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An In-Well Hydrolysis Solid Phase Extraction Method Using EVOLUTE® HYDRO CX for 96 Licit and Illicit Drugs of Abuse from Urine Prior to UPLC-MS/MS

Introduction

This application note describes the extraction of 96 licit and illicit drugs of abuse from urine prior to UPLC-MS/MS analysis using EVOLUTE® HYDRO CX 96-well plates.

EVOLUTE® HYDRO CX plates offer an efficient way to perform hydrolysis in the well of the extraction plate. This method provides high analyte recovery, reduced extraction time due to the elimination of a sample transfer step as well as the elimination of the column conditioning and equilibration steps, and a reduced risk for sample carryover or cross-contamination due to the elimination of the sample transfer step.

Analytes

6-Acetylcodeine, 6-Acetylmorphine, 7-Aminoclonazepam, 7-Aminoflunitrazepam, Alprazolam, Amitriptyline, Amphetamine, Aripiprazole, Atomoxetine, Benzoyllecgonine, Buprenorphine, Bupropion, Buspirone, Butalbital, Carbazepine, Carisoprodol, Chlordiazepoxide, Chlorpheniramine, Chlorpromazine, Clomipramine, Clonazepam, Clonidine, Clozapine, Cocaethylene, Cocaine, Codeine, Cyclobenzaprine, Desalkylflurazepam, Dextromethorphan, Diazepam, Dihydrocodeine, Duloxetine, EDDP, Ethyl Sulfate, Fentanyl, Fluoxetine, Gabapentin, Haloperidol, Hydrocodone, Hydromorphone, Hydroxylprazolam, Hydroxybupropion, Hydroxymidazolam, Hydroxytriazolam, Imipramine, Ketamine, Lamotrigine, Levetiracetam, Lidocaine, Lorazepam, mCPP, MDA, MDEA, MDMA, Meperidine, Meprobamate, Methadone, Methamphetamine, Methaqualone, Methcathinone, Midazolam, Morphine, N-desmethylclomipramine, N-desmethylnortriptyline, N-desmethylpentadolate, Naloxone, Norbuprenorphine, Nordiazepam, Norfentanyl, Norhydrocodone, Norketamine, Normeperidine, Noroxymorphone, Norpropoxyphene, Nortriptyline, Oxazepam, Oxcarbazepine, Oxycodone, Oxymorphone, PCP, Pentobarbital, Phenobarbital, Phenytoin, Pregabalin, Quetiapine, Risperidone, Ritalinic Acid, Secobarbital, Tapentadol, Temazepam, THC-COOH, Topiramate, Tramadol, Triazolam, Trimipramine, Venlafaxine, Zolpidem, Zolpidem-phenyl-4-carboxylic acid

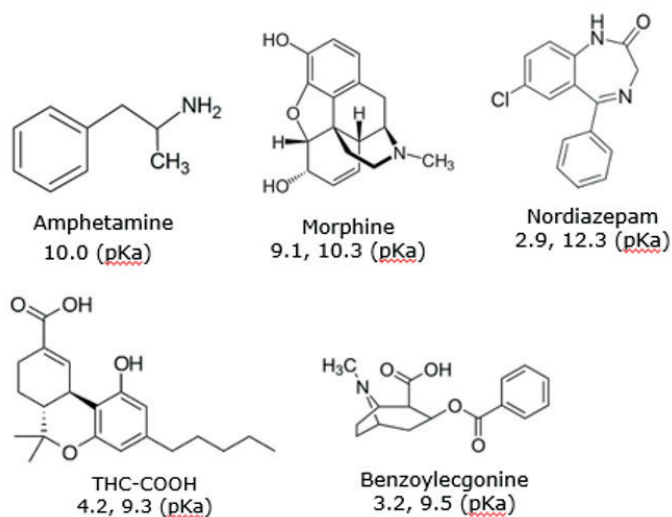


Figure 1. Structures Amphetamine, Morphine, Nordiazepam, THC-COOH, Benzoyllecgonine.

Sample Preparation Procedure

Format

EVOLUTE® HYDRO CX 10 mg 96-well plates, part number 601-0010-PZ01

EVOLUTE® HYDRO CX 30 mg 96-well plates, part number 601-0030-PZ01

Sample Pre-treatment

Add 100 μ L urine to wells of EVOLUTE® HYDRO CX plate. Choose an enzyme to use for hydrolysis. For Kura BGTurbo, add 200 μ L of the master mix (master mix contains 100 μ L 150 mM sodium phosphate buffer, pH 6.8, 15 μ L internal standard mix in methanol, 30 μ L BGTurbo and 55 μ L water per sample). For IMCSzyme, add 25 μ L IMCS buffer, 55 μ L water, 10 μ L internal standard mix in methanol, and 20 μ L IMCSzyme. For Kura BG100 or Campbell enzymes, add 100 μ L 100 mM ammonium acetate buffer, pH 4.0 and 20 μ L enzyme. Mix, then cover plate and incubate at 55 $^{\circ}$ C for 30 minutes. Allow samples to cool to room temperature and pretreat with 100 μ L 4% phosphoric acid (aqueous).

Analyte Extraction

Load

Load samples by applying positive pressure.

Wash 1

Elute interferences with 4% phosphoric acid (aqueous) (1 mL). Apply positive pressure to push wash solvent through columns.

Wash 2

Wash columns with 50:50 MeOH/water (1 mL). Apply positive pressure to push wash solvent through columns. Apply full pressure to the columns and dry for 60 seconds.

Analyte Elution

10 mg plate: Apply DCM/MeOH/NH₄OH (78:20:2, v/v, 500 µL)

30 mg plate: Apply DCM/MeOH/NH₄OH (78:20:2, v/v, 750 µL)

Apply positive pressure to push the elution solvent into the collection plate. Apply a second aliquot of DCM/MeOH/NH₄OH (78:20:2, v/v) and apply positive pressure to push the elution solvent through the columns.

Biotage® PRESSURE+ 96 and Biotage® Extrahera™ conditions for the load, wash, and elution steps are shown in Table 1 (10 mg plate) and Table 2 (30 mg plate). When using the Extrahera to extract, the pretreatment and loading of samples was performed in the “Equilibration” step of the software. This allowed for the addition of the pretreatment solvent to the wells of the EVOLUTE® HYDRO plate and then positive pressure to allow for loading of the samples onto the plate.

Post Elution and Reconstitution

Evaporate extracts in a stream of nitrogen using a Biotage® SPE Dry 96 (40 °C, 20-40 L/min).

Reconstitute extracts with 100 µL of 90:10 mobile phase A/ mobile phase B (0.1% formic acid in water/0.1% formic acid in MeOH) and vortex. Transfer collection plate to LC-MS/MS autosampler.

UHPLC Conditions

Instrument

Shimadzu Nexera x2

Column

Restek Raptor Biphenyl 50 x 3 mm, 2.7 µm

Column oven

40°C

Injection Volume

2.5 µL

Mobile Phase

A: 0.1% formic acid in water

B: 0.1% formic acid in MeOH

Flow rate: 0.45 mL/min

Table 1. EVOLUTE® HYDRO CX 10 mg 96-Well Plate Parameters.

Biotage® PRESSURE+ 96 Parameters		Biotage® Extrahera™ Parameters	
Step	Pressure	Step	Pressure
Sample Load	2 psi (30 sec)	Sample Load	0.8 bar (60 sec)
	5 psi (30 sec)		1.0 bar (30 sec)
	8 psi (60 sec)		
Wash 1	2 psi (30 sec)	Wash 1	1.0 bar (45 sec)
	5 psi (30 sec)		2.0 bar (45 sec)
	8 psi (60 sec)		
Wash 2	2 psi (30 sec)	Wash 2	1.0 bar (45 sec)
	5 psi (30 sec)		2.0 bar (45 sec)
	8 psi (60 sec)		
Elute 1	2 psi (30 sec)	Elute 1	0.5 bar (30 sec)
	4 psi (30 sec)		0.8 bar (30 sec)
	6 psi (60 sec)		1.0 bar (30 sec)
Elute 2	2 psi (30 sec)	Elute 2	0.5 bar (30 sec)
	4 psi (30 sec)		0.8 bar (30 sec)
	6 psi (60 sec)		1.0 bar (30 sec)
Plate Dry	40 psi (60 sec)	Plate Dry	5.0 bar (60 sec)

Table 2. EVOLUTE® HYDRO CX 30 mg 96-Well Plate Parameters.

Biotage® PRESSURE+ 96 Parameters		Biotage® Extrahera™ Parameters	
Step	Pressure	Step	Pressure
Sample Load	4 psi (30 sec)	Sample Load	0.8 bar (60 sec)
	6 psi (30 sec)		1.5 bar (30 sec)
	10 psi (60 sec)		
Wash 1	4 psi (30 sec)	Wash 1	1.0 bar (45 sec)
	6 psi (30 sec)		2.0 bar (30 sec)
	10 psi (60 sec)		
Wash 2	4 psi (30 sec)	Wash 2	1.0 bar (45 sec)
	6 psi (30 sec)		2.0 bar (30 sec)
	10 psi (60 sec)		
Plate Dry	40 psi (60 sec)	Plate Dry	5.0 bar (60 sec)
Elute 1	4 psi (30 sec)	Elute 1	0.8 bar (45 sec)
	6 psi (30 sec)		1.0 bar (45 sec)
	8 psi (60 sec)		
Elute 2	4 psi (30 sec)	Elute 2	0.8 bar (45 sec)
	6 psi (30 sec)		1.0 bar (45 sec)
	8 psi (60 sec)		
Plate Dry	40 psi (60 sec)	Plate Dry	5.0 bar (30 sec)

Table 3: UHPLC Gradient Conditions.

Time	%B
0.01	5
0.50	15
5.25	45
7.50	95
7.70	95
7.75	5
9.25	STOP

MS/MS Conditions

Instrument

Sciex 5500 Triple Quadrupole

Mode

ESI (+/-)

Temperature

600°C

Ionspray Voltage

1500 V

Results and Discussion

This EVOLUTE® HYDRO CX method resulted in the majority of analyte recoveries ranging from 60–100%, as can be seen in Figure 2 (several compounds are shown to represent each drug class). There are several exceptions to this as some compounds only bind to the columns using reverse-phase interactions as opposed to cation exchange interactions. The higher amount of methanol in wash 2 leads to some of these compounds being washed away. However, the higher methanol wash also results in cleaner extracts.

Matrix effects for several of the compounds in the panel to represent each drug class can be seen in Figure 3. Matrix effects for most compounds are below 15%.

Hydrolysis efficiency of each compound was also investigated and is shown in Figure 4. As is shown in the Figure, different enzymes result in different hydrolysis efficiencies.

Ordering Information

Part Number	Description	Quantity
601-0010-PZ01	EVOLUTE® HYDRO CX 10 mg 96-Well Plate	1
601-0030-PZ01	EVOLUTE® HYDRO CX 30 mg 96-Well Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
414001	Biotage® Extrahera™ Automation System	1
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120V	1

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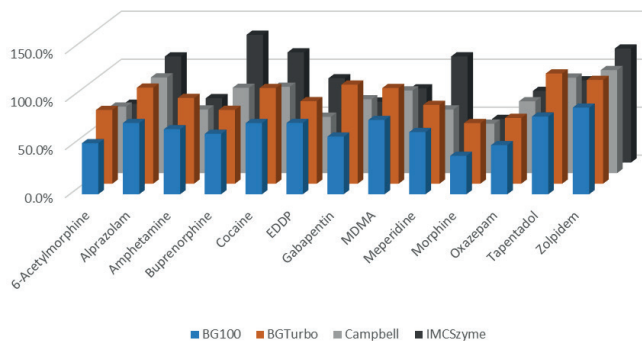


Figure 2. Recovery Using EVOLUTE® HYDRO CX 30 mg Plate with Various Enzymes.

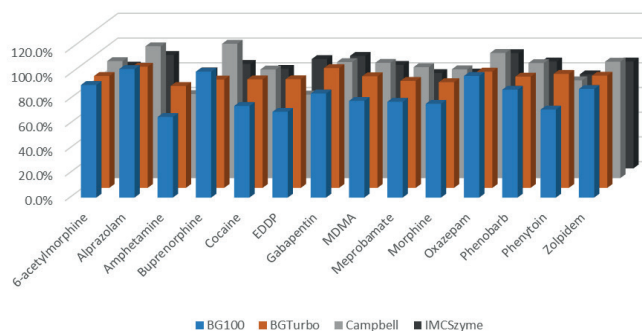


Figure 3. Matrix Effects Using EVOLUTE® HYDRO CX 30 mg Plate with Various Enzymes.

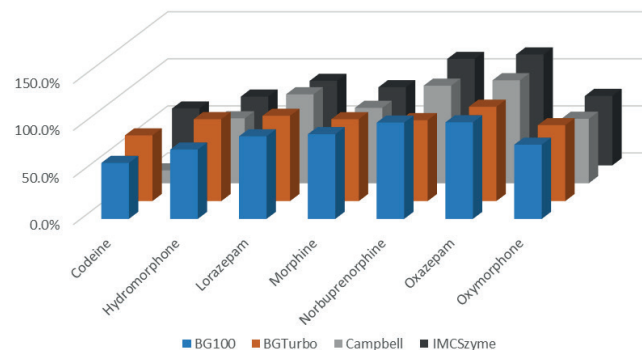


Figure 4. Hydrolysis Efficiency Using EVOLUTE® HYDRO CX 30 mg Plate.

Part Number: AN886.V.1

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