Determination of Optimal Sample Size with Microelution without Dry Down Using Solid Phase Extraction for a Drugs of Abuse Panel

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Introduction

Drugs of abuse (DOA) testing is vital for many clinical, forensic, and pain management laboratories One hindrance with common testing methods is that patient samples are not always available in high quantities. Therefore, it is important to use as little sample as possible during the extraction process, without detrimentally effecting DOA recoveries. Many laboratories find it necessary to include as many DOA analytes as possible within a single extraction method to maximize time and cost savings during analysis. An extraction protocol using a 10 mg mixed-mode cation exchange sorbent (EVOLUTE® EXPRESS CX) was developed and various sample sizes were assessed to determine the optimal sample volume for a 98-compound DOA panel. A method involving microelution off of the sorbent requiring no evaporation or reconstitution steps were also developed.

Methods

Reagents and Materials

Standards

All standards were purchased from Cerilliant (Round Rock, TX). HPLC grade water and methanol (MeOH) were purchased from Sigma Aldrich (St. Louis, MO) in addition to reagent grade dichloromethane (DCM), formic acid, phosphoric acid (H_3PO_4), and ammonium hydroxide (NH_4OH). EVOLUTE® EXPRESS CX (10 mg bed) extraction plate (601-0010-PX01), Biotage® PRESSURE+ 96 position positive pressure manifold (PPM-96), and Biotage® SPE Dry 96 (SD-9300-DHS-NA) were supplied by Biotage. Restek provided the LC column.

Sample Preparation

Urine Sample Preparation

A drug-free urine sample was donated. This urine was spiked with 100 ng/mL of the 98 drugs of abuse compounds. These DOA compounds included drugs from multiple drug classes: anticonvulsants, barbiturates, benzodiazepines, opioids, antidepressants, stimulants, hallucinogens, cannabinoids and antipsychotics.

Hydrolysis Parameters

Four different hydrolysis enzymes were tested: Campbell (Campbell Science, Rockford, IL), BG100 (Kura Biotech, Rancho Dominguez, CA), BGTurbo (Kura Biotech, Rancho Dominguez, CA), and IMCSzyme (IMCS, Irmo, SC). Sample sizes of 25 µL, 50 µL, 75 µL, and 100 µL were tested to determine the optimum sample size. The different sample sizes required different hydrolysis preparations shown in table 1.

	25 µL sample	50 µL sample	75 µL sample	100 µL sample
Campbell/BG100				
100 mM ammonium acetate buffer, pH 4.0	25 µL	50 µL	75 µL	100 µL
Enzyme	10 µL	10 µL	15 µL	20 µL
IMCSzyme				
IMCS buffer	10 µL	15 µL	20 µL	25 µL
Water	15 µL	30 µL	40 µL	55 µL
Methanol	10 µL	10 µL	10 µL	10 µL
enzyme	10 µL	10 µL	15 µL	20 µL
BGTurbo				
150 mM sodium phosphate buffer, pH 6.8	25 µL	50 µL	75 µL	100 µL
Water	15 µL	30 µL	40 µL	55 µL
Methanol	10 µL	10 µL	10 µL	10 µL
enzyme	10 µL	15 µL	20 µL	25 µL

Table 1. *Hydrolysis parameters for different sample sizes.*

EVOLUTE® EXPRESS CX SPE Procedure

The extraction method for the samples that underwent evaporation and reconstitution is shown in table 2. The microelution combinations tested are shown in table 3. The microelution protocol utilized a 50 µL sample size and involved the same sample load, wash #1, wash #2 and plate dry steps as did the sample size determination protocol.

Step	Volume (µL)	Solvent	Time (min)	Pressure (psi)
Sample Load	ALL	Sample	1-2	2-4
Wash #1	1000	4% H_3PO_4	1-2	2-4
Wash #2	1000	50% Methanol (aq)	1-2	2-4
Plate Dry	N/A	N/A	1	20
Elution	2 x 500	DCM/MeOH/NH_4OH [78:20:2]	2-3	2-4
Plate Dry	N/A	Quick Pulse	x2	20

Table 2. *Biotage 96 Positive Pressure Processing Parameters.*

	98:2 MeOH/NH_4OH	2.5% formic acid in water
Combination 1	1 x 150 µL	1 x 100 µL
Combination 2	2 x 75 µL	2 x 75 µL
Combination 3	2 x 50 µL	2 x 50 µL
Combination 4	1 x 100 µL	1 x 100 µL

Table 3. *Microelution combinations.*

Dry Down and Sample Reconstitution: Elution solvent was collected into a collection plate. For the samples that used the microelution technique described in table 3, the plate was covered and analyzed via LC-MS/MS. All other samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage® SPE Dry. Extracts were then reconstituted with 100 µL of 90:10 mobile phase A/mobile phase B and analyzed via LC/MS-MS.

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 4. *Shimadzu Nexera X2 UPLC.*

Mass Spectrometry Parameters

Instrument: SCIEX 5500 triple quadrupole Mass Spectrometer with Turbo Ionspray® Ion interface (Foster City, CA). Optimized source parameters shown in table 5 (sMRM transition parameters not shown, but available upon request). Retention window for sMRM set at 45 seconds with target scan time at 2.85 seconds.

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

Table 5. *SCIEX 5500 Triple Quadrupole ESI (+/-) Turbo Ionspray® Source Parameters.*

Results

Sample Size Determination

Varying sample sizes did have an effect on the recoveries and matrix effects of compounds in the DOA panel. As can be seen in figure 1, a 50-75 µL sample size yielded the highest recoveries and lowest matrix effects. Most recoveries were greater than 80%. Matrix effects were within 10% for most compounds, which means that there was very little suppression or enhancement, as is seen in figure 2. Figures 1 and 2 show recoveries and matrix effects for the IMCSzyme for a sampling of the DOA to cover all drug classes. The other enzymes tested exhibited similar results.

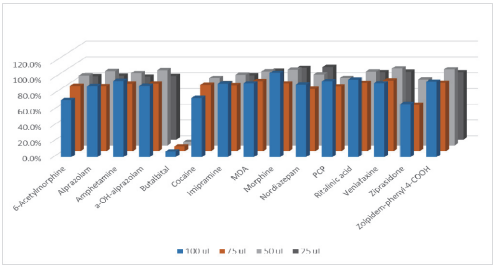

Figure 1. *Variations in recoveries seen using different sample sizes using a 10 mg sorbent bed and the IMCSzyme.*

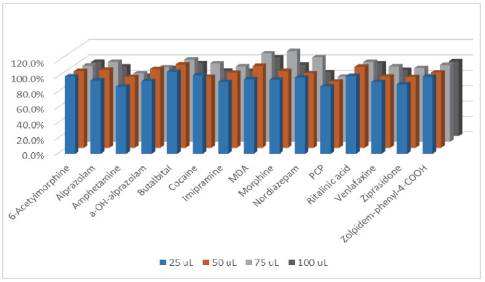

Figure 2. *Variations in matrix effects seen using different sample sizes using a 10 mg sorbent bed and the IMCSzyme.*

Microelution with no Evaporation

The highest recoveries were obtained using a volume of 150 µL of 98:2 methanol/ammonium hydroxide, followed by 100 µL of 2.5% formic acid in water for elution. Many compounds (opiates, benzodiazepines, stimulants) had recoveries of at least 60% with this protocol, as is seen in figure 3. Several compound classes, including tricyclic antidepressants (nortriptyline), selective serotonin and norepinephrine reuptake inhibitors (duloxetine), and some opioids (EDDP), had decreased recoveries with the microelution protocol. These recoveries ranged from 5-20%. When using a traditional SPE elution with evaporation and reconstitution, recoveries of these compounds were all greater than 80%.

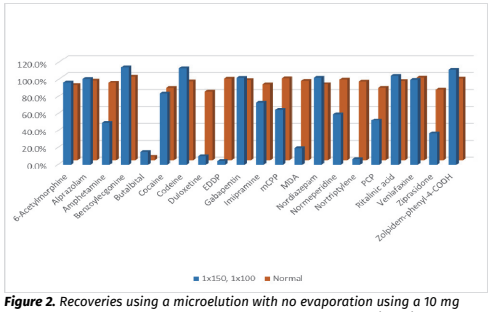

Figure 2. *Recoveries using a microelution with no evaporation using a 10 mg sorbent bed and the IMCSzyme compared to a normal SPE elution with dry down.*

Matrix effects when using a microelution were the same as matrix effects seen when using a normal SPE extraction protocol, as can be seen in figure 4, which shows that the elution of 2.5% formic acid in water is not pulling any additional interferences off of the extraction plate.

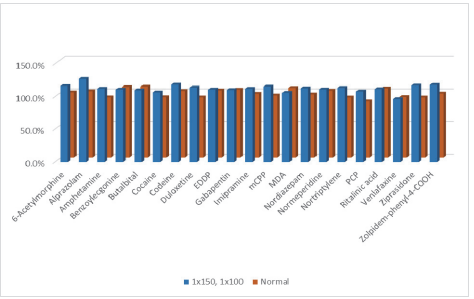

Figure 4. *Matrix effects using a microelution protocol with no evaporation using a 10 mg sorbent bed and the IMCSzyme compared to a normal SPE elution with no dry down.*

When an excess of organic solvent is injected on the LC-MS/MS, there is an increased chance of peak broadening and peak fronting/tailing. Using the microelution method, the peak shape was investigated to determine if it was still acceptable. Figure 5 shows peak shapes for 6-acetylmorphine when looking at various injection solvents. As can be seen, an increased amount of organic solvent in the injection solvent does not seem to increase the peak width of 6-acetymorphine.

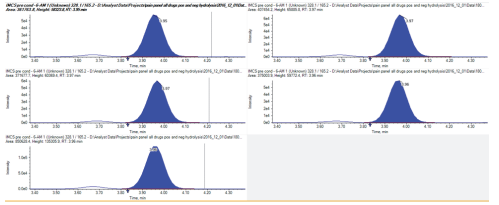

Figure 5. *Peak shape for 6-acetylmorphine using (1) combination 1, (2) combination 4, (3) combination 3, (4) combination 2, and (5) 2 x 500 µL 78:20:2 DCM/MeOH/NH_4OH with evaporation and reconstitution.*

Conclusions

- The EVOLUTE® EXPRESS CX 10 mg is a great option for extraction of 98 drugs of abuse from urine.
- EVOLUTE® EXPRESS CX 10 mg produced excellent recoveries for most compounds. The optimal sample size for the 10 mg sorbent bed is 50 µL.
- Using a 50 µL sample size with a microelution without evaporation or reconstitution resulted in recovery of all compounds in the DOA panel.
- LC-MS/MS peak shapes were not affected by the increase in organic solvent in the sample injection.
- Using a microelution protocol allows for a time and cost savings for laboratories as less solvent is used for the elution and no time is spent on the evaporation or reconstitution of samples.

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