Conversion of drug metabolites due to contaminants in commercial β-glucuronidase products

Pongkwan (Nikki) Sitasuwan*, Cathleen Melendez, Margarita Marinova, L. Andrew Lee
Integrated Micro-Chromatography Systems, LLC - Irmo, SC

ABSTRACT

Small molecule drugs undergo phase II glucuronidation and sulfation in the liver to facilitate excretion into various biological fluids. β-glucuronidase and sulfatase are used to hydrolyze the metabolites, enabling more sensitive and accurate quantitation of the metabolites by mass spectrometry (MS). Any contamination in these enzymes affects not only the effectiveness of the enzyme but may also alter the analytes, skewing the outcome. We demonstrated esterase contamination in various commercial β-glucuronidase enzymes by utilizing a fluorescent substrate. Contaminated enzymes showed the conversion of 6-monoacetylmorphine (6-MAM), a specific metabolite of heroin, to morphine, which can skew the readout to false negative for heroin usage.

INTRODUCTION

Small molecule drugs are cleared from the body by being glucuronidated or sulfated in the liver. These phase II conjugated metabolites then travel through the circulatory system and are excreted in biological fluids, including saliva, blood, and urine. Clinical laboratories utilize a hydrolysis process to liberate these molecules from their glucuronide or sulfate conjugates to facilitate their detection by a range of analytical techniques including liquid chromatography tandem mass spectrometry (LC-MS/MS). Glucuronide hydrolysis can be achieved with strong acid but this can lead to analyte degradation and conversion[1]. β-glucuronidase is a cleaner option to hydrolyze glucuronides without a non-specific compound degradation.

In the case of heroin usage, drug testing began by monitoring morphine, a known metabolite of heroin[2]. However, morphine is also a metabolite of codeine and can be found from ingestion of poppy seeds. False positives for heroin use quickly became a problem. Therefore, the Department of Defense (in 1994) and the Department of Health and Human Services (in 1998) mandated testing for 6-MAM because it is a definitive metabolite of heroin[3]. The cut-off concentration is as low as 10 ng/mL.

We demonstrate that esterase contamination is present in many of the commercially available β-glucuronidases. This contamination correlates with conversion of acetylated opiates, such as 6-MAM to morphine. This is significant because initial 6-MAM levels in urine are often low [4]. If significant amount of 6-MAM is de-esterified, then 6-MAM may fall below the cut-off level. Another possible effect of esterase contamination is the conversion to benzoylegnine, an active metabolite of cocaine found in blood and urine, to ecgonine (***Figure 1c***). Ecgonine methyl ester, an inactive metabolite of cocaine, could also be converted to ecgonine in the presence of contaminated esterase (***Figure 1c***). Therefore, it is important to note the presence of esterase when using β-glucuronidase or sulfatase to monitor the pharmacokinetics of esterified drugs and steroids.

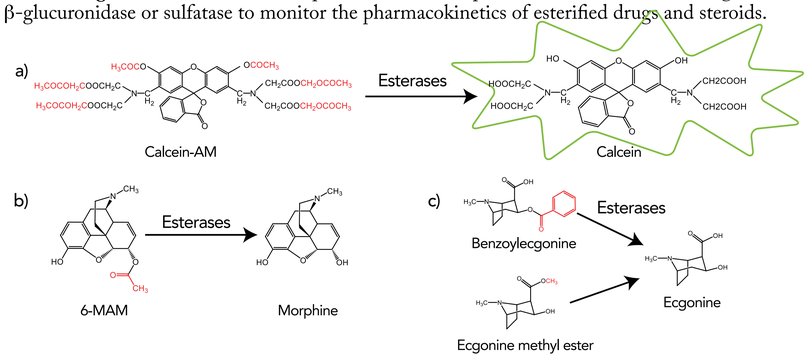

Figure 1. *Schematic representation of (a) fluorogenic dye calcein-acetoxymethyl (Calcein-AM), (b) 6-MAM undergoing de-esterification, (c) Benzoylecgonine and ecgonine methyl ester undergoing de-esterification.*

For this poster, a fluorogenic substrate (***Figure 1a***) as well as 6-MAM (***Figure 1b***) were employed to demonstrate esterase presence in various commercially available β-glucuronidases. Fluorogenic assay shows a strong correlation with 6-MAM conversion. Among those enzymes with low esterase contamination, performance to hydrolyze morphine-6-β-D-glucuronide (M6G), codeine-6-β-D-glucuronide (C6G), and norbuprenorphine glucuronide was assessed.

MATERIALS AND METHODS

Five commercially available β-glucuronidases (crude snail, crude limpet, crude abalone, purified abalone (100 kU/mL), and purified abalone (50 kU/mL)) as well as genetically modified β-glucuronidase (IMCSzyme) were investigated for esterase contamination. Purified β-glucuronidases from abalone were purchased from two different commercial vendors. Purified abalone 1 was provided at >50,000 U/mL and purified abalone 2 was provided at >100,000 U/mL. Calcein-AM (AnaSpec, Inc.) was used in the fluorescence assay to detect esterase activity. To detect the effect of esterase on 6-MAM conversion, drug-free urine was spiked with 500 ng/mL of 6-MAM. 50 µL of urine was hydrolyzed with 75 µL of master mix containing buffer, enzyme, and internal standard (codeine-D6). Each master mix contained the same amount of enzyme (40 µL). The hydrolysis buffer for five enzymes was sodium acetate pH 4.5, while the buffer for IMCSzyme was Rapid Hydrolysis Buffer. The incubation temperature was fixed at 55°C for 0, 1, or 2 hours. The hydrolyzed samples were extracted with DPX CX tips and eluted with 5% ammonium hydroxide in methanol. The eluent was diluted 4-fold in mobile phase A (0.1% formic acid in water). Analyses were performed using a Thermo TSQ Endura triple quadrupole system with a Thermo Vanquish UPLC equipped with a Millipore Chromolith RP-18 column (5 x 4.6 mm). The protocol is represented in ***Figure 2***.

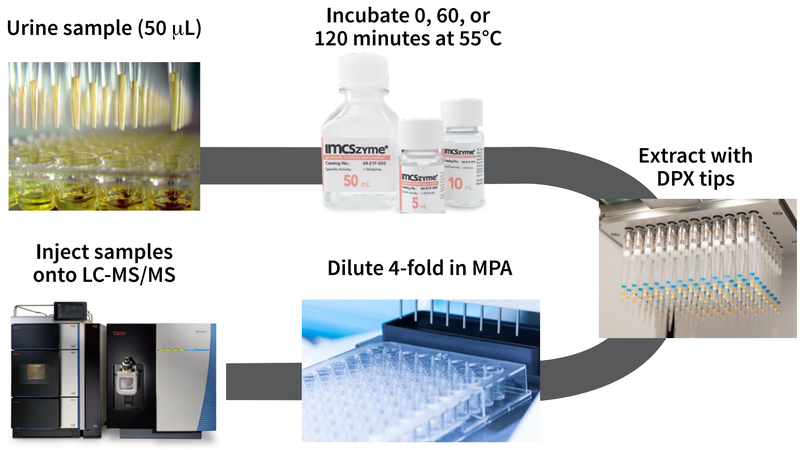

Figure 2. *Schematic representation of hydrolysis and sample preparation procedures*

To challenge the purified β-glucuronidase hydrolysis performance, drug-free urine was spiked with 500 ng/mL of each M6G, C6G, and NBG. 80 µL of urine was hydrolyzed with 90 µL of master mix solution which contained buffer, enzyme, water, and internal standards. The incubation temperature was fixed at 58°C for 30 or 90 minutes. The hydrolyzed samples were extracted with DPX WAX/RP tips and eluted with 60 µL of 1% formic acid in methanol. The eluent was diluted 8-fold in mobile phase A (2.5 mM ammonium formate and 0.1% formic acid in water). Analyses were performed using Waters Xevo TQ-S tandem mass spectrometer with Waters Acquity UPLC equipped with Waters BEH C18 column (2.1 x 50 mm, 1.7 µm).

RESULTS

The purity of β-glucuronidases was visualized by running 4 µL of each enzyme on SDS-PAGE along with a molecular weight marker (***Figure 3***). Protein concentrations were calculated using a Bradford assay: IMCSzyme 1 mg/mL, purified abalone (50 kU/mL) 1.2 mg/mL, purified abalone (100 kU/mL) 0.2 mg/mL, crude abalone 7.3 mg/mL, crude limpet 18.6 mg/mL, and crude snail 47.9 mg/mL. Based on the results from Bradford assay and SDS-PAGE, IMCSzyme and purified abalone (100 kU/mL) were > 95% purity, whereas the other enzymes had < 50% purity.

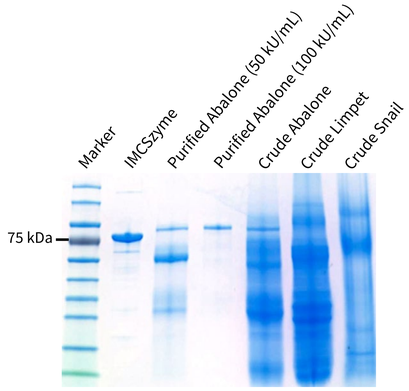

Figure 3. *SDS-PAGE image of several commercially available β-glucuronidases (4 µL was loaded for each enzyme)*

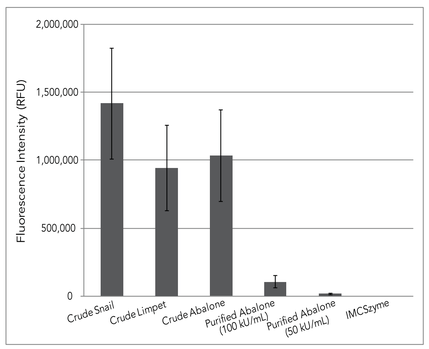

Figure 4. *Relative fluorescence intensities from β-glucuronidases incubated with calcein-AM, showing the fluorescence measured from cleaved calcein.*

To rapidly check for esterase activity, we adapted a fluorogenic assay commonly used for cell viability test. Calcein-AM is an acetoxymethyl ester derivative of fluorescent calcein. The AM ester is colorless and non-fluorescent until de-esterified. The dye has been extensively used to label live cells which contain endogenous intracellular esterases[5]. In a similar manner, various β-glucuronidases are incubated with calcein-AM and the presence of esterases can be observed with fluorescent intensity measurement. High levels of fluorescence were measured in all crude enzymes, implying high levels of esterases (***Figure 4***).

Drug-free urine spiked with 500 ng/mL of 6-MAM was incubated with different β-glucuronidases for 0, 1, and 2 hours. The levels of 6-MAM and morphine were monitored to trace the conversion of 6-MAM to morphine facilitated by the contaminant esterase. Even without incubation, crude abalone shows 20% loss of 6-MAM, and crude snail and crude limpet show 40% loss of 6-MAM (***Figure 5***). On the other hand, purified enzymes still maintained > 90% of 6-MAM. At the end of the 2-hour incubation, all crude enzymes resulted in > 50% loss of 6-MAM, but purified enzymes show > 75% of 6-MAM present (***Figure 5***).

The loss of 6-MAM highly correlates with the increase of morphine level. Among the crude enzymes, crude snail shows the highest loss of 6-MAM (400 ng/mL) and the highest gain of morphine (300 ng/mL) after 2-hour incubation (***Figure 6***). The purified enzymes show minimal 6-MAM conversion and the morphine amount is lower than 30 ng/mL throughout the incubation duration (Figure 6). Comparing calcein (***Figure 4***) and morphine measurements (***Figure 6***) reveals the same trend, showing crude snail enzyme with the highest levels of conversions and both purified abalone (50 kU/mL) and IMCSzyme with no conversion.

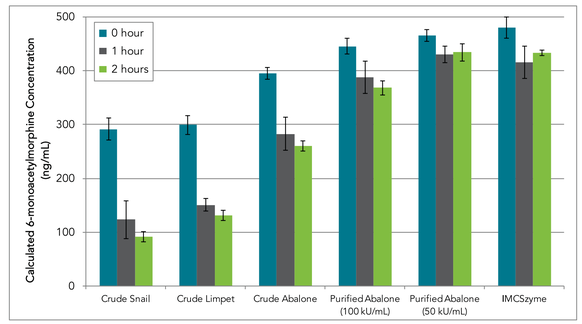

Figure 5. *Average calculated concentrations of 6-MAM after 0, 1, and 2 hours of incubation with the respective enzymes in drug-free urine spiked with 500 ng/mL of 6-MAM*

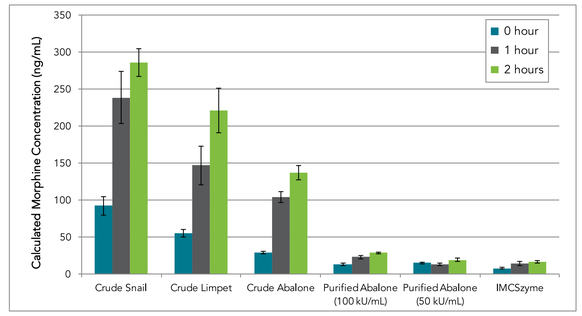

Figure 6. *Average calculated concentrations of morphine after 0, 1, and 2 hours of incubation with the respective enzymes in drug-free urine spiked with 500 ng/mL of 6-MAM*

The purified enzymes, which show relatively low to no esterase contamination, were examined for their glucuronide hydrolysis performance. IMCSzyme achieved 90% hydrolysis within a 30-minute incubation, while both purified abalone β-glucuronidases required a 90-minute incubation to achieve the same hydrolysis percentage.

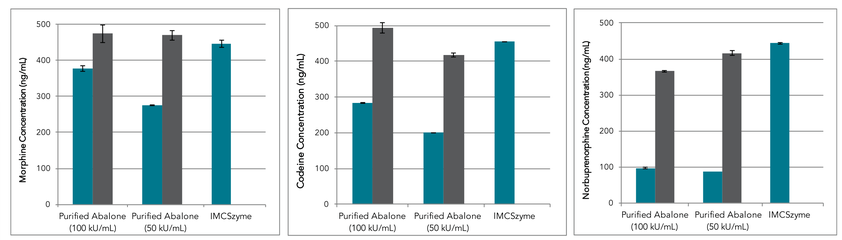

Figure 7. *Average calculated concentrations of morphine, codeine, and norbuprenorphine after 30 and 90 minutes of incubation with the respective purified enzymes in drug-free urine spiked with 500 ng/mL of M6G, C6G, and NBG.*

CONCLUSION

- All crude β-glucuronidases from snail, limpet, and abalone show high fluorescence from calcein-AM conversion, indicating the presence of esterases.
- Enzyme with a high level of calcein fluorescence correlates to 6-MAM conversion to morphine.
- Among the enzymes tested, IMCSzyme is the only one with no fluorescence from calcein conversion.
- Among the purified β-glucuronidases, IMCSzyme requires only 30 minutes of incubation while the other enzymes require a 90-minute incubation for complete glucuronide hydrolysis.
- Preliminary data (not shown) suggests the conversion of benzoylecgonine and ecgonine methyl ester to ecgonine during β-glucuronidase treatment in crude enzymes.

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**Contact: Nikki Sitasuwan - nikki@imcstips.com*
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