Better Hydrolysis and Increased Efficiency for Urine Drug Testing Using Mixed-Mode Solid Phase Extraction with In-well Hydrolysis

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Introduction

Most drugs and metabolites are conjugated prior to excretion in the urine. Methods to detect drug analytes in urine usually include enzyme hydrolysis to convert glucuronide metabolites back to their free form prior to LC-MS analysis to increase sensitivity. Optimum hydrolysis conditions depend upon the compounds of interest and the chosen enzyme. Here, we present a method for detection of approximately 100 drugs and metabolites in urine using mixed-mode polymeric cation exchange solid phase extraction (SPE). Four different glucuronidase enzymes and several incubation times and temperatures were evaluated to determine optimum hydrolysis conditions for seven commonly detected glucuronide metabolites. Samples were subjected to offline hydrolysis and SPE (EVOLUTE® EXPRESS CX, Biotage, Charlotte, NC) and results compared to an SPE plate that incorporates in-well hydrolysis (EVOLUTE HYDRO CX, Biotage).

Methods

Samples

Four beta-glucuronidase enzymes:

- abalone (Campbell Science, Rockford, IL)
- red abalone (BG100, Kura Biotec, Rancho Dominguez, CA)
- recombinant (BG Turbo, Kura Biotec)
- recombinant IMCSzyme (IMCS, Irmo, SC)

Three sets of hydrolysis conditions:

- 55°C for 10 minutes
- 55°C for 30 minutes
- room temperature for 30 minutes

Seven glucuronide metabolites (Cerilliant, Round Rock, TX) fortified in drug-free urine at 100 ng/mL:

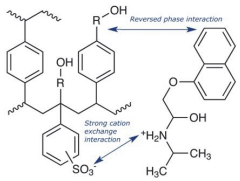
- codeine-6-ß-D-glucuronide
- hydromorphone-3-ß-D-glucuronide
- lorazepam glucuronide
- morphine-3-ß-D-glucuronide
- norbuprenorphine glucuronide
- oxazepam glucuronideoxymorphone-3-ß-D-glucuronide

Fortified Specimens:

- 0.1 mL fortified urine, analyzed in triplicate
- Samples fortified with free drugs (Cerilliant) and metabolites were also hydrolyzed and analyzed
- Hydrolysis efficiency was determined by comparing area counts of free drug analyte to hydrolyzed drug analyte
- Sample set 1 hydrolyzed off-line in a collection plate with internal standard (IS), buffer and enzyme, then loaded onto wells of an EVOLUTE EXPRESS CX 30 mg 96 well plate for SPE
- Sample set 2 added specimen, IS, buffer and enzyme onto wells of the EVOLUTE HYDRO CX 30 mg plate and hydrolyzed in well prior to SPE

Solid Phase Extraction:

- EVOLUTE® EXPRESS and HYDRO CX polymeric mixed mode phase have reversed phase and cation exchange interaction
- Optimized for small molecules
- 30, 50 µm particle size
- 40Å pore size



SPE Extraction Procedure

Step	Reagent	Volume or Time
Pretreatment	4% H_3PO_4	0.1 mL
Wash 1	4% H_3PO_4	1 mL
Wash 2	50% MeOH (aq)	1 mL
Dry	Nitrogen at 30 psi	1 minute
Elute	78:20:2 CH_2Cl_2:MeOH:NH_4OH	2 x 0.75 mL
Dry	Nitrogen at 40°C	approx 20 min
Reconstitute	90:10 MPA:MPB	0.1 mL

SPE was performed using a Biotage Extrahera™

Instrument Parameters

Mobile phase A (MPA): 0.1% formic acid (FA) in water
Mobile phase B (MPB): 0.1% FA in MeOH
Column: Restek Raptor biphenyl 50 x 3 mm, 2.7 µm
LC: Shimadzu Nexera UPLC
Flow Rate: 0.45 mL/min
Injection volume: 2.5 µL
LC Gradient

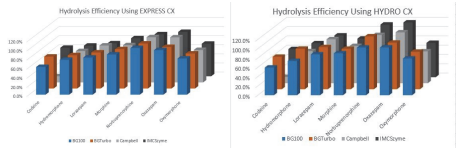
Time (min)	MPB Concentration (%)
0.01	5
2.20	40
4.50	95
5.50	95
5.60	5
6.5	STOP

MS/MS: Sciex 5500 QQQ

Results and Discussion

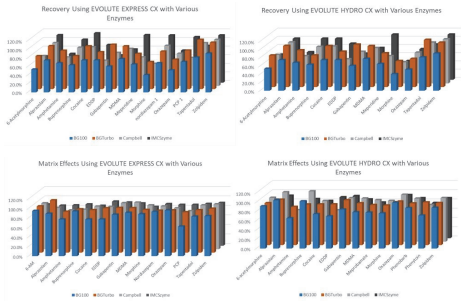
- 55°C for 30 minutes provided the best hydrolysis for all seven glucuronides studied
- Excellent agreement was observed for hydrolysis efficiencies between the EVOLUTE EXPRESS CX and the EVOLUTE HYDRO CX
- Recovery and matrix effect were similar for non-conjugated compounds between the EVOLUTE EXPRESS CX and the EVOLUTE HYDRO CX
- 55°C for 10 min and room temp for 30 min resulted in lower hydrolysis efficiencies for some compounds

Hydrolysis Efficiency 55°C 30 min EVOLUTE® EXPRESS CX vs EVOLUTE EXPRESS HYDRO CX

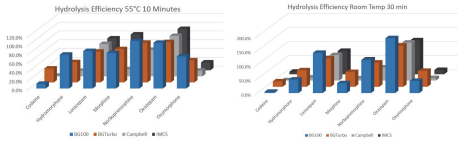


Recovery and Matrix Effect 55°C 30 min

Representative compounds



Hydrolysis Efficiency 55°C 10 min and Room Temp 30 min



Conclusions

- All four enzymes performed well at 55°C for 30 minutes, except the Campbell enzyme on codeine-6-ß-D-glucuronide.
- Hydrolysis efficiencies for many compounds decreased significantly at room temperature for 30 minutes; only the benzodiazepines had good hydrolysis efficiencies.
- Hydrolysis results for opioids at 55°C for ten minutes were mixed, but generally lower than at 30 minutes.
- No significant differences were observed between the EVOLUTE EXPRESS CX plate with off-line hydrolysis and the EVOLUTE HYDRO CX plate with in-well hydrolysis.
- Recovery and matrix effect for all compounds were acceptable.
- The enzyme and conditions for hydrolysis of glucuronide metabolites and recovery of non-conjugated compounds should be selected based on the compounds of interest and required sensitivity.

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