

Automated Extraction of 12 Drugs of Abuse from Human Breast Milk Using 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 Benzoyllecgonine, (-)-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.

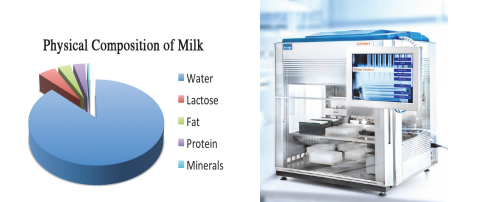


Figure 1. Breast Milk Composition.



Figure 2. Biotage® Extrahera™ Automation System.

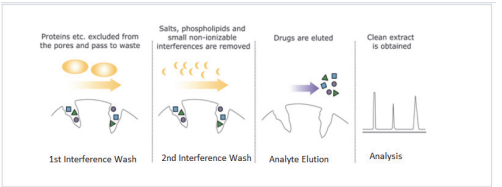


Figure 3. EVOLUTE® EXPRESS CX Interferences Removal.

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 (CH₃COOH), Acetonitrile (ACN), ammonium hydroxide (NH₄OH) Hexane (C₆H₁₄), ethyl acetate (EtOAc). EVOLUTE® EXPRESS CX (30 mg bed) extraction plate (601-0030-PX01), and Biotage® SPE Dry 96 (SD-9300-DHS-A), Biotage® 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 min. 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 the following extraction and elution protocol (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
Benzoyllecgonine	5	10	15	20	30	40	10	20
Cotinine	1.25	2.5	3.75	5	7.5	10	2.5	5
Caffeine	125	250	375	500	750	1000	250	500
d-Amphetamine	5	10	15	20	30	40	10	20
d-Methamphetamine	5	10	15	20	30	40	10	20
Methadone	6.25	12.5	18.75	25	37.5	50	12.5	25
Morphine	5	10	15	20	30	40	10	20
Oxazepam	25	50	75	100	150	200	50	100
Oxycodone	2.5	5	7.5	10	15	20	5	10
Phencyclidine (PCP)	2.5	5	7.5	10	15	20	5	10
Secobarbital	10	20	30	40	60	80	20	40
THCOOH	2.5	5	7.5	10	15	20	5	10

Table 1. Spiked Concentrations in Cals and QCs.

EVOLUTE® EXPRESS CX SPE/ Biotage® Extrahera™ Procedure

The extraction method for the samples that underwent evaporation and reconstitution is shown in table 2. **Dry Down and Sample Reconstitution:** Elution solvents 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% MEOW in water and analyzed via LC/MS-MS.

Step	Volume (µL)	Solvent/Equipment	Time (min)	Pressure (psi)
Condition	1000	Methanol	1-2	1.5
Equilibration	1000	4 % Formic Acid	1-2	1.5
Sample Load	300	Sample	1-2	1.5
Wash #1	300	4 % Formic Acid	1-2	1.5
Wash #2	300	70:30 Hexane/ EtOAc	1-2	1.5
Elution 1	600	2% Acetic Acid/ACN	2-3	1.5
Evaporation	N/A	Using SPE Dry	5-10	N/A
Elution 2		DCM/IPA/NH ₄ OH [78:20:2]	2-3	1.5
Evaporation	N/A	Using SPE Dry	5-10	N/A

Table 2. Biotage® Extrahera™ Extraction protocol.

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	0.4 mL/min
Column Temp.	40 °C
Sample Temp.	20 °C
Injection Volume	20 µL

Table 3. Shimadzu Nexera X2 UPLC set-up.

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 (MRM transition parameters not shown, but available upon request). Retention window for MRM 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 5 minutes' gradient. The DOA standard curves in breast milk had excellent LOQ and a linearity within the measured range (Table 1) with an r² value greater than 0.995 (figure 4). When an excess of aqueous 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).

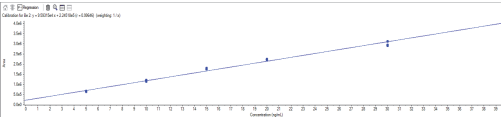


Figure 4. Calibration curve for BE.

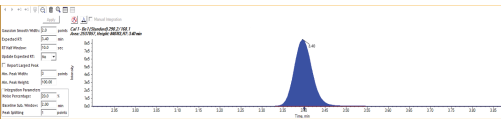


Figure 5. Peak Shape at lowest calibration for BE at 5 ng/mL.

Carryover was assessed by analyzing the AUC 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 Hexane/EtOAc mixture eliminated interferences from the matrix and elimination of lipids and proteins left in the sample.

The QC were analyzed to obtain inter- and intra-day precision and accuracies 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 of 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)
Benzoyllecgonine	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	92.5	2.4
Caffeine	250	100.3	8.2	93.8	6.3
	500	97.2	3.6	95.4	3.4
d-Amphetamine	10	98.6	8.1	98.8	5.8
	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	6.7
	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	6.7
	10	97.1	5.8	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 and 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 Using 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 during the extraction helped recovery of all analytes from different classes detected using positive and negative mode on the mass spec.
- » Automating this extraction protocol can provide savings in analysis time, solvent consumption, and will minimize 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.