APPLICATION NOTE

Hydrolyzing 30,000 ng/mL of codeine-6-β-glucuronide in 15 minutes with greater than 80% efficiency using IMCSzyme®

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Overview

Enzymatic hydrolysis of glucuronides is common practice in many of the urine drug testing laboratories utilizing LC-MS/MS. Limitations on directly monitoring glucuronides on tandem mass spectrometry are higher cost of glucuronide standards or lack of certified reference materials of the glucuronidated drug. However, a major historical challenge for enzymatic hydrolysis has been in opiates. In particular codeine-6-β-D-glucuronide is challenging and generally has poor yield even with incubation time over 2 hours. Although the cutoff concentration of codeine in urine is 2,000 ng/mL according to the guideline from the US Substance Abuse and Mental Health Services Administration (SAMHSA), the various combinations of drugs and their glucuronidated metabolites detected in actual patient urine samples could be as high as 100,000 ng/mL. This study examines the genetically modified β-glucuronidase, IMCSzyme®, and its ability to achieve over 80% recovery of glucuronides in as little as 15 minutes for over 30,000 ng/mL of various glucuronidated opiates in urine.

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Materials and Method

Standards were purchased from Cerilliant and DPX WAX tips were purchased from DPX Labs, LLC (Columbia, SC). Genetically modified β-glucuronidase (IMCSzyme®) is from IMCS, LLC.

30 µL of urine was hydrolyzed with 270 µL of hydrolysis solution (containing rapid hydrolysis buffer, IMCSzyme®, water, and internal standards) with shaking (Vortemp incubator shaker). The enzyme amounts in each hydrolysis reaction ranging from 30 µL to 80 µL were evaluated (Table 1). The incubation temperature was fixed at 55 °C for 15 or 30 minutes. The hydrolyzed samples were extracted with DPX WAX tips and eluted with 1% formic acid in acetonitrile. The eluent was dried under nitrogen and reconstituted with 100 µL of 5% methanol in water. The samples were analyzed on Thermo TSQ Vantage triple quadrupole instrument coupled with an Agilent 1260 HPLC using an Agilent Poroshell EC-C18 column (3.0 x 50 mm, 2.7 µm) heated to 50°C. Mass spectrometer parameters were as follow: electrospray voltage, 4000 V; gas pressure, 60 psi.

Table 1. Ratios for hydrolysis reaction

IMCSzyme® (µL)	Water (µL)	Urine Sample (µL)	Rapid Hydrolysis Buffer (µL)	Internal Standards (in 50% methanol) (µL)	Total Volume (µL)
30	120	30	90	30	300
40	110				
50	100				
60	90				
70	80				
80	70				

Results

The calibration and quality control curves were generated from drug free urine spiked with unconjugated parent analytes. The experimental values correlated with the expected concentrations with R2 values greater than 0.99 (**Figure 1**). To determine the minimum enzyme amount required for the complete hydrolysis of drug free urine spiked with 500 ng/mL of morphine-6-β-D-glucuronide, 500 ng/mL of oxymorphone-3-β-D-glucuronide, 500 ng/mL of hydromorphone-3-β-D-glucuronide, and 30,000 ng/mL of codeine-6-β-D-glucuronide, the drug free urine spiked with glucuronidated analytes was hydrolyzed with 30, 40, 50, 60, 70, or 80 µL of IMCSzyme® for 15 and 30 minutes at 55 °C with shaking. The hydrolyzed compounds for 15 and 30 minute incubation were calculated using the generated calibration curve (**Table 2** and **Table 3**, respectively). The results illustrated that at least 60 µL of the enzyme was needed to achieve greater than 80% hydrolysis of 30,000 ng/mL codeine-6-β-D-glucuronide within 15 minutes (**Figure 2**). For 30-minute incubation, greater than 90% hydrolysis was achieved with 30 µL of IMCSzyme®.

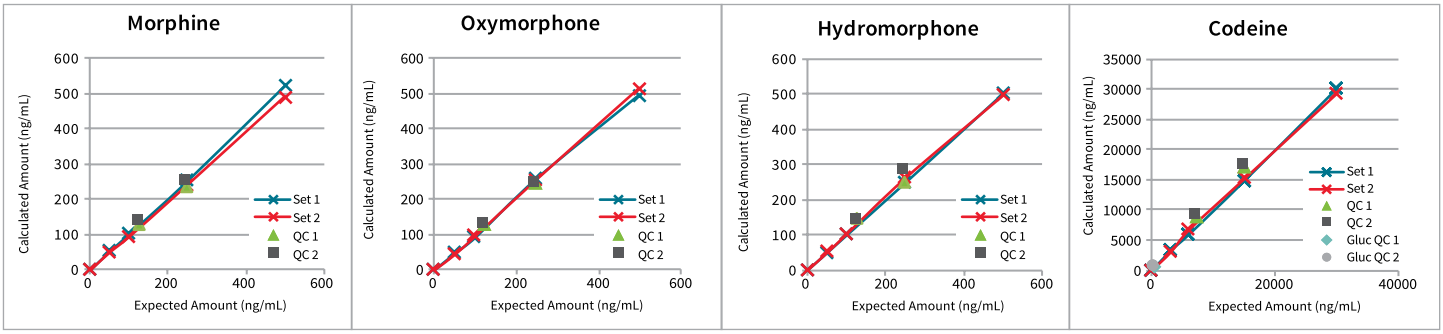

Figure 1. Calibration and quality control curves of morphine (50 – 500 ng/mL), oxymorphone (50 – 500 ng/mL), hydromorphone (50 – 500 ng/mL), and codeine (3,000 – 30,000 ng/mL) in drug free urine using IMCSzyme®. The correlation of the calculated amount with the expected amount was greater than 0.99.

Table 2. Calculated amount of morphine, oxymorphone, hydromorphone, and codeine after 15 minuted incubation with different amounts of enzyme used. Errors are ± 1 standard deviation.

IMCSzyme® Used (µL)	Morphine (ng/mL)	Oxymorphone (ng/mL)	Hydromorphone (ng/mL)	Codeine (ng/mL)
30	430 ± 25.2	368 ± 11.8	403 ± 18.2	17,994 ± 1,664
40	475 ± 21.6	427 ± 14.0	461 ± 11.9	21,132 ± 2,091
50	493 ± 23.0	447 ± 8.9	495 ± 20.5	24,167 ± 960
60	508 ± 28.6	471 ± 16.3	489 ± 1.8	25,306 ± 1639
70	517 ± 35.0	448 ± 23.5	502 ± 21.3	28,134 ± 2504
80	527 ± 25.5	448 ± 9.9	505 ± 14.5	27,373 ± 1477
Expected Concentration	500	500	500	30,000

Table 3. Calculated amount of morphine, oxymorphone, hydromorphone, and codeine after 30 minute incubation with different amounts of enzyme used. Errors are ± 1 standard deviation.

IMCSzyme® Used (µL)	Morphine (ng/mL)	Oxymorphone (ng/mL)	Hydromorphone (ng/mL)	Codeine (ng/mL)
30	512 ± 6.8	468 ± 14.6	491 ± 21.4	27,734 ± 918
40	541 ± 15.3	481 ± 16.1	532 ± 37.8	29,615 ± 669
50	538 ± 11.1	480 ± 36.7	523 ± 11.9	28,476 ± 1068
60	534 ± 15.0	466 ± 11.7	510 ± 5.4	32,942 ± 3100
70	528 ± 3.0	464 ± 11.6	495 ± 9.4	31,123 ± 1203
80	555 ± 16.8	468 ± 17.4	492 ± 9.8	29,712 ± 664
Expected Concentration	500	500	500	30,000

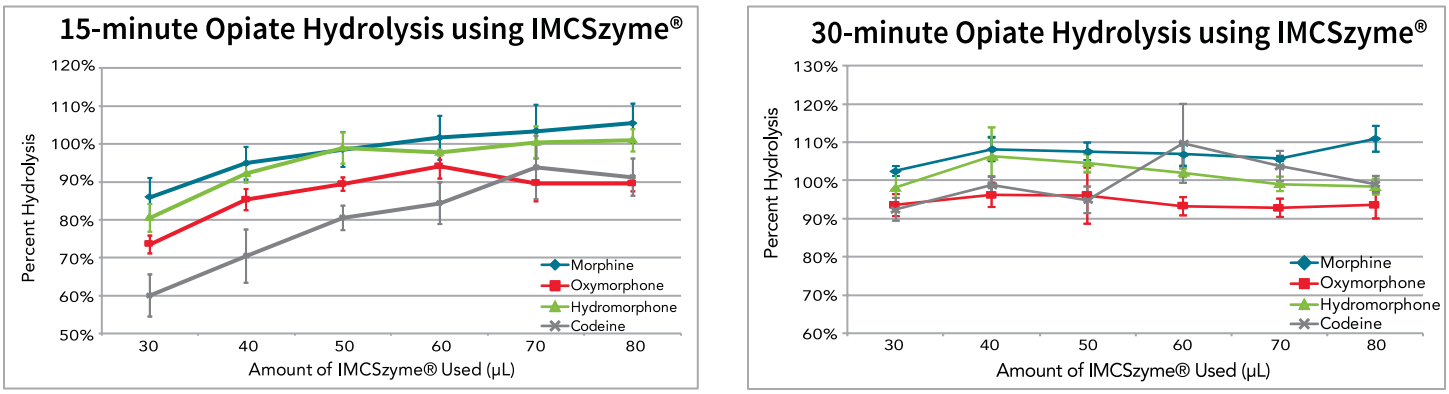

Figure 2. Percent recovery of opiates for 15 minute (*left*) and for 30 minute (*right*) incubation using various amounts of IMCSzyme®. Drug-free urine contains 500 ng/mL of morphine-6-β-D-glucuronide, 500 ng/mL of oxymorphone-3-β-D-glucuronide, 500 ng/mL of hydromorphone-3-β-D-glucuronide, and 30,000 ng/mL of codeine-6-β-D-glucuronide.

Conclusion

Greater than 90% hydrolysis was achieved using 30 µL of IMCSzyme® to hydrolyze 30 µL of urine sample for 30 minutes. This experiment demonstrated that by doubling the enzyme amount to 60 µL, the hydrolysis time to achieve >80% recovery of codeine at 30,000 ng/mL could be cut down to 15 minutes. This reduction in hydrolysis time from 30 minutes to 15 minutes for such a large quantity of glucuronide is essential for increasing throughput while maintaining accuracy. Hydrolysis process is easily adapted for full automation.

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