Solid Phase Extraction of Cocaine and Metabolites from Biological Specimens

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1 INTRODUCTION

Cocaine is a naturally occurring stimulant that is found in the leaves of the *Erythroxylon coca* plant. The primary metabolites of cocaine in humans are benzoylecgonine and ecgonine methyl ester. Cocaethylene is another biotransformation product of cocaine that is produced when cocaine and ethanol are used together.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate (1:1 in deionized water).
Analytes	Cocaine (COC), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME)
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. The specimens are prepared for solid phase extraction (SPE) via centrifugation and/or dilution. Cocaine and metabolites are eluted from the SPE cartridge using a mixed solvent system of methylene chloride, isopropanol, and ammonium hydroxide. The eluent is taken to dryness, reconstituted, and analyzed directly by liquid chromatography-mass spectrometry (LC-MS).

4 SPECIMEN CRITERIA

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate (1:1 in deionized water). Typically, 1 mL of specimen is per replicate. In instances where sample volume is limited or there is reason to suspect a sample of being a strong positive a smaller volume of specimen, diluted to 1.0 mL with deionized water, may be used.

5 EQUIPMENT

5.1 Equipment

A. Centrifuge
B. Evaporator with nitrogen
C. Miscellaneous routine laboratory glassware and supplies
D. Rotator
E. Solid phase extraction vacuum manifold or positive pressure manifold
F. Vortexer

5.1.1 Column

A. Grace 5 µm particle silica HPLC column, 2.1 x 150 mm (or equivalent)
B. Appropriate guard/frit

5.2 Consumables

A. 12 x 75 mm test tubes
B. 16 x 100 mm test tubes
C. Clean Screen DAU SPE Cartridges (United Chemical Technologies, Bristol, PA)
D. pH paper

5.3 Instruments

A. Thermo LTQ Orbitrap XL Hybrid Ion Trap/Fourier Transform Mass Spectrometer
B. Shimadzu HPLC

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1 / XP Professional
Mass Spectrometer	Foundation	1.0.2 or higher
	Xcalibur	2.1.0 SP1 / 2.0.7
	LTQ Tune Plus	2.5.5
	Shimadzu LC Controller	5.4 / 6.5

5.5 Chemicals/Reagents

Storage/stability determined by manufacturer unless otherwise noted.

5.5.1 Purchased

A. Acetonitrile	≥ LC/MS grade
B. Ammonium hydroxide, concentrated	ACS grade
C. Hydrochloric acid, concentrated	ACS grade
D. Isopropanol	≥ HPLC grade

E. Methanol	≥ GC^2 grade, HPLC, Optima grade
F. Methylene chloride	≥ HPLC grade
G. Sodium phosphate dibasic heptahydrate	
H. Sodium phosphate monobasic monohydrate	
I. Water	≥ 18mΩ, HPLC, Optima, or UPLC grade

5.5.2 *Prepared*

A. 100 mM Phosphate Buffer (pH 6.0)

To a 500 mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify 5.8<pH<6.1. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.

B. Elution Solvent (Methylene Chloride/Isopropanol/Ammonium Hydroxide (78/20/2))

Combine 20 mL isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL methylene chloride and mix well. Store in glass at room temperature. Prepare fresh daily.

C. 0.1 M Hydrochloric Acid

To a 100-mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well. Store in glass at room temperature. Stable 6 months.

D. 95:5 Methanol:Water

Combine 95 mL methanol (HPLC grade) and 5 mL deionized water in a graduated cylinder. Mix well. Store in glass or plastic at room temperature. Stable 12 months.

E. LC Mobile Phase (95:5:0.03 Methanol:Water:Ammonia)

Combine 950 mL HPLC grade methanol and 50 mL deionized water. Mix well. Add 0.3 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 2 weeks.

5.6 Standards/Controls

Storage/stability determined by manufacturer unless otherwise noted.

5.6.1 *Purchased*

A. Negative Control

Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Clinical Controls International, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control is extracted and analyzed with every assay. The Negative Control will be matrix matched, when possible.

B. Internal Standard Components (100 µg/mL)

1. d3-cocaine
2. d3-ecgonine methyl ester
3. d3-cocaethylene
4. d8-benzoylecgonine

Purchased from Cerilliant Corporation or equivalent.

C. Control Components (1.0 mg/mL)

1. cocaine
2. ecgonine methyl ester
3. cocaethylene
4. benzoylecgonine

Purchased from Cerilliant Corporation, Lipomed or equivalent.

5.6.2 Prepared

5.6.2.1 Internal Standard Working Solution

Analyte (0.1 mg/mL stock)	**Aliquot (mL)**	**Final Conc. (µg/mL)**
A. D3-cocaine	1.0	10
B. D3-ecgonine methyl ester	1.0	10
C. D3-cocaethylene	1.0	10
D. D8-benzoylecgonine	1.0	10
i. Add components to		10 mL volumetric flask
ii. Add 0.1 M HCl	0.020	
iii. QS	10.0	acetonitrile
Mix well. Store below 0°C. Stable for at least 2 years.		

5.6.2.2 Intermediate Control Standard

Analyte (1.0 mg/mL stock)	**Aliquot (mL)**	**Final Conc. (µg/mL)**
A. cocaine	0.800	80
B. cocaethylene	0.400	40
C. benzoylecgonine	0.800	80
D. ecgonine methyl ester	0.400	40
i. Add components to		10 mL volumetric flask
ii. Add	0.050	0.1 M HCl

iii. QS	10.0	acetonitrile
Mix well. Store below 0°C. Stable for at least 1 year.		

5.6.2.3 Working Control Standard

Solution	Aliquot (mL)	Final Conc. (µg/mL)
A. Intermediate Control Standard	1.0	8 and 4
i. Add component to		10 mL volumetric flask
ii. Add	0.050	0.1 M HCl
iii. QS	10.0	Deionized water
Mix well. Prepare fresh.		

5.6.2.4 Positive Control Scheme

Level	Working Control Standard to add to 1 mL blood (µL)	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)	Cocaethylene (ng/mL)	Ecgonine Methyl Ester (ng/mL)
Low Control	20	160	160	80	180
High Control	100	800	800	400	400

5.6.2.5 LC/MS Performance Standard

Analyte (1.0 mg/mL stock)	Aliquot (mL)	Final Conc. (µg/mL)
A. cocaine	0.025	1.0
B. cocaethylene	0.025	1.0
C. benzoylecgonine	0.025	1.0
D. ecgonine methyl ester	0.025	1.0
i. Add components to		25 mL volumetric flask
ii. Add	0.050	0.1 M HCl
iii. QS	25.0	acetonitrile
Mix well. Store below 0°C. Stable for at least 1 year. A 5 µl portion of this mixture is analyzed by LC/MS/MS.		

6 PROCEDURE

	Step	Note	Reference/Lot
	A. **Samples**		
☐	1. To labeled 16 x 100 mm screw-top tubes add:		
	☐ i. 1 mL of biological fluid		
	☐ ii. 1 g of a prepared tissue homogenate		
	B. **Controls**		
☐	1. Prepare Negative Control(s)		
☐	2. Prepare Positive Control(s) i. Working Control Standard		
	ii. Control Scheme		
	C. **Internal Standard(s)**		
☐	1. Add 25 µL of Internal Standard Working Solution (250 ng/mL final concentration)		
☐	2. Bring all samples to ~5 mL with deionized water and vortex		
☐	3. Let all samples stand for 5 minutes		
☐	4. Centrifuge at ~3000 rpm for 10 minutes.		
	D. **Buffer**		
☐	1. Add 2 mL of 100mM phosphate buffer		
☐	2. Vortex		
☐	3. Check pH: 6 ± 0.5		
	E. **Extract** (SPE, sorbent should not be dried until step 4)		
	1. Condition cartridges (1 mL/min)		
☐	i. Add 3 mL Elution Solvent		
☐	ii. Add 3 mL methanol		
☐	iii. Add 3 mL deionized water		
☐	iv. Add 1 mL 100mM phosphate buffer		
☐	2. Load samples (1 mL/min). Do not transfer any pelleted material resulting from centrifugation.		
	3. Wash cartridges (1 mL/min)		
☐	i. Add 2 mL of deionized water		
☐	ii. Add 2 mL 100mM HCl		

☐	iii. Add 3 mL of methanol		
☐	4. Dry cartridge under full vacuum for 90 seconds		
	5. Elute (1 mL/min)		
☐	i. Add 3 mL Elution Solvent		
☐	ii. Collect eluent in 12 x 75 mm tubes		
	F. **Concentrate**		
☐	1. Evaporate to dryness under nitrogen at 40°C		
	G. **Reconstitute**		
☐	1. Add 100 µL of 95:5 methanol:water 2. Vortex 3. Transfer to ALS vial and cap		
	H. **Instrumental Analysis**		
☐	1. LC/MS: analyze 5 µL		
☐	i. Analyze LC/MS Performance Standard prior to batch analysis		
	ii. Mobile Phase 1 (mixed)		
	iii. LC Column		

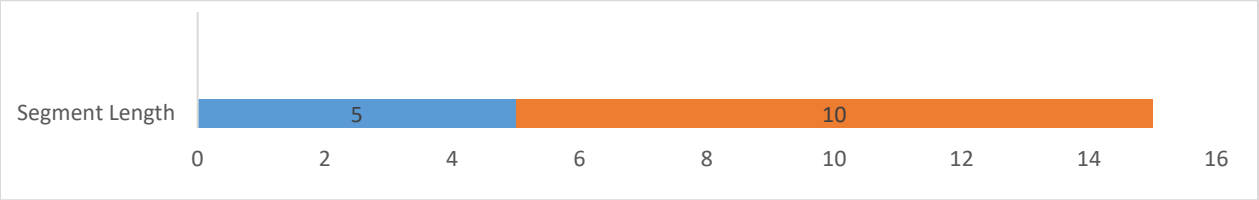
7 ANALYTICAL PARAMETERS

7.1 Shimadzu HPLC Gradient/Conditions

Time (min)	Flow Rate			
	Mobile Phase %	(mL/min)	Column Heater (^{o}C)	30
0	100	0.3	Autosampler (^{o}C)	ambient
15	100	0.3	Run Time (min)	15

7.2 LTQ-XL Orbitrap

A. Source Mode: ESI (+)



Segment	Event	Mode	Range (m/z)	Analyzer	Resolution
1	1	Full Scan	260-360	ITMS	unit
	2	MS/MS (event 1) m/z: 290, 304, 318 45% relative CE Isolation width 1.5 m/z	Software control	ITMS	unit
2	1	Full Scan	170-230	ITMS	unit
	2	MS/MS (event 1) m/z: 200 40% relative CE Isolation width 1.5 m/z	70-230	ITMS	unit

8 DATA ANALYSIS

8.1 Decision Criteria

8.1.1 LC/MS Performance Standard Decision Criteria

8.1.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should generate Gaussian shaped chromatographic peaks. The following molecular ions should be traced for each analyte: cocaine – 304, ecgonine methyl ester – 200, cocaethylene – 318, benzoylecgonine – 290.

The retention times of the 4 analytes should be within ± 5 % of the previous run of the performance standard. Minor changes in mobile phase percentage may account for slight retention time shifts.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

8.1.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the four analytes in the performance standard should be present. The following molecular ions should be present as the base peak for each analyte: cocaine – 304, ecgonine methyl ester – 200, cocaethylene – 318, benzoylecgonine – 290.

8.1.2 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All analytes should be detected in the Positive Control.

8.1.3 Analyte Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, the criteria in sections 8.1.3.1 through 8.1.3.2 should be met in order to identify cocaine, benzoylecgonine, ecgonine methyl ester, or cocaethylene within a biological specimen:

8.1.3.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

8.1.3.1.1 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

8.1.3.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

8.1.3.2 Mass Spectrometry

The MS/MS fragmentation spectra should meet the following independent criteria for each compound identified.

A. Cocaine: (fragments of m/z 304) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.

B. Cocaethylene: (fragments of m/z 318) The base peak should be m/z 196, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.

C. Benzoylecgonine: (fragments of m/z 290) The base peak should be m/z 168, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 150 and m/z 272.

D. Ecgonine methyl ester: (fragments of m/z 200) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 82 and m/z 156.

9 REPORTING

9.1 Cocaine

To report cocaine qualitatively based upon this method, the area of the M+H peak for cocaine must be greater than or equal to 5% of the M+1 peak for benzoylecgonine.

10 CORRECTIVE MEASURES

Refer to TOX-101 for guidance on action steps in the event of a quality control failure.

11 PERFORMANCE CHARACTERISTICS

11.1 LOD

Cocaine:	10 ng/mL, or lower
Benzoylecgonine:	10 ng/mL, or lower
Ecgonine methyl ester:	5 ng/mL, or lower
Cocaethylene:	5 ng/mL, or lower

12 LIMITATIONS

12.1 Interferences

None known. Grossly decomposed or putrefied samples may affect both detection limits.

13 SAFETY

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

14 REVISION HISTORY

Revision	Issued	Changes
07	02/11/2022	Document reformat. Minor updates to wording in section 2, 3 and 4. Simplified procedure in 6-D-2 (all sample types stand for five minutes).
08	07/15/2022	Removed all quantitative references (multiple sections).