SOP 403 - Cocaine and Metabolites Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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	10.1.1.5 – Updated RT acceptance range 10.1.2.1 – Updated IRC acceptance range 10.1.3.2 – Updated Calibrator acceptance range 10.1.4.1 – Updated QC acceptance range	MSF – 05/07/2015
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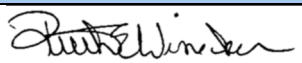
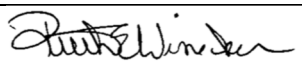
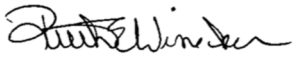
Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 403 - Cocaine and Metabolites Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. Principle of Assay

1.1. This method is designed to confirm and quantitate cocaine (COC), benzoylecgonine (BE), and cocaethylene (CE) in biological specimens by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC/MS/MS). The drugs are extracted from their biological matrix by protein precipitation with acetone and identified by the retention times of precursor ions and ion ratios of the product ions.

Cocaine is an alkaloid that is present in the coca plant, a bush native to the South American Andean region and countries of Bolivia, Colombia and Peru. The stimulant properties of the coca plant were well known to locals for thousands of years, but it was not until 1859 that the primary alkaloid, cocaine, was isolated. Twenty-five years later, in 1884, Sigmund Freud became the first to use cocaine as a local anesthetic. The clinical use of cocaine spread quickly, but was tempered by its recreational use, leading to restrictions and prohibition throughout the early 1900's (1).

Cocaine's primary mode of action centrally is as an inhibitor of the dopamine transport (reuptake) protein, thus increasing extracellular concentrations of dopamine. The effects of cocaine intoxication include increased alertness and euphoria, which in turn can lead to addiction. Potentially fatal side effects include heart attack and heart failure, and are due to cocaine's effect on the cardiac action potential, leading to cardiac arrhythmias as well as stroke and aneurysm due to adverse effects of cocaine on blood pressure (2). Because of the high potential for abuse and possibility of severe dependence, yet still maintaining an accepted medical use, cocaine is defined as a Schedule II drug by the Controlled Substances Act.

The most common routes of administration are insufflation and smoking, though other routes such as intravenous injection and oral are used. Regardless of the route of administration, the primary metabolite, and marker for prior cocaine usage, is benzoylecgonine. There is little postmortem redistribution, as well as no specific pattern for cocaine or benzoylecgonine concentration with regards to site of specimen collection or magnitude with time (3).

The preferred specimen for the quantitation of cocaine, and its metabolites, is blood from a peripheral specimen. Other valid specimens include urine, vitreous, liver, and other tissues.

2. Specimens

2.1. This procedure is applicable to urine, blood, serum, properly prepared tissue specimens (typically 1:4 homogenates), bile*, vitreous and gastric contents*.

2.2. A 0.5 mL (g) sample size (in duplicate) is generally employed, unless a dilution is required, so that the drug concentration in the unknown specimens falls within the range of the calibration curve.

2.2.1. *For non-typical matrices, an additional 0.5mL aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. **Reagents and Materials**

3.1. DI water, HPLC grade

3.2. Methanol, HPLC grade

3.3. Acetone, HPLC grade

3.4. Acetonitrile, HPLC grade

3.5. Deuterated Cocaine Internal Standard

3.6. Cocaine calibration solution

3.7. Cocaine positive control solution

3.8. Drug Free Blood, Urine, Liver Homogenate

3.9. Water with 0.1% formic acid

3.10. Acetonitrile with 0.1% formic acid

4. **Standards, Controls, and Solutions**

4.1. Prepare Calibration, IS, and Control standards, as needed, according to SOP 010.

4.2. **Cocaine Internal Standard Stock Solution (10µg/mL)**

4.2.1. Into a 10mL volumetric flask, add the following:

4.2.1.1. 1 ampule (~1mL) of Benzoylecgonine-d3 (Cerilliant - 100µg/mL)

4.2.1.2. 1 ampule (~1mL) of Cocaine-d3 (Cerilliant - 100µg/mL)

4.2.1.3. 1 ampule (~1mL) of Cocaethylene-d8 (Cerilliant - 100µg/mL)

4.2.1.4. Fill to the line with acetonitrile, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test

tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.3. **Cocaine Internal Standard Working Solution (1000ng/mL)**

4.3.1. Into a 10mL volumetric flask, add 1mL of Cocaine Internal Standard Stock Solution (10µg/mL) with a micropipette.

4.3.2. Fill to the line with acetonitrile, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.4. **Cocaine Calibrators and Positive Controls –** these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.5. **Water with 0.1% formic acid**

4.5.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid

4.5.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

4.6. **Acetonitrile with 0.1% formic acid**

4.6.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid

4.6.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

5. **Equipment and Special Supplies**

5.1. Test Tubes, 13 x 100 mm

5.2. 10-100µL micropipette

5.3. 100-1000µL micropipette

5.4. Centrifuge 2000 x g

5.5. Vortex mixer

5.6. Nitrogen evaporator

5.7. LC autosampler vials, 12 x 32 mm

5.8. Polyspring inserts, 5 mm O.D.

6. **Instrumentation and Parameters**

6.1. Windows PC with Thermo LCQuan and Xcaliber software
  6.1.1. Instrument method (TSQ01 & TSQ02): "Cocaine"
  6.1.2. Click here for instrument parameters.
6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

  6.4.1. Click here for instrument parameters

7. **Target Ions** (± 1 nominal mass)

7.1.	Cocaine-d3	(**307** 185 85)
7.2.	Cocaine	(**304** 182 82)
7.3.	Benzoylecgonine-d3	(**293** 171 105)
7.4.	Benzoylecgonine	(**290** 168 105)
7.5.	Cocaethylene-d8	(**326** 204 85)
7.6.	Cocaethylene	(**318** 196 82)

  7.6.1. Note: The precursor ion of each analyte is listed first and bolded, the first product ion- used for quantification-is second, followed by the second product ion-used for qualification/confirmation.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 13 x 100 mm test tubes.

  8.1.1. **Note: follow the tube labeling and tape transfer procedure as directed in the Quality Assurance and Quality Control manual.**

8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Deuterated Cocaine Internal Standard Mix to all the tubes.

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Cocaine calibration solution and the Cocaine positive control solution to the tubes labeled as standards and control, respectively, labeling test tubes as you go. Only internal standard should be present in the test tube(s) labeled "Blank".

8.4. Add 0.5 mL blank blood to each of the standards and controls. Include a urine blank/control (0.5 mL) and/or a liver homogenate blank/control (0.5 g) if appropriate.

8.5. Add the appropriate amount of predetermined unknown specimen, labeling test tubes as you go. (See Specimens section).

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 3.5mL acetone to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top acetone layer into clean and labeled 13x100 test tubes, place in nitrogen evaporator, and evaporate under a stream of nitrogen at 55° C, to dryness.

8.10. Remove dried specimens from nitrogen evaporator and reconstitute with 300µL of methanol. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.11. Transfer ~100 µL of each extract into appropriately labeled autosampler vials fitted with 100 µL polyspring insert and place in the autosampler tray of the Thermo TSQ triple-quadropole LC/MS/MS.

8.12. Build and initiate sequence as directed in SOP 053.

9. Calculations

9.1. Quantitative Response ratio

9.1.1. The method for processing the data using the Thermo LCQuan software is "Cocaine" (SOP 055). It is used to calculate the internal standard response ratio, raw amounts, concentration, and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's.

9.1.2.1.2. Response Ratio = QN_a / Qn_{istd}

9.1.2.1.2.1. QN_a = response of the quantitative ion of the analyte

9.1.2.1.2.2. QN_{istd} = response of the quantitative ion of the internal standard Amount

9.2. Calibration

9.2.1. A linear regression resulting from the 6 standards is used to quantitate the analytes in the case. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.3. Dilution Factor

9.3.1. D = Total volume/Sample volume

9.4. Multiplier for homogenates, dilutions, and non-standard volumes

9.4.1. $M = (V_{curve} / V_{samp}) \times D$

9.4.1.1. M = Multiplier
9.4.1.2. D = dilution factor

9.4.1.3. V_{curve} = matrix volume of calibration curve

9.4.1.4. V_{samp} = matrix volume of specimen

9.5. Concentration

9.5.1. C = (A / V) * M

9.5.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.

9.5.1.2. A = Amount of drug in sample

9.5.1.3. V = Volume of sample

9.5.1.4. M = Multiplier

9.6. Average

9.6.1. Average = $(R_1 + R_2) / 2$

9.6.1.1. R_1 = first result

9.6.1.2. R_2 = second result

9.7. Qualifier Ion Ratios

9.7.1.1.1. Ratio 1 = QL_1/QN

9.7.2. QL_1 = response of the quantifying product ion

9.7.3. QN = response of the qualifying product ion

10. **QA/QC**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

10.1.1.3. Peaks sharing parent/product ions must have baseline resolution.

10.1.1.4. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.1.5. Retention time must not deviate outside ± 3% of target, based upon the retention time of the calibrators and controls.

10.1.2. Mass spectroscopy

10.1.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio, as determined by a mid-level calibrator (CAL 4).

10.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.1.3. Calibrators

10.1.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (± 25% if at LOQ).

10.1.3.3. If the above criteria are not met, the calibration curve shall be determined to be invalid for that analyte and all affected cases shall be repeated or reported qualitatively at the discretion of a toxicologist (refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual).

10.1.4. Controls

10.1.4.1. Controls must calculate within ± 20% of the target value.

10.1.4.2. If the above criteria are not met, the batch shall be determined to be invalid for that analyte and all affected cases shall be repeated or reported qualitatively at the discretion of a toxicologist.

10.1.5. Blanks

10.1.5.1. Matrix specific negative controls (blanks) must not contain the analyte of interest at an area response ratio (IS area/Target area) greater than $1/10^{th}$ that of the lowest positive calibrator.

10.1.5.2. If the above criteria are not met, the batch shall be determined to be invalid for that analyte and all affected cases shall be repeated.

10.1.6. Any deviation from the above criteria must be approved by a senior chemist.

11. **Validation of Method**

Parameter	Result
Bias	Benzoylecgonine - L = -1.74% H = -6.15%
	Cocaethylene - L = -0.94% H = -5.94%
	Cocaine - L = -6.68% H = -10.53%
Precision	Benzoylecgonine - L = 3.20% H = 3.49%
	Cocaethylene - L = 2.41% H = 3.55%
	Cocaine - L = 2.93% H = 3.75%
Calibration model	Benzoylecgonine – Linear 1/x

	Cocaethylene - Linear 1/x
	Cocaine - Linear 1/x
Carryover	*Cocaine/Cocaethylene - no CO @ 4000ng/mL Benzoylecgonine - no CO @ 8000ng/mL
Interference Studies	No interfering signals observed
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	N/A - Analyte matching deuterated internal standards used (ΔRT ≤ 0.01 min)
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	BE - 2.5 ng/mL CE - 2.0 ng/mL COC - 2.0 ng/mL
LOQ (Set to lowest calibrator with acceptable Accuracy/Precision).	Cocaine - 10ng/mL Cocaethylene - 10ng/mL Benzoylecgonine - 20ng/mL
Dilution Integrity	N/A - Specimen dilution not routinely performed
Processed Sample Stability - (re-analyze after 8 days)	Extracts stable for at least 7 days

12. **Reporting**

12.1. When entering results in Toxlog, click on the 'Add Cocaine Analytes' button to automatically add Benzoyecgonine, Cocaethylene, and Cocaine to the analyte column of the result form. If any additional analytes are added (EME), delete that row from the table.

12.2. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section).

12.3. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 1

Dil / Scenario	1	1	REPORT
A	In curve	In curve	Average
B	In curve	AQL or BQL	"In" value
C	In curve	ND *	Repeat
D	AQL/BQL	AQL/BQL	Less than/ Greater than
E	BQL	ND	ND

* ND = None Detected, due to IRC, S/N threshold, r.t., or other

12.3.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration range

12.3.2. AQL = Measured concentration (pre-multiplier) falls Above Quantitation Limit

12.3.3. BQL = Measured concentration (pre-multiplier) falls Below Quantitation Limit

12.3.4. ND = None Detected

12.4. Averaging reportable values

12.4.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.4.2. Enter calculated concentration for each specimen into toxlog.

12.5. Significant figures

12.5.1. Concentrations are truncated and reported with two significant figures in mg/L (maximum of three decimal places).

12.6. Reinjection:

12.6.1. A sample may be re-injected due to autosampler failure, apparent low recovery, to check for carry-over or to meet ion ratio and/or retention time criteria. Re-injected sample(s) must be followed by reinjection of either the duplicate case sample(s) or matrix-matched calibrator or control. All re-injected samples must meet QA/QC criteria.

12.7. See the QA/QC Manual for laboratory guidelines.

13. **Preparation of Load**

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data –paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data - stapled

14. **References**

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14.2. Benowitz, Neal L. "Clinical Pharmacology and Toxicology of Cocaine." Pharmacology & Toxicology 72.1 (1993): 3-12.

14.3. Logan, B. K., D. Smirnow, and R. G. Gullberg. "Lack of Predictable Site-Dependent Differences and Time-Dependent Changes in Postmortem Concentrations of Cocaine, Benzoylecgonine, and Cocaethylene in Humans." Journal of Analytical Toxicology 21.1 (1997): 23-31.

14.4. Erin Chambers, Diane M. Wagrowski-Diehl, Ziling Lu, Jeffrey R. Mazzeo, Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analysis. Journal of Chromatography B 852(2007) 22-34.