An Automated Approach to Urine Sample Preparation Employing Room Temperature Enzymatic Hydrolysis

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Introduction

Routine testing for drugs of abuse (DOA) in urine is commonly performed by many clinical, forensic, and pain management laboratories. The method of testing varies but can often provide unwanted load stress for day-to-day operation in labs. Most seek simplified yet reliable and robust modes of sample preparation and analysis. Many laboratories also desire automation for simpler, more reliable methods of extraction that reduce labor costs. Hydrolysis was conducted using the Kura Biotec B-One enzyme and the manufacturer's suggested conditions on the Biotage® Extrahera™ Automated Extraction System. A panel of 10 conjugated metabolites of common DOAs were hydrolyzed and extracted by supported liquid extraction using ISOLUTE® SLE+. Hydrolysis efficiencies were calculated to assess method functionality.

The B-One enzyme from Kura Biotec is an enhanced recombinant enzyme that allows for a short, room temperature hydrolysis. The enzyme comes as a mix with a buffer and is added to the urine sample. It was recommended that the enzyme be added in a 1:1 ratio to the urine sample by the manufacturer.

The Biotage® Extrahera™ Automated Extraction System provides automation of the extraction process for supported liquid extraction, solid phase extraction, phospholipid depletion, protein precipitation, or dual mode extraction methods. The full extraction process from sample pretreatment and loading onto an extraction plate/cartridge to washing of the plate/cartridge and elution can be performed on the system without the need for analyst intervention.

Methods

Reagents and Materials

All standards were purchased form Cerilliant (Round Rock, TX). LC-MS grade water and methanol (MeOH) were purchased from Fisher Scientific (Waltham, MA). Reagent grade dichloromethane (DCM), formic acid, ammonium hydroxide (NH_4OH), and isopropanol (IPA) were purchased from Sigma Aldrich (St. Louis, MO). B-One enzyme was supplied by Kura Biotec (Rancho Dominguez, CA). ISOLUTE SLE+ 400 µL extraction plates (820-0400-P01), Biotage® Extrahera™, and Biotage® SPE Dry 96™ (SD-9600-DHS-NA) were supplied by Biotage (Charlotte, NC). The LC column was provided by Restek (Bellefonte, PA). Human urine was provided by a drug-free donor.

Sample Preparation

A sample containing 10 glucuronidated DOA compounds (codeine-6-β-D glucuronide, morphine-3-β-D glucuronide, hydromorphone glucuronide, oxymorphone glucuronide, norbuprenorphine glucuronide, naloxone glucuronide, lorazepam glucuronide, oxazepam glucuronide, amitriptyline glucuronide, and THC-COOH glucuronide) was prepared in drug free urine so that the final concentration of non-conjugated drug in a fully hydrolyzed sample was 100 ng/mL. A separate sample containing the non-conjugated form of the 10 DOA compounds at 100 ng/mL was also made.

Extraction Procedures

Both the hydrolysis and extraction were performed on the Biotage® Extrahera™ automated system. Urine samples (100 µL) were aliquoted into a 96-well plate. The plate was loaded onto the Extrahera™ system (Figure 1).

Figure 1. *Biotage® Extrahera™ Automated Extraction System.*

Methanol (10 µL) was added to the urine sample to mimic the addition of internal standard. B-One enzyme was then added to the urine samples at a 1:1 ratio and samples were incubated at room temperature on the system for 10 minutes. After incubation, 100 µL of 0.1% NH_4OH (aqueous) was added to each sample as a pretreatment buffer. The samples were then loaded onto the ISOLUTE SLE+ 400 µL extraction plate. Elution was performed using 2 x 750 µL of 95:5 DCM/IPA (see Figure 2 for the ISOLUTE® SLE+ procedure).

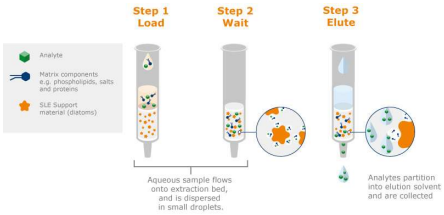

Figure 2. *Schematic of ISOLUTE® SLE+ Supported Liquid Extraction procedure.*

Extrahera™ parameters are shown in table 1.

Step	Solvent	Pressure (bar)	Time
Pretreatment	100 µL B-One enzyme mix	N/A	2.5 min (time to aliquot)
Pause	Incubation		7.5 min
Pretreatment	0.1% NH_4OH	N/A	N/A
Load	Full 300 µL sample	5.0	30 sec
Wait	N/A	N/A	5 min
Elution	2 x 750 µL 95:5 DCM/IPA	5.0	20 sec

Table 1. *Extrahera™ parameters*

Dry Down and Sample Reconstitution

Samples were eluted into a 2 mL collection plate on the Extrahera™ system. All samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage SPE Dry™ (shown in Figure 3). Extracts were then reconstituted with 100 µL of 90:10 mobile phase A/mobile phase B and analyzed via LC-MS/MS.

Figure 3. *Biotage® SPE Dry™ evaporator.*

Chromatography Parameters

UPLC	Parameter
Column	Restek Raptor Biphenyl 2.7 µm, 50 x 3.0 mm
MPA	0.1% formic acid (aq)
MPB	0.1% formic acid in MeOH
Flow Rate	0.45 mL/min
Column Temp	40°C
Sample Temp	20°C
Injection Volume	2.5 µL

Table 2. *Shimadzu Nexera X2 UPLC Parameters*

The LC gradient started at 95% aqueous and gradually decreased to 5% aqueous over a 9.0-minute total run time. This allowed for full separation of all compounds in the panel.

Mass Spectrometry Parameters

A SCIEX 5500 triple quadrupole mass spectrometer with Turbo IonSpray Ion Interface (Foster City, CA) was used. Optimized source parameters are shown in table 3 (sMRM transition parameters are not shown but are available upon request). Retention windows for sMRM were set at 45 seconds with a target scan time of 2.85 seconds.

Ionization Spray Voltage	+1500 (V)
Source Temp	600°C
Curtain Gas	30 (V)
CAD	Medium
GS1	50
GS2	70

Table 3. *SCIEX 5500 Triple Quadrupole ESI (+/-) Turbo Ionspray Source Parameters*

Results

Hydrolysis Efficiencies

A full 96 well plate was used. Rows A through D on the plate contained urine samples with the glucuronidated compounds (labeled as gluc in Figure 4 and shown in blue). Rows E through H contained urine samples with the non-conjugated compounds (labeled as non-gluc in Figure 4 and shown in orange).

	1	2	3	4	5	6	7	8	9	10	11	12
A	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc
B	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc
C	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc
D	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc	gluc
E	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc
F	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc
G	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc
H	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc	non-gluc

Figure 4. 96-well plate map.

Hydrolysis efficiencies (area counts of hydrolyzed samples/area counts of non-conjugated samples) were calculated across the entire plate. They were also calculated separately for each column in the plate, as the B-One hydrolysis enzyme was added to each column separately when using the Extrahera™ system. Percent CVs were within 17% for all 10 glucuronidated compounds across the plate. Table 4 shows the average hydrolysis efficiencies, standard deviations, and % CVs for the 10 glucuronidated compounds.

Compound	Average Hydrolysis Efficiency	Standard Deviation	% CV
Amitriptyline	104.7%	0.073	7.0%
Codeine	77.1%	0.070	9.1%
Hydromorphone	108.8%	0.145	13.4%
Lorazepam	115.2%	0.051	4.4%
Morphine	99.4%	0.163	16.5%
Naloxone	115.5%	0.112	9.7%
Norbuprenorphine	100.2%	0.051	5.1%
Oxazepam	113.1%	0.047	4.2%
Oxymorphone	110.8%	0.124	11.2%
THC-COOH	94.4%	0.158	16.8%

Table 4. *Hydrolysis efficiencies across the plate.*

Figure 5 shows the hydrolysis efficiencies for all columns in the 96-well plate. Column 10 had slightly higher hydrolysis efficiencies than the other columns in the 96-well plate, however, % CVs were all still within 20% when including the data from column 10 (see Table 4).

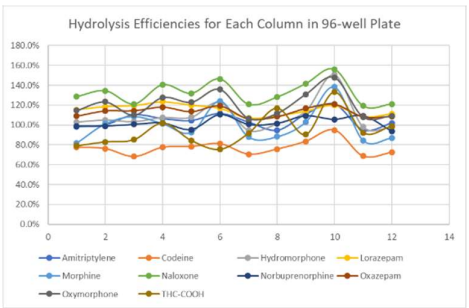

Figure 5. *Hydrolysis efficiencies for each column in a 96-well plate.*

Conclusions

The B-One enzyme resulted in almost complete hydrolysis of all glucuronidated compounds in the panel. The B-One enzyme used with the Biotage® Extrahera™ Automated Extraction System provided complete unattended processing: hydrolysis, pretreatment, and SLE+ extraction of an entire 96 well plate in 54 minutes.

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