Small Molecules Automated Extraction from Human Breast Milk Using the Biotage® Extrahera™ and EVOLUTE® EXPRESS CX Prior to LC-MS/MS Analysis

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Introduction

Breast feeding is beneficial in meeting the nutritional and immunological needs of infants. Using illegal drugs while breast feeding can have severe consequences for both infant and mother. At Biotage, we developed a new extraction protocol for 12 common drugs of abuse (DOA) to be detected in breast milk (Figure 1) using mixed-mode polymeric cation exchange solid phase extraction (SPE). The EVOLUTE® EXPRESS CX (figure 3) extraction allowed for highly-sensitive detection of analytes including Benzoylecgonine, (-)-Cotinine, Caffeine, d-Amphetamine, d-Methamphetamine, Methadone, Morphine, Oxazepam, Oxycodone, Phencyclidine (PCP), Secobarbital, and (l)-9-Carboxy-11-nor-Delta-9-THC (THC-COOH). Extrahera (figure 2), a bench top automated extraction system, provided minimal sample intervention and high throughput for the analysis of these DOA. Using the combination of reliable automation and SPE sample preparation techniques, a method was developed demonstrating the precision, accuracy, linearity, and sensitivity necessary for a robust quantitative workflow.

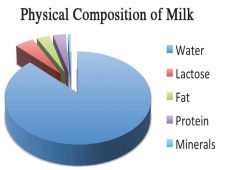

Figure 1. Breast Milk Composition.

Figure 2. Extrahera Automation System.

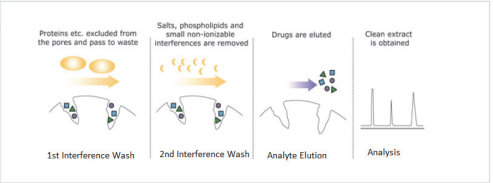

Figure 3. EVOLUTE EXPRESS CX Interferences Removal.

Experimental

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, acetic acid (CH_3COOH), Acetonitrile (ACN), ammonium hydroxide (NH_4OH) Hexane (C_6H_{14}), ethyl acetate (EtOAc). EVOLUTE® EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), and Biotage® SPE Dry 96 (SD-9300-DHS-NA), Extrahera (414001) were supplied by Biotage. The LC column was provided by Restek Corp. QC materials were generously donated from UTAK labs (product # 21801-4).

Sample Preparation

Breast Milk Sample Pre-treatment and Preparation

Calibrators were prepared by spiking the target analytes into drug-free human breast milk. Serial dilutions were used to achieve the remaining standard calibration concentrations (Table 1). In 1.5 mL micro-centrifuge tubes, 200 µL of methanol was added to 200 µL of each calibrator and control and vortexed for 1 minute then centrifuged for 10 minutes at 13,000 rpm. 300 µL of the resulting supernatant was then loaded onto the EVOLUTE® EXPRESS CX 30 mg plate, and went through extraction and elution (Table 2). Conditioning and equilibration steps are optional to reach a lower limit of detection for THCOOH.

Analyte conc. In ng/mL	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2
Benzoylecognine	5.0	10.0	15.0	20.0	30.0	40.0	10.0	20.0
Cotinine	1.3	2.5	3.75	5.0	7.5	10.0	2.5	5.0
Caffeine	125	250	375	500	750	1000	250	500
d-Amphetamine	5.0	10.0	15.0	20.0	30.0	40.0	10.0	20.0
d-Methamphetamine	5.0	10.0	15.0	20.0	30.0	40.0	10.0	20.0
Methadone	6.3	12.5	18.75	25.0	37.5	50.0	12.5	25.0
Morphine	5.0	10.0	15.0	20.0	30.0	40.0	10.0	20.0
Oxazepam	25.0	50.0	75.0	100.0	150.0	200.0	50.0	100.0
Oxycodone	2.5	5.0	7.5	10.0	15.0	20.0	5.0	10.0
Phencyclidine(PCP)	2.5	5.0	7.5	10.0	15.0	20.0	5.0	10.0
Secobarbital	10.0	20.0	30.0	40.0	60.0	80.0	20.0	40.0
THCOOH	2.5	5.0	7.5	10.0	15.0	20.0	5.0	10.0

Table 1. Spiked Concentrations In Cals and QCs.

EVOLUTE® EXPRESS CX SPE/Extrahera Procedure

The extraction method is shown in (Table 2).

Dry Down and Sample Reconstitution: Eluted samples were collected into a collection plate. Samples were evaporated to dryness at 40°C with 20 L/min of nitrogen using a Biotage® SPE Dry. Extracts were then reconstituted with 200 µL of 5% MeOH in water and analyzed via LC/MS-MS.

Step	Volume (uL)	Solvent/Equipment	Time (min)	Pressure (psi)
Condition	1000	Methanol	1-2	0.5-3
Equilibration	1000	4 % Formic Acid	1-2	0.5-3
Sample Load	300	Sample	1-2	0.5-3
Wash #1	300	4 % Formic Acid	1-2	0.5-3
Wash #2	300	70:30 Hexane/EtOAc	1-2	0.5-3
Dry	N/A	N/A	5	20
Elution 1	600	2% Acetic Acid in ACN	2-3	0.5-3
Evaporate	N/A	Using SPEDry @ 40°	5-10	N/A
Elution 2	600	DCM/IPA/NH4OH[78:20:2]	2-3	0.5-3
Evaporate	N/A	Using SPEDry @ 40°	5-10	N/A

Table 2. *Biotage® Extrahera™ Extraction protocol.*

Chromatography Parameters

HPLC	Parameters
Column	Restek Raptor Biphenyl 2.7 µm, 50 x 3.0mm
MPA	0.1 % Formic acid in H2O
MPB	0.1 % Formic acid in MeOH
Flow Rate	0.4 mL/min
Column Temp.	40°C
Sample Temp	10°C
Injection Vol.	20 µL

Table 3. *Shimadzu Nexera X2 UPLC setup.*

Mass Spectrometry Parameters

Instrument: A SCIEX 5500 triple quadrupole Mass Spectrometer with Turbo Ionspray® Ion interface (Foster City, CA) was used. Optimized source parameters are shown in table 5 (MRM transition parameters not shown, but available upon request). Retention window for MRM was set at 60 seconds with target scan time at 2.5 seconds.

Ionization Spray Voltage	+1500(V)	**CAD**	Medium
Source Temp	600 °C	**GS1**	50
Curtain	30 (V)	**GS2**	50
Pos & Neg mode switching			

Table 4. *SCIEX 5500 Triple Quadrupole ESI (+/-) Turbo Ionspray® Source Parameters.*

Results

Analytes were extracted from the calibrators and QC material by protein precipitation followed by SPE. Samples were then analyzed via an LC/MS-MS system and a biphenyl column using a 5-minute gradient. The DOA standard curves in breast milk had excellent linearity within the measured range (Table 1) with R^2 values greater than 0.995 (figure 4). When an excess of organic solvent is injected on the LC-MS/MS, there is an increased chance of peak broadening and peak tailing. Using the EVOLUTE® EXPRESS CX method, the peak shape was investigated to determine if it was still acceptable (Figure 5).

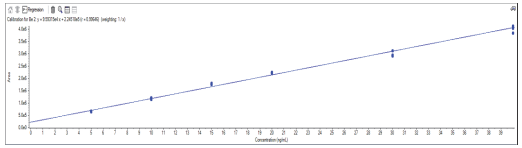

Figure 4. *Calibration curve for benzoylecgonine (BE)*

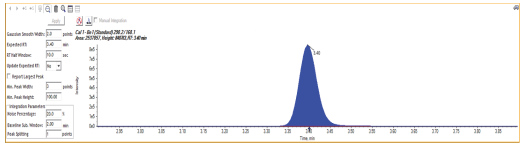

Figure 5. *Peak Shape at lowest calibration for BE at 5 ng/mL.*

Carryover was assessed by analyzing the area count of the blank calculated as percentage of the mean peak area of the lowest calibrator concentration. No significant carryover (0%) was determined for all analytes. Matrix effects were also investigated for all analytes by comparing the AUCs of standard curves prepared in 100% water to those in drug-free human breast milk. No significant matrix effects were observed (<10%). Washing the plate with a Hexane/EtOAc mixture eliminated interferences from the matrix and eliminated lipids and proteins left in the sample.

The QC samples were analyzed to obtain inter- and intra-day precision and accuracy values. Accuracies determined were within (10%) and coefficient of variation values were all (<10%) for the concentrations within the measured range (Table 5).

All analytes had excellent process efficiency within ± 10% indicating efficiency in sample clean up to eliminate the lipids and the proteins. Using two different elution solvents helped increase recovery for analytes from different classes.

Analyte	QC Conc. In ng/mL	Intraday % Accuracy (n=3)	Intraday % Precision (n=3)	Interday % Accuracy (n=3)	Interday % Precision (n=3)
Benzolyecognine					
	10	100.3	7.6	95.8	6.3
	20	91.9	3.4	92	3.3
Cotinine					
	2.5	93.6	6.7	90.6	1.9
	5	94.5	2.9	62.5	2.4
Caffeine					
	250	100.3	8.2	93.8	5.3
	500	97.2	3.6	95.4	3.4
d-Amphetamine					
	10	98.6	8.1	96.8	5.6
	20	90.7	4.6	93	3.7
d-Methamphetamine					
	10	93.6	6.7	90.6	2.2
	20	94.5	2.9	92.5	2.9
Methadone					
	12.5	98.2	7.9	92.6	5.4
	25	97.2	3.9	94.4	3.5
Morphine					
	10	93.5	7.9	95.3	5.7
	20	91.7	5.4	92.1	4.8
Oxazepam					
	50	94.9	4.6	92.1	2.8
	100	93.5	3.5	94.8	2.9
Oxycodone					
	5	96.2	4.7	92.6	4.9
	10	92.1	3.9	93.5	3.9
Phencyclidine (PCP)					
	5	92.3	8.1	95.8	5.7
	10	97.1	5.6	91.2	4.8
Secobarbital					
	20	95.1	4.6	91.3	2.9
	40	92.4	3.5	93.5	3.1
THCOOH					
	5	98.1	4.3	92.6	5.1
	10	93.1	3.8	93.5	4.2

Table 5. *Inter & intra-day precision and accuracy.*

Conclusions

- Drugs of abuse in human breast milk can be automated and quantified accurately with this method. This analytical method employs protein precipitation followed by EVOLUTE® EXPRESS CX. Interferences including proteins, sugars, non-ionisable molecules and phospholipids are removed during the elution step, ensuring extremely clean extracts and enabling analysis of 12 DOA from different classes.
- Using commercial quality controls ensured the integrity of making in-house calibrators
- Using two different elution solvents allowed for recovery of analytes from different classes that were detected using positive and negative mode on the mass spec.
- Automating this extraction protocol can provide savings in analysis time, solvent consumption, and minimizes manual sample intervention when compared to traditional sample preparation techniques.

References

Marchei, E., Escuder, D., Pallas, C., Garcia-Algar, O., Gómez, A., Friguls, B., Pellegrini, M. and Pichini, S. (2019). Simultaneous analysis of frequently used licit and illicit psychoactive drugs in breast milk by liquid chromatography tandem mass spectrometry.

ASMS 2019, Atlanta, GA.
Part Number: P203