

# Analysis of Commercially Available Beta-Glucuronidase Enzymes and Optimum Hydrolysis Conditions in Urine for Licit and Illicit Drugs Using In-well Hydrolysis

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## Introduction

Most drugs, both licit and illicit, are excreted in urine as glucuronide conjugates. Hydrolysis using beta-glucuronidase converts the glucuronidated metabolites back to their “free” or non-conjugated form, increasing sensitivity for LC-MS/MS analysis. Hydrolysis using red abalone, abalone, and recombinant beta-glucuronidase enzymes was evaluated using an in-well hydrolysis plate to determine which provided the most efficient hydrolysis of glucuronide metabolites without affecting overall recovery of non-conjugated compounds. A glucuronide control containing 8 glucuronide compounds was used to determine the extent of hydrolysis that occurred. A non-conjugated control containing 96 licit and illicit drugs of abuse was evaluated to determine if signal suppression occurred as a result of enzyme hydrolysis.

## Experimental

### Reagents & Materials

Standards: all standards were purchased from Cerilliant (Round Rock, TX). IMCSzyme β-glucuronidase and buffer were provided by IMCS. BGTurbo and BG100 enzymes were provided by Kura Biotech. Campbell enzyme was purchased from Campbell Science. HPLC grade water, methanol (MeOH), and acetonitrile (ACN) were purchased from Sigma Aldrich (St. Louis, MO) in addition to reagent grade, dichloromethane (DCM), phosphoric acid, formic acid, ammonium acetate, and ammonium hydroxide (NH<sub>4</sub>OH). Urine was supplied from healthy drug-free personnel. EVOLUTE® HYDRO CX 30 mg 96-well plates (601-0030-PZ01), EVOLUTE® EXPRESS CX 30 mg 96-well plates (601-0030-PX01) Biotage® Extrahera™ Sample preparation (414001), and Biotage® SPE Dry 96 (SD-9600-DHS-NA) were supplied by Biotage.

### Sample Preparation

#### Standards and Enzyme Hydrolysis

All extracted samples were from a 20 mL working stock of drug free urine spiked with all analytes to yield a final concentration of 100 ng/mL. A glucuronide control was used containing 8 glucuronides (oxazepam, lorazepam, morphine-3-β-D, codeine-6-β-D, hydromorphone-3-β-D, oxymorphone-3-β-D, norbuprenorphine, and THC-COOH). This glucuronide control was spiked so that the final concentration with full hydrolysis would be 100 ng/mL. For each sample analyzed, 100 μL of spiked urine was loaded onto a EVOLUTE® HYDRO CX 96-well plate (or into a 96-well collection plate for the samples undergoing extraction using EVOLUTE® EXPRESS CX). For BGTurbo, 135 μL of master mix was added (master mix contained 100 μL 150 mM ammonium acetate buffer pH 6.8, 10 μL MeOH, 25 μL BGTurbo for each sample). For IMCSzyme, 25 μL IMCSzyme buffer, 55 μL water, 10 μL MeOH, and 20 μL IMCSzyme was added. For BG100 and Campbell enzymes, 100 μL 100 mM ammonium acetate buffer pH 4.0 and 20 μL enzyme was added. All samples were incubated for 30 minutes at 55°C and allowed to cool to room temperature prior to pre-treatment.

#### EVOLUTE® HYDRO CX SPE Procedure

96-well Plates: All extractions were performed using 30 mg mixed-mode cation exchange in-well hydrolysis EVOLUTE® HYDRO CX 96-well plates (P/N: 601-0030-PZ01) or EVOLUTE® EXPRESS CX 96-well plates (P/N: 601-0030-PX01)

#### Biotage® Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocol was carried out using Extrahera, an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The protocol is detailed in **Table 1**. The Sample Load

step was done in the “Equilibration” step of the Software. This allowed for adding of the pretreatment solvent to the samples in the in-well plate and loading of the sample onto the extraction sorbent.

**Sample Pre-treatment:** All samples were acidified with 100 μL of 4% phosphoric acid (4% H<sub>3</sub>PO<sub>4</sub>, aqueous) after reaching room temperature, post hydrolysis.



**Table 1.** Extrahera Processing Parameters for EVOLUTE® HYDRO CX

Step	Volume (μL)	Solvent	Time (sec)	Pressure (Bar)
<b>Load</b>	Whole Sample	N/A	60	0.8
			30	1.2
<b>Wash #1</b>	1000	4% H <sub>3</sub> PO <sub>4</sub>	45	1.0
			30	2.0
<b>Wash #2</b>	1000	50% MeOH	45	1.0
			30	2.0
<b>Plate Dry</b>	N/A	N/A	60	5.0
<b>Elution #1</b>	750	DCM/MeOH/NH <sub>4</sub> OH [78:20:2]	45	0.8
			45	1.0
<b>Elution #2</b>	750	DCM/MeOH/NH <sub>4</sub> OH [78:20:2]	45	0.8
			45	1.0
<b>Plate Dry</b>	N/A	N/A	45	5.0

**Dry Down and Sample Reconstitution:** The eluents were evaporated under a stream of heated (40 °C) nitrogen at 40 L/min using a Biotage® SPE Dry 96. All extracts were reconstituted with 100 μL of 90:10 MOB A/MOB B and analyzed via LC-MS/MS.

### Chromatography Parameters

**Table 2.** Shimadzu Nexera X2 UHPLC Parameters

<b>Column</b>	Restek Raptor Biphenyl 2.7 μm, 50 x 3.0 mm
<b>MOB A</b>	0.1% Formic Acid (aq)
<b>MOB B</b>	0.1% Formic Acid in MeOH
<b>Flow Rate</b>	0.45 mL/min
<b>Column Temp</b>	40 °C
<b>Sample Temp</b>	15 °C
<b>Inj. Volume</b>	2.5 μL

**Table 3.** Shimadzu Nexera X2 UHPLC Gradient

Time	%A	%B
0.01	95	5
0.50	85	15
5.25	65	45
7.5	5	95
7.7	5	95
7.75	95	5
9.25	-	STOP

### Mass Spectrometry Parameters

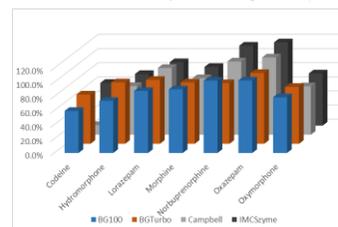
Instrument: SCIEX 5500 triple quadrupole Mass Spectrometer with Turbo Ionspray® Ion interface (Foster City, CA). Optimized source parameters are shown in **Table 4**. Retention window for sMRM was set at 60 seconds with target scan time at 1 second.

**Table 4.** SCIEX 5500 ESI (+) Turbo Ionspray® Source Parameters.

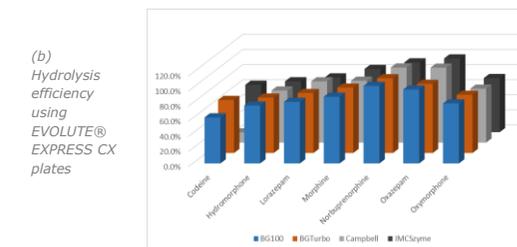
<b>Ionization Spray Voltage</b>	+1500(V)	<b>CAD</b>	8 (V)
<b>Source Temp</b>	600 °C	<b>GS1</b>	50
<b>Curtain</b>	30 (V)	<b>GS2</b>	50

## Results

Hydrolysis efficiency, recovery, and matrix effects were all investigated when using the EVOLUTE® HYDRO CX 30 mg 96-well plates. The EVOLUTE® HYDRO CX plates were also compared to the EVOLUTE® EXPRESS CX plates to ensure that the chemistry remained unchanged. Hydrolysis efficiency can be seen in **figure 2a** for the EVOLUTE HYDRO CX plates and in **figure 2b** for the EVOLUTE® EXPRESS CX plates. **Table 5** shows the hydrolysis efficiency for the EVOLUTE® HYDRO CX plates using all enzymes.



**Figure 2. (a)** Hydrolysis efficiency using EVOLUTE® HYDRO CX plates for in-well hydrolysis.

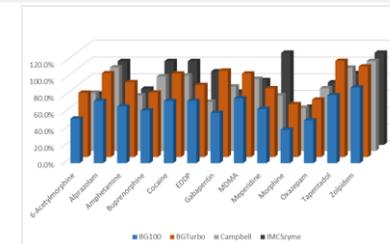


**(b)** Hydrolysis efficiency using EVOLUTE® EXPRESS CX plates

**Table 5.** Hydrolysis Efficiency Using EVOLUTE® HYDRO CX 30 mg Plates

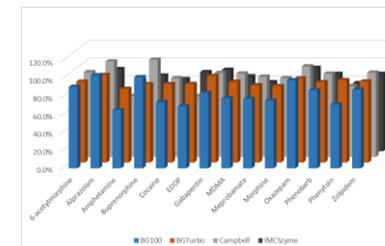
	BG100	BGTurbo	Campbell	IMCSzyme
<b>Codeine</b>	59.5%	69.8%	13.7%	60.4%
<b>Hydromorphone</b>	73.7%	87.0%	69.0%	73.1%
<b>Lorazepam</b>	87.8%	90.6%	94.5%	89.8%
<b>Morphine</b>	90.1%	87.0%	80.1%	83.0%
<b>Norbuprenorphine</b>	102.4%	85.9%	103.7%	113.1%
<b>Oxazepam</b>	102.6%	100.2%	109.4%	117.8%
<b>Oxymorphone</b>	78.9%	80.6%	68.5%	73.8%

As can be seen, the hydrolysis efficiencies between the in-well hydrolysis plate (EVOLUTE® HYDRO CX) and the out-of-well hydrolysis plate (EVOLUTE® EXPRESS CX) are nearly identical. This indicates that there is no difference between the chemistries of the plates, which results in the same hydrolysis efficiencies and capabilities of the plates. Represented in **figure 3** are the recoveries for several compounds in the 96 compound panel. These compounds are a representation of the majority of the drug classes that are present in the method. Most recoveries ranged from 60-100%. Several compounds had lower recoveries. These compounds bind to the sorbent by reverse phase interactions as opposed to cation exchange interactions. **Figure 4** shows the matrix effects for several compounds in the method. As can be seen, some enzymes caused more suppression than other enzymes for the analytes shown.



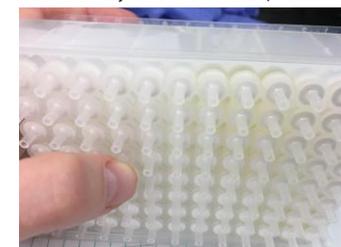
**Figure 3:** Recoveries using EVOLUTE® HYDRO CX 30 mg 96-well plates for all drug classes

**Figure 4:** Matrix Effects using EVOLUTE® HYDRO CX 30 mg 96-well plates for all enzymes.



Use of different enzymes results in different recoveries and matrix effects. This could be caused by the type of beta-glucuronidase that was used. The Campbell Science enzyme is from abalone; the Kura

**Figure 5:** EVOLUTE® HYDRO CX plate after hydrolysis incubation showing that sample is held above the sorbent for the duration of hydrolysis



BG100 enzyme is from red abalone; the Kura BGTurbo and IMCSzyme enzymes are both recombinant. **Figure 5** depicts samples being held in the EVOLUTE® HYDRO CX in-well hydrolysis plate. This picture was taken after a 30 minute, 55°C incubation. As can be seen, there is no breakthrough of sample when using the EVOLUTE® HYDRO CX plates.

## Conclusions

- » EVOLUTE® HYDRO is an efficient way to perform in-well hydrolysis for a 96 analyte panel.
- » EVOLUTE® HYDRO CX results in a time savings as the conditioning and equilibration steps are eliminated. There is also a reduced risk for carryover and contamination as a sample transfer step is eliminated.
- » When comparing the 4 beta-glucuronidase enzymes, it seems that the BGTurbo enzyme results in the best overall hydrolysis efficiency, especially when opiates are included in the panel. The Campbell enzyme resulted in the lowest hydrolysis efficiency when analyzing a drug panel containing opiates.