultra-performance liquid chromatography was performed on a Thermo Scientific™ Vanquish™ system over a 6 minute gradient using a Phenomenex Kinetex® Phenyl-Hexyl 100 Å column (4.6 x 50 mm, 2.6 µm) heated to 40°C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The system was equilibrated with 5% B for the first 0.5 minutes and then the gradient transitioned from 5 to 95% B from 0.5 to 3.0 minutes. The gradient remained at 95% B from 3.0 to 3.8 minutes and re-equilibrated at initial conditions from 4.0 to 6.0 minutes. The liquid chromatography system was coupled to a Thermo Scientific™ TSQ Endura™ MS using the following parameters: electrospray voltage: 1,000 V; sheath gas: 55 arb; auxiliary gas: 11 arb; sweep gas: 1 Arb; ion transfer tube temperature: 300°C and vaporizer temperature: 300°C. Detection was performed by multiple-reaction monitoring analysis of the most intense transitions originating from the protonated molecular ion $[M+H]^+$ of each analyte (Supplementary Table SI).

### Enzyme activity root sum calculation

In addition of using average to calculate enzyme activity, we also use root sum activity. A root sum activity takes the product of activities on *n* substrates and then calculates the *n*th root of that value. This contrasts with an average, where the values are summed and then divided by *n*. While the average and the root sum will be proportional, the root sum will have a higher value when the standard deviation is lower (i.e., the range of values is narrower), all else being equal. The equation used to calculate root sum activity is as follows: $\sqrt[n]{X1 * X2 * Xn}$

## Results and Discussion

### Enzyme comparisons for different substrates and across pH

Enzyme pH optima are determined based on assays that use colorimetric substrates such as phenolphthalein glucuronide (20). In this work, we took a different approach using a panel of 13 common glucuronides drugs and measured their respective free analyte amounts by LC–MS-MS across a range of pH and enzyme quantities. This approach revealed that the activities of enzymes toward actual drug substrates vary significantly across pH and that each drug substrate exhibits its own optimum pH (Figure 1). Enzyme activities on three drug substrates out of the 13 glucuronides tested over a pH range of 4.0–7.0 are shown for clarity. Activities for all 13 substrates are provided in Supplementary Table SII. Activity is measured as picomole of free analyte per minute of reaction per milligram of enzyme at room temperature.

Amitriptyline glucuronide shows a trend where enzyme activities are below 500 pmol/min/mg between pH 4 and 5 for *RT* and *Bp* and below 800 pmol/min/mg for *Ee* (Figure 1A). Activities for all three enzymes quickly increase starting at pH 5.5, and hydrolysis efficiency increasing as the pH of the reaction buffer rises from acidic to neutral (Supplementary Table SII). This pH profile is distinct from other substrates and may relate to the fact that amitriptyline is an *N*-linked rather than *O*-linked glucuronide. *Ee* exhibits the highest activity toward amitriptyline glucuronide among the three recombinant enzymes and drops from 13,000 pmol/min/mg at pH 7.0 to 1,600 pmol/min/mg at pH 5.5. Similarly, enzyme activity of *Bp* drops from 3,800 to 850 pmol/min/mg between pH 7.0 and 5.5 and enzyme activity of *RT* drops from 4,600 to 1,260 pmol/min/mg from pH 7.0 to 5.5, respectively. Of the three enzymes, *Ee* also exhibits the highest activity toward codeine glucuronide, across the tested pH range (Figure 1B), with maximum activity around pH 5.0 measured at 1,630 pmol/min/mg. Enzyme activity of *RT* toward codeine glucuronide peaks at pH 5.5 and then drops at pH above this optimum. Enzyme activity of *Bp* did not exceed 60 pmol of codeine/min/mg at pH 6.5–7.0 or 14.5-fold lower activity relative to *Ee*. For oxymorphone glucuronide conversion (Figure 1C), *RT* has

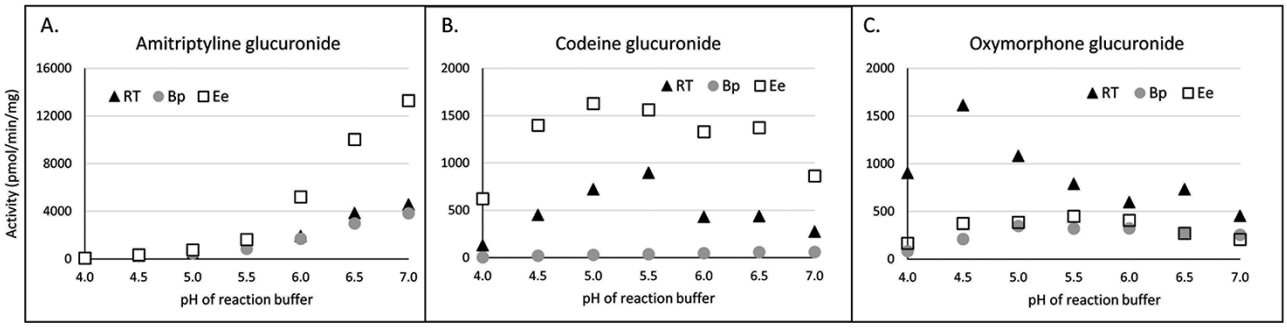


**Figure 1.** Enzyme activity comparison of β-glucuronidases on three drug metabolites from pH 4 to pH 7. Enzyme activity is defined as picomole of glucuronide hydrolyzed per minute per milligram of enzyme. (A) The common trend for all the enzymes is their activity toward amitriptyline glucuronide increases as the pH rises from acidic to neutral. (B) Among the three enzymes, *Ee* exhibits the highest activity towards codeine glucuronide across the pH range tested, *RT* is the second best and presents an optimum pH of 5.5, and *Bp* shows almost no activity toward this drug, with only noticeable amounts of 60 pmol of codeine/min/mg at pH 6.5 to 7.0. (C) *RT* has the highest activity toward oxymorphone glucuronide across all pH tested, with maximum activity peaking at pH 4.5, whereas for *Ee* and *Bp* have very similar recoveries across the entire pH range tested. The maximum activity value for *Ee* hovers around 450 pmol/min/mg at pH 5.5 and for *Bp* is around 400 pmol/min/mg at pH 5–6.

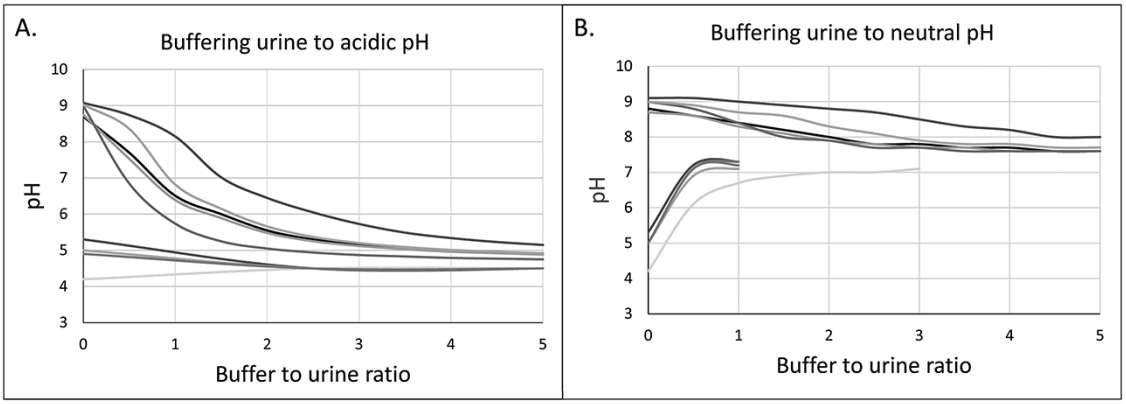


**Figure 2.** Maintaining adequate pH for optimum hydrolysis requires the use of buffers. Ten patient urine samples were selected for titration with buffers to two pH value targets: 4.5 and 7.4. Among the 10 urine patient samples, five had low pH values (~4 to 5) and the other five samples had high pH values (~9). 0.2 M acetate buffer was used to titrate samples to pH values near 4.5, and 0.2 M phosphate buffer was used to titrate samples near pH value of 7.4. (A) Urine samples with high pH required at least 3:1 buffer:urine ratio to adjust the pH to 4.5. One of the basic pH urine sample remained above pH 5 despite using a 5:1 buffer:urine ratio. (B) Samples with acidic pH values needed small amounts of buffer to adjust the pH to the 7.4 value, mostly 1:1 buffer:urine ratio, with only one urine sample requiring more buffer at the 3:1 buffer:urine ratio. Samples with basic pH values required more buffer than their acidic counterparts to adjust the pH. Most of the basic samples required at least 3:1 buffer:urine ratio, with one of the samples requiring a 5:1 buffer:urine ratio to lower the pH below 8. Such challenges in urine pH adjustment can further compromise enzyme hydrolysis efficiency, potentially leading to low recovery and inaccurate quantitation.

highest activity (1,600 pmol/min/mg) at acidic pH of 4.5, tapering from this pH optimum and exhibiting a third of its maximum activity at pH 7.0 (~450 pmol/min/mg). Enzymatic activity of *Ee* is remarkably unchanged for this drug substrate in the pH range tested, and it never increases beyond its maximum of 450 pmol/min/mg at pH 5.5, while *Bp* has the lowest activities across the tested pH ranging from 80 to 350 pmol/min/mg, having the highest activity at pH 5.0.

## Urine titrations with buffer

Normal urine pH ranges from 4.5 to 8.0, and addition of buffers to adjust urine pH toward the enzyme optimum for hydrolysis is a common practice. Adjusting to pH 4.5 with 0.2 M acetate buffer requires less buffer for samples that are already acidic, but basic samples require three to five times the volume of buffer to reduce pH to ≤5.0 (Figure 2A). The neutral pH of 7.4 was achieved with 0.2 M phosphate buffer and required at least 3x volume of buffer to drop pH below 8 in basic samples, except for one of the five urine specimens, where a 5x volume of buffer was needed (Figure 2B). Effective pH adjustment has direct impact on enzyme hydrolysis activity. In the case of *RT*, half unit pH shift from 4.5 to 5.0 reduces activity on oxymorphone glucuronide by 30% (cf. Figure 1C) and increases activity on codeine glucuronide by 20%. Such subtlety of pH can be lost when using synthetic urine or DFU controls, where extreme pH is unusual and minimal buffering is necessary to titrate to target pH. Substrate biases and pH optima are a feature of all enzymes, not just β-glucuronidases. Common adaptations to non-ideal conditions include the use of more enzyme to compensate lower activity at sub-optimum conditions, that is, a buffered urine sample is titrated with enzyme until all analytes are effectively hydrolyzed. When taking this approach, several different glucuronidated analytes should be fortified in DFU and challenged with varying amounts of enzyme.

## Root sum: meaningful value for comparing β-glucuronidases

Given the wide variability between different substrates and pH conditions, comparing enzymes across such broad ranges can be challenging. Averaging the enzyme activities on multiple substrates might offer one solution. However, averages are skewed by high activity on one or few substrates and can hide the low activities on other substrates. That range of activities would be reflected in the variance or standard deviation of activity, but now a second parameter must be calculated and evaluated. An alternative approach is a term called "root sum", where the activities across a range of *n* substrates are multiplied, and then, the *n*th root is calculated to generate a value that reflects both an average and variation.

The three enzymes can be compared based on this root sum of activities across the pH range (Supplementary Table SII) and focus on the activities at the respective pH optima (5.5 for *RT*, 6.5 for *Bp*, and 6.0 for *Ee*) as shown in Table I. While the average activity for *Bp* is higher than *Ee*, the root sum of activity is higher for *Ee* than *Bp*. As described, this relationship is also reflected by the high relative standard deviation percentage of *Bp* activities compared to *Ee*. Root sums of activities, which for *RT*, *Bp* and *Ee* are 3,079, 1,384, and 2,709, respectively, provide a single value for comparing different enzymes that will represent the average catalytic activity with a lower RSD value. A higher root sum value indicates a higher average catalytic activity with lower variance across substrates.

## Enzyme performance in synthetic and DFU

The enzyme activities for 13 glucuronide standards fortified in Surine were measured (Table I). The enzyme activities are ranked by intensity in gray scale for each of the drug substrates for comparison between the enzymes, with the highest activity in lightest background, mid-level in darker gray and lowest among the three enzymes in darkest gray. Activity profiles for these three enzymes demonstrate that β-glucuronidases, while active toward a broad range of glucuronidated substrates,

**Table I.** Enzyme Activities for 13 Substrates

| pH | 5.5 | 6.5 | 6.0 |
|---|---|---|---|
| | *RT* | *Bp* | *Ee* |
| Substrate | Enzyme activity at 500 ng/mL of substrate | | |
| Amitriptyline | 1,256 | 2,973 | 5,198 |
| Buprenorphine | 8,876 | 7,992 | 2,485 |
| Codeine | 896 | 58 | 1,329 |
| Dihydrocodeine | 936 | 375 | 1,737 |
| Hydromorphone | 1,062 | 275 | 551 |
| Lorazepam | 39,278 | 22,328 | 15,890 |
| Morphine | 2,090 | 629 | 1,852 |
| Norbuprenorphine | 30,296 | 2,562 | 2,101 |
| O-desmethyltramadol | 1,144 | 336 | 2,605 |
| Oxymorphone | 789 | 272 | 405 |
| Oxazepam | 7,586 | 16,647 | 8,139 |
| Tapentadol | 2,900 | 1,449 | 5,352 |
| Temazepam | 4,561 | 6,051 | 9,055 |
| Average | 7,821 | 4,765 | 4,362 |
| %RSD | 158% | 148% | 101% |
| Root sum | 3,079 | 1,384 | 2,709 |

pmol/min/mg

each have unique substrate biases. One recalcitrant substrate for one enzyme may be a preferred substrate for another enzyme, as shown for codeine glucuronide being a poor substrate for *Bp* measured at 58 pmol/min/mg but ideal for *Ee* at 1,329 pmol/min/mg. Compared to buprenorphine glucuronide, *Ee* was measured at 2,485 pmol/min/mg, and *Bp* was measured at 7,992 pmol/min/mg.

An ideal enzyme should have uniform activities across multiple substrates and a broad pH range. Among the 13 glucuronides, *RT* has its lowest activity toward oxymorphone glucuronide (789 pmol/min/mg) and highest toward lorazepam glucuronide (39,000 pmol/min/mg). Lowest and highest activities of *Bp* stretches from 58 pmol/min/mg for codeine glucuronide to 22,323 pmol/min/mg for lorazepam glucuronide. The activity for *Ee* ranges from 405 pmol/min/mg for oxymorphone glucuronide to 15,890 pmol/min/mg for lorazepam glucuronide. Among the three enzymes, *Bp* exhibits the lowest activities for seven of the tested glucuronides, whereas *RT* and *Ee* only show the lowest activities for three of glucuronides, suggesting *Bp* has the lowest performance among the three enzymes tested (Table I). However, such metrics are lost in averaging the activities (4,765 average value for *Bp*) but captured in the root sum value (1,384).

Among the 13 glucuronides, oxymorphone glucuronide appeared to be more challenging substrate for all three enzymes. This is more apparent when the averages of the three enzyme activities for codeine glucuronide and oxymorphone glucuronide are considered (Table I). Average for codeine glucuronide is 761 and for oxymorphone glucuronide, it is 489. Consequently, the catalytic activities toward oxymorphone glucuronide were further evaluated using *RT* and *Bp*, which have the highest and the lowest catalytic activities toward this substrate. These two enzymes were tested in two different matrices, synthetic urine (Surine, indicated with solid lines) and DFU (indicated with dashed lines) (Figure 3). This comparison shows how the kinetic assay matches the enzyme with higher activity (*RT*, marked as open circles), achieving nearly complete hydrolysis with lower enzyme amount, whereas a lower activity enzyme (*Bp*, marked as solid triangles) requires at least four times more enzyme to achieve similar recovery (Figure 3). The results correlate to the catalytic rate difference

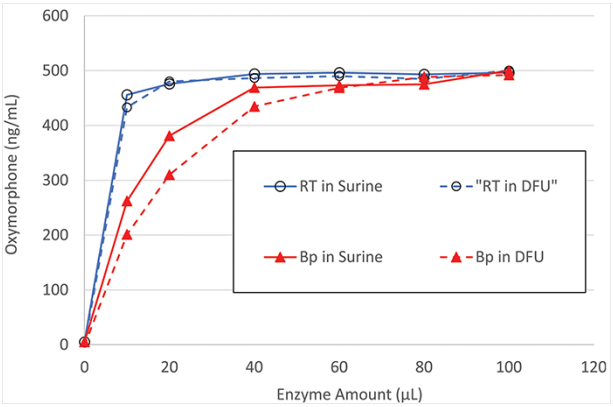


**Figure 3.** Synthetic urine (Surine, solid line) and certified DFU (dashed line) were fortified with oxymorphone glucuronide equivalent to 500 ng/mL of free base. The increasing amounts of enzyme correlate to higher recovery of oxymorphone; however, *Bp* (filled triangles) needed larger enzyme amounts to achieve similar recoveries than *RT* (open circles) in both matrices (Surine and DFU). Catalytic rate for *RT* against oxymorphone glucuronide (790 pmol/min/mg enzyme) is ~3-fold higher than *Bp* (270 pmol/min/ mg enzyme). These previous results correlate well with those obtained here in Surine where *RT* achieved near complete hydrolysis at 10 µL and *Bp* required at least 30–40 µL to obtain similar recoveries. In contrast to Surine, *Bp* exhibited reduced hydrolysis, possibly due to biological and chemical elements present in DFU that compromise enzyme activity, whereas *RT* has higher tolerance and exhibits no significant change in performance in Surine or DFU.

between enzymes *RT* and *Bp* on oxymorphone, where 789 pmol/min/mg is approximately three times higher than 272 pmol/min/mg (Table I).

The matrix itself has significant effects on hydrolysis efficiency. Approximately 20% lower recovery is observed in the DFU matrix compared to Surine for 10 and 20 µL of *Bp* (Figure 3). This difference is associated with elements specific to DFU that compromise the performance of the enzyme. Unlike Surine, DFU is pooled human urine sample that contains additional chemicals or metabolites that may inhibit or inactivate enzymes. These components reduce recoveries for *Bp* in DFU if insufficient enzyme is used.

## Variability of urine samples adds further complexity

The difference in enzyme performance between Surine and DFU was further explored by testing the two enzymes (*Bp* and *RT*) on individual urine patient specimens ($n = 19$). These specimens were selected based on pH extremes to assess the performance of the two enzymes using the 3-fold volume ratio of buffer to urine. The specimens were fortified with oxymorphone glucuronide equivalent to 500 ng/mL of free base concentration to compare enzyme performances (Figure 4). The samples are ordered from lowest pH (sample 1) to highest pH (sample 19). Specific gravity and creatinine levels were also measured (Supplementary Table SIII), but no correlation between urine pH, specific gravity or creatinine levels to enzyme performance is observed. Recoveries from the samples processed with *RT* show smaller deviation (~9% relative standard deviation, RSD) across all 19 samples, (dark gray bars) indicating less sample-to-sample effect on the enzyme activity. The lower recoveries for samples treated with *Bp* were expected, as its activity is at a third of *RT*. However, a high variation was also observed

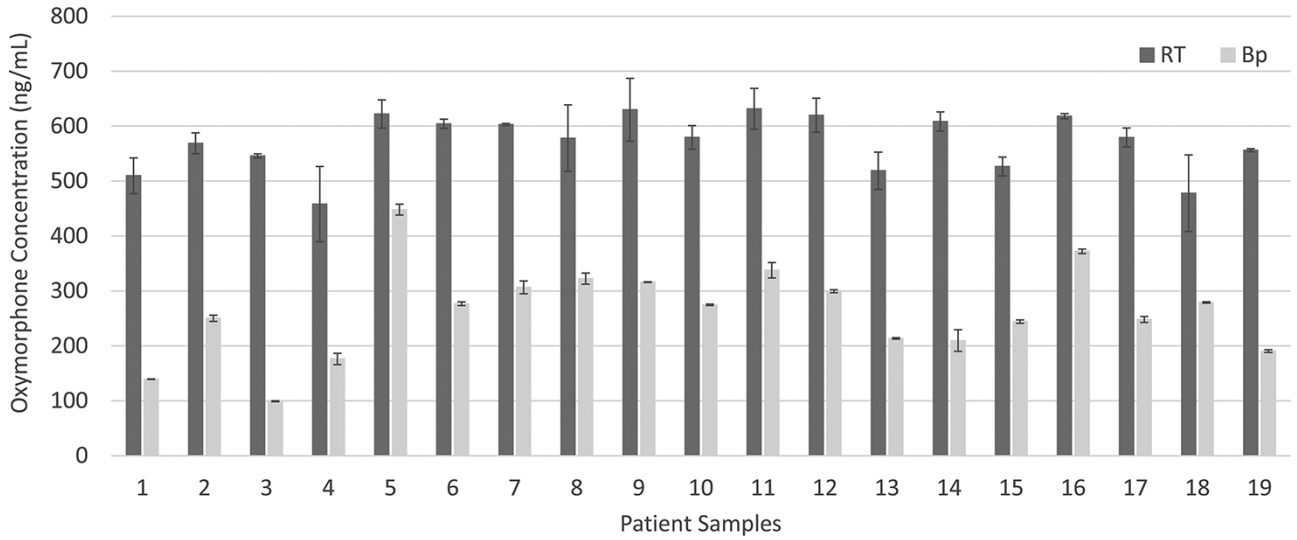


**Figure 4.** Comparison of *RT* (dark gray) and *Bp* (light gray) enzyme hydrolysis of oxymorphone glucuronide on 19 patient urine samples with pH extremes. Urine samples were fortified with oxymorphone glucuronide equivalent to 500 ng/mL of free base. Oxymorphone recovery was calculated by subtracting the oxymorphone amount obtained on the unfortified urine samples from the oxymorphone amount obtained in the fortified urine samples. All samples were processed identically, where 10 μL of enzyme were combined with 100 μL of urine, 300 μL of hydrolysis buffer and 20 μL of internal standard. All samples were incubated at room temperature (20–25° C) for 15 minutes. Recoveries of oxymorphone for *RT* were between ±20% of the nominal value (500 ng/mL) with a %RSD value of 9%. In contrast, *Bp* exhibits reduced and variable oxymorphone recoveries. For instance, sample number three has the lowest recovery with less than 20% of the nominal value (~100 ng/mL), and sample number five has the highest recovery with nearly 80% of the nominal value (>400 ng/mL), resulting on a %RSD value of 31%. Samples three and five highlight the presence of chemicals that compromise *Bp* hydrolysis performance. A robust and more active enzyme, such as *RT*, exhibits more consistent performance regardless of the heterogeneity of the urine samples.

**Table II.** Binary table of 90 specimens treated with *RT* or *Bp*

| | | Specimens treated with *Bp* | |
|---|---|---|---|
| | | **>100 ng/mL** | **<100 ng/mL** |
| Specimens treated with *RT* | >100 ng/mL | 26 | 33 |
| | <100 ng/mL | 0 | 31 |

from 20% recovery (~100 ng/mL) for sample #3 to > 80% (> 400 ng/mL) for sample #5, indicating a high sensitivity to the composition of urine samples that compromise *Bp* hydrolysis performance (light gray bars). The standard deviation of recoveries for *Bp* at ± 80 ng/mL, with an average recovery of 260 ng/mL resulting in ~30% RSD. Extended incubation time (or increased enzyme dose) may improve recoveries for *Bp*, assuming enzyme inactivation is not the issue. Elevating reaction temperature from ambient may increase recovery, but higher temperatures may exacerbate inactivation events (21). Future studies will be needed to parse out thermal and chemical factors leading to inactivation of β-glucuronidases, as well as inhibition by metabolites.

To validate our conclusions, we examined additional 90 urine specimens that had previously been screened positive for opiates (Table II), applying the same LC–MS-MS method and same enzyme-to-sample ratio as 19 sample set, but without fortification of oxymorphone glucuronide. We chose a cutoff threshold for samples at 100 ng/mL of oxymorphone and processed the samples identically with two different enzymes (*RT* and *Bp*). In the analysis of 19 samples, only 1 of 19 samples fell below the 100 ng/mL cutoff when *Bp* was used, that is, 5.2% disagreement between the two enzymes, which appears to be insignificant. However, differences in the larger study were more pronounced. Of the 90 samples, agreement between the enzymes was observed in 57 out of the 90 samples, with 26 above the cutff (>100 ng/mL) and 31 below 100 ng/mL for 63% agreement (Table II). The 37% disagreement resulted from 33 samples treated with *Bp* falling below cutoff, whereas the same samples treated with *RT* were above the 100 ng/mL cutoff. A lower cutoff or increasing the amount of enzyme and/or the length of incubation to improve oxymorphone hydrolysis in samples with *Bp* may increase agreement, but these changes come with additional costs in materials or time.

Successful hydrolyses depend upon a complex interaction between enzyme, substrates and matrix to achieve quantitative yield of target analytes. Purified β-glucuronidases were compared to show enzyme-dependent biases on a panel of common drug targets, including opioids (semi-synthetic or non-synthetic), benzodiazepines, illicit substances and tricyclics. In addition, a range of pHs were tested to show the interdependence of pH optimum on both enzyme and substrate. The substrate preferences and pH optimum of a system complicate enzyme and buffer choices, depending upon the critical analytes for detection. Synthetic urine, commonly used for standardization of protocols, is free from endogenous metabolites that can interfere with drug target hydrolyses, either inhibiting activity and/or inactivating the enzyme. By contrast, pooled urine samples present a more realistic view of matrix impacts upon hydrolysis. The unique pH and composition of individual urine samples that occur in the clinical setting further complicate testing. The results from this study systematically deconvolute these factors and provide evidence illuminating the issues at stake.

Our findings challenge some assumptions about β-glucuronidases used in urine drug testing. Codeine glucuronide is not always the most recalcitrant substrate, as

shown for two other enzymes with lower activities on oxymorphone glucuronide relative to codeine glucuronide. Catalytic rates vary by at least two orders of magnitude for the 13 different glucuronidated analytes tested at an optimal pH. Unsuccessful pH adjustment after buffer addition to clinical samples is likely to occur in some clinical samples that exhibit abnormally high or low pH levels. These samples require larger amounts of buffer to titrate the pH, and the enzyme performance will be impacted differently for each substrate. This implies that monitoring a single analyte as an internal control for enzyme performance could easily be misleading, as a shift of 0.5 pH units can significantly alter recovery for several analytes, possibly in opposite directions. Lastly, the use of Surine or DFU to establish optimum hydrolysis parameters comes with caveats. Surine does not contain chemical or biological elements found in clinical samples, whereas DFU or pooled urine samples may more closely resemble clinical samples. The apotheosis of these interacting variables is highlighted where "identical" treatment (same enzyme load, time, temperature, and optimal pH) can result in near complete recovery of fortified oxymorphone glucuronide from one sample but only 20% recovery in another sample for one of the β-glucuronidases. Many unanswered questions remain, such as the nature of the components in clinical samples that inhibit and/or inactivate β-glucuronidases (22–24), the role of heat inactivation, and the possible conversions of drug metabolites (25, 26). Next generation enzymes should be designed to hydrolyze the broadest range of substrates under the least forgiving conditions in the shortest time possible.

## Conclusion

In evaluating new β-glucuronidases, we identified some key metrics to ensure that enzyme performances are properly assessed to provide higher fidelity in urine drug tests. We demonstrated that β-glucuronidases have different pH and substrate profiles and not one substrate reflects enzyme performance. Relying on a single substrate in synthetic matrix or single pH as a benchmark for enzyme performance is at best unreliable. Clinical urine specimen pH ranges from 4.5 to 8.0, and a shift of 0.5 pH unit can alter enzyme performance by 20% or more. This reiterates the need for proper buffering to achieve optimum pH for hydrolysis. Unfortunately, synthetic urine is devoid of additional chemicals excreted during natural metabolism and is not ideal for challenging β-glucuronidase hydrolysis. In conclusion, we propose an alternative metric for comparing different β-glucuronidases with a root sum of activities, a single value to demonstrate both average activities and its relative standard deviation across multiple analytes. In order to obtain such value, a range of conditions must be evaluated during enzyme discovery before applying the enzyme toward clinical settings.

## Supplementary Data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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