Evaluation of three beta-glucuronidase enzymes to determine the best hydrolysis conditions for urine samples in clinical toxicology and pain management

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Introduction

Most drugs are excreted in urine as glucuronide conjugates. Hydrolysis using a beta-glucuronidase enzyme to convert the metabolites to their "free" form for analysis increases sensitivity. Red abalone (Kura Biotech), abalone (Campbell Scientific), and recombinant (IMCSzyme) beta-glucuronidase enzymes were evaluated to determine which provided the most complete hydrolysis of glucuronide metabolites without effecting the overall recovery of non-conjugated compounds.

Methods

Extraction Parameters

Four glucuronides were included in a urine glucuronide control to determine the extent of hydrolysis by each enzyme: morphine-3-beta-D-glucuronide, norbuprenorphine glucuronide, oxazepam glucuronide, and 11-nor-9-carboxy-THC glucuronide (THC-COOH) (Cerilliant, Round Rock, TX). The control was prepared so that the amount of non-conjugated drug would equal 100 ng/mL upon complete hydrolysis. A spiked urine sample containing 56 non-conjugated drugs and metabolites at 100 ng/mL was also analyzed to calculate hydrolysis efficiency and compare differences in matrix effects among the 3 enzymes and different hydrolysis conditions. 200 µL of buffer (IMCS buffer for the IMCSzyme or 0.1M ammonium acetate buffer pH 4.0 for the Kura and Campbell enzymes) were added to 200 µL of sample. Next, enzyme at 6250 units/mL was added (25 µL of IMCSzyme or 13 µL of Kura or Campbell). The samples were incubated at either 55°C or 65°C for 30 or 60 minutes. The samples were then pretreated with 4% aqueous phosphoric acid (H_3PO_4). Samples were extracted using EVOLUTE® EXPRESS CX 30 mg 96-well plates using the method shown in Table 1.

Table 1: EVOLUTE® EXPRESS CX Extraction Method

Step	Solvent/Amount
Condition	0.5 mL Methanol (MeOH)
Equilibration	0.5 mL 4% H_3PO_4
Load samples	550 µL sample loaded
Wash #1	1 mL 4% H_3PO_4
Wash #2	1 mL 50:50 MeOH/Water
Dry Columns	1 minute
Elute #1	0.5 mL 78:20:2 DCM/IPA/NH_4OH
Elute #2	0.5 mL 78:20:2 DCM/IPA/NH_4OH
Dry Columns	45 seconds

Samples were dried down under nitrogen and reconstituted in 150 µL 90:10 Mobile Phase A/Mobile Phase B.

Instrument Parameters

Mobile phase A (MOB A): 0.1% formic acid (FA) in water
Mobile phase B (MOB B): 0.1% FA in MeOH
Column: Phenomenex Kinetex 2.6 µm, phenyl hexyl 50 x 4.6 mm
LC: Shimadzu UPLC
Injection volume: 2 µL

Table 2: LC Gradient

Time (min)	MOB B Concentration (%)
0.01	5
2.20	40
4.50	95
5.50	95
5.60	5
6.5	STOP

MS/MS: Sciex 5500 QQQ

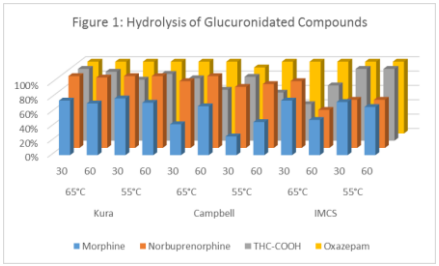
Table 3: MS/MS Transitions and Collision Energies (CE)

Compound	Q1	Q3	CE	Q3	CE
6-monoacetylmorphine	328.1	165.2	60	211.2	30
7-aminoclonazepam	286.0	121.2	50	222.2	30
α-OH-alprazolam	325.1	297.0	40	216.1	60
alprazolam	309.1	281.1	40	205.1	60
amitriptylene	278.1	105.1	50	202.2	70
amphetamine	136.1	119.0	20	91.0	20
benzoylecgonine	290.2	168.1	30	105.0	50
buprenorphine	468.3	396.2	60	414.2	50
carisoprodol	261.2	97.2	20	176.2	10
chlordiazepoxide	299.9	226.9	10	241.0	10
clonazepam	316.1	102.1	32	123.3	7
cocaine	304.1	182.1	30	77.0	70
codeine	300.1	152.1	70	115.1	80
diazepam	285.1	154.1	40	193.0	40
dihyhdrocodeine	302.1	201.1	40	145.1	60
EDDP	278.3	234.2	40	186.2	50
fentanyl	337.2	105.1	50	188.1	40
gabapentin	172.1	154.1	30	137.1	20
hydrocodone	300.1	199.1	40	128.1	70
hydromorphone	286.2	185.1	40	128.0	70
ketamine	238.1	125.1	50	179.2	40
lorazepam	321.0	275.1	50	229.1	40
MDMA	194.1	163.2	20	105.2	40
meperidine	248.2	220.0	30	174.1	30
meprobamate	219.2	158.2	10	97.1	20
methadone	310.2	265.2	20	105.0	20
methamphetamine	150.1	91.2	20	119.2	10
morphine	286.2	152.0	80	165.0	60
naloxone	328.0	128.2	80	115.0	80
N-des-tapentadol	208.2	107.1	50	121.1	20
norbuprenorphine	414.3	83.1	70	101.1	50
nordiazepam	271.1	140.0	50	165.1	50
norfentanyl	233.2	84.1	20	150.0	20
norhydrocodone	286.1	199.1	40	128.1	70
nor-ketamine	224.2	125.1	50	179.2	20
normeperidine	234.2	160.1	20	188.1	20
nortriptylene	264.2	91.1	60	117.1	20
oxazepam	287.1	241.0	30	269.1	20
oxycodone	316.2	241.0	50	256.0	30
oxymorphone	302.1	227.0	50	198.1	60
PCP	244.3	91.0	60	159.3	20
pregabalin	160.2	142.2	15	55.0	35
ritalinic acid	220.1	84.1	50	56.1	60
tapentadol	222.2	107.1	50	121.1	30
temazepam	301.1	255.1	50	177.1	60
THC-COOH	345.2	327.0	23	193.1	38
tramadol	264.2	58.1	60	42.1	80
zolpidem	308.1	235.1	50	236.2	40
zolpidem-phenyl-4-COOH	338.1	265.1	50	266.1	40
butalbital	223.0	42.0	-70	179.8	-20
phenobarb	230.9	42.0	-70	187.9	-10
secobarb	237.0	42.0	-70	193.8	-20
THC-COOH	343.0	299	-30	245.0	-40

Results and Discussion

Enzyme Hydrolysis of Glucuronide Control

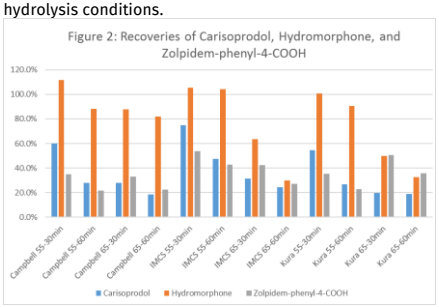
Figure 1 shows the percent hydrolysis (calculated as the ratio of glucuronide/free) for each of the compounds in the glucuronide control for all enzymes and hydrolysis conditions.



The results indicate that there is no one enzyme or incubation time/temperature that is optimal for all four glucuronides tested. The Campbell enzyme did not fully hydrolyze morphine under any conditions. Hydrolysis of THC-COOH is temperature and time dependent for all three enzymes (the amount of hydrolysis is higher at lower times and temperatures). Oxazepam was completely hydrolyzed under all conditions.

Recovery of Non-Conjugated Control

The recoveries of most analytes were consistent (within ±10%) among the three enzymes at the various times and temperatures. Carisoprodol, hydromorphone, and zolpidem-phenyl-4-COOH showed some variability among different enzymes and incubation parameters. Figure 2 shows the recoveries for these compounds for the three enzymes at all hydrolysis conditions.



Conclusions

Based on these results, the Campbell enzyme provided adequate results for most of the glucuronide compounds but hydrolysis of morphine glucuronide was low. The Kura and IMCSzyme enzymes resulted in the most complete hydrolysis of all four glucuronides. The majority of compounds in the non-conjugated control yielded consistent recovery among all enzymes at all hydrolysis conditions. The enzyme and conditions for both hydrolysis of glucuronide metabolites and recovery of non-conjugated compounds should be selected based on the compounds of interest and the required sensitivity.

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