

# Detection and Quantification of Cocaine and Benzoylcegonine in Meconium Using Solid Phase Extraction and UPLC/MS/MS

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## Abstract

A methodology for the selective determination and quantification of cocaine and its major metabolite benzoylcegonine in meconium using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is described. Past studies indicate that up to 40% of neonates dying within two days of birth with no apparent cause of death have cocaine and/or benzoylcegonine in their blood, and rates of infants exposed to cocaine prenatally has been estimated to be between 2.6% and 11% of all live births[1]. Ultra performance liquid chromatography (UPLC) is an emerging analytical technique which draws upon the principles of chromatography to run separations at higher flow rates for increased speed, while simultaneously achieving superior resolution and sensitivity. Extraction of both analytes was achieved using a preliminary protein crash followed by solid-phase extraction (SPE) employing UCT Clean-screen columns with reversed phase and ion-exchange retention mechanisms. The column was conditioned with sequential washes of methanol, DI water and 0.1M phosphate buffer (pH 6.0). Samples were then loaded onto the columns and following washes with DI water, 1M HCl and methanol, analytes were eluted with 3 mL of dichloromethane: 2-propanol: ammonium hydroxide (78:20:2) elution solvent. Samples were dried down under a gentle stream of nitrogen and reconstituted in 200µL of DI water: acetonitrile (75:25). Limits of detection for both analytes were 3 ng/g and the lower limit of quantitation (LLOQ) was 30 ng/g. Linearity was achieved over the range 30 to 250 ng/g. The methodology showed excellent intra run precision with % CV values ranging from 1-11% for cocaine and 1-16% for benzoylcegonine. Inter run precision was evaluated and experiments produced % CV values ranging from 3-5 % for cocaine and 4-9% for benzoylcegonine. The increased speed and separation efficiency offered by UPLC, allowed for the separation and subsequent quantification of both analytes in less than 2 minutes. Dramatic increases in separation speed such as those afforded by UPLC translate into increased samples per unit time in high throughput toxicology laboratories. Development of sensitive analytical methodologies capable of detecting low levels of such drugs in meconium will prove beneficial for the identification of prenatal substance abuse.

Keywords: Cocaine, meconium, UPLC-MS/MS

## Experimental

### Chemicals and reagents

Cocaine, benzoylcegonine, cocaine-d<sub>3</sub>, and benzoylcegonine-d<sub>3</sub> standards (1mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). ISOLUTE® HM-N supported liquid-liquid extraction columns were purchased from Biotage (Charlottesville, VA). All solvents were HPLC grade and obtained from Fisher Scientific (Pittsburgh PA).

### Calibration curve matrix

As certified drug free meconium is not commercially available, the suitability of negative blood for constructing calibration curves was investigated by preparing spiked meconium samples at varying concentrations (n=10) and quantifying them using a calibration curve made up in negative meconium and a calibration curve made up in negative blood. Meconium specimens which had previously screened negative for cocaine using a 50 ng/g cutoff at AIT laboratories (Indianapolis, IN) were collected and spiked with both cocaine and benzoylcegonine to give concentrations of 15 ng/mL (n=5) and 125 ng/mL (n=5). Spiked meconium was then quantified using a calibration curve constructed in negative meconium and a calibration curve constructed in certified drug free blood. Quantitative results obtained using the meconium calibration curve showed excellent correlation (<15% CV) with those obtained using the calibration curve made up in negative blood and as a result, all subsequent method validation experiments were performed using calibration curves prepared in certified negative blood.

### Calibration curves

Calibration curves for all experiments were prepared according to Table 1.

Table 1. Preparation of cocaine and benzoylcegonine calibration curves.

Standard Concentration (ng/mL)	Volume of Working Standard (µL)	Volume of Deionized water (µL)
250	500	500
100	200	800
50	100	900
25	50	950
10	20	980
5	10	990
Negative	0	1000

## Sample Preparation

Meconium samples were accurately weighed and then diluted by a factor of 3 (w/v) with 50:50 methanol/water to assist with the sonication procedure. Samples were shaken and sonicated for 10-15 minutes. Following sonication, 1 mL of the meconium sample was added to appropriately labeled culture tubes. Cocaine, benzoylcegonine and deuterated internal standards were added to samples which were then block vortexed for 5 minutes. Analytes were extracted by first adding 2 mL of cold acetonitrile while simultaneously vortexing each sample. Samples were then centrifuged for 10 minutes at 3000 rpm. Following centrifugation, the organic layer was transferred to a clean labeled large screw top test tube. 3 mL of 0.1 M phosphate buffer (pH 6.0) was added to each sample followed by the addition of 1 mL of concentrated ammonium hydroxide. Samples were then vortexed by hand for 10-15 seconds followed by a 5 minute centrifugation at 3000 rpm.

### Solid Phase Extraction

Analytes were selectively extracted using a solid phase extraction employing UCT clean-screen ZSDAU020 columns with reversed phase and ion-exchange retention mechanisms. Columns were first conditioned with sequential washes of methanol (3 mL), deionized water (3 mL) and 0.1 M phosphate buffer, pH 6.0 (1 mL). All solvents were allowed to drip through the columns slowly under gravity and waste containers were interchanged accordingly. Following column conditioning, samples were poured onto the columns and allowed to drip through unassisted for 15 minutes before any pressure was applied. Following sample loading, sequential wash steps were performed using deionized water (1 mL), 1.0 M HCl (1 mL), and methanol (3 mL). All wash solvents were allowed to drip through the columns unassisted for 5-10 minutes after which time any remaining solvent was assisted through the columns using a positive pressure manifold. Following column wash steps, positive pressure was applied for 5-10 minutes to ensure complete elution of wash solvents. Waste containers were then exchanged for small, labeled elution test tubes and analytes were eluted with 3 mL of 78:20:2 (dichloromethane: 2-propanol: ammonium hydroxide) elution solvent which was made fresh daily. Samples were then dried down under a gentle stream of nitrogen and reconstituted in 200 µL of DI water:ACN (75:25). Samples were transferred to appropriately labeled plastic vials and injected for analysis.

## Liquid Chromatography

Liquid chromatographic separations were performed on a Waters ACQUITY™ ultra performance liquid chromatograph (UPLC) (Waters Corp., Milford, MA, USA). Separations were achieved on an ACQUITY UPLC® HSS T3 column (2.1x 50mm) packed with 1.8µm bridged ethyl hybrid (BEH) particles and maintained at 35°C. The mobile phase consisted of deionized water containing 0.1% formic acid (solvent A), and acetonitrile containing 0.1% formic acid (solvent B). Analytes were eluted from the UPLC column using the following step-wise binary elution gradient: Initial mobile phase composition was 75:25 (H<sub>2</sub>O:ACN). Initial conditions were held constant for 0.5 mins after which the composition of solvent B was linearly increased to 50% over 1.5 mins, finally conditions were returned to their initial composition of 75:25 (H<sub>2</sub>O:ACN) over the next 0.01 mins and held for 0.49 min to equilibrate the column before the next injection in the sequence. The total run time was 2.50 mins. Samples were maintained at 7.5°C in the sample organizer and sample injection volumes were 5µL for all analyses. Flow rates were maintained at 0.5 mL/min for the first 0.50 mins after which they were increased to 0.6 mL/min for the remainder of the chromatographic separation. All flow was directed into the ESI source of the mass spectrometer.

## Mass spectrometry

Mass spectrometric detection was performed using a Waters TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source operating in positive ion mode. MS/MS conditions were as follows: capillary voltage 0.80 kV, cone voltage 20 V, extractor voltage 3.0 V, RF lens voltage 0 V. The source temperature was 120°C while the desolvation temperature was set at 350°C. Cone gas was set at a flow of 100 L/Hr while the desolvation gas flow was 900 L/Hr. The collision gas flow was set to 0.10 mL/min. Nitrogen (99.995% purity) was used as the desolvation gas, and ultra-pure argon (99.999% purity) was used as the collision gas. Appropriate quantifier and qualifier mass transitions were identified for each analyte by directly infusing a 10 µg/mL solution of each compound into the mass spectrometer ionization source at a flow rate of 20 µL/min (Table 2).

Table 2. MS/MS parameters used for each analyte and deuterated internal standard

Compound	Mass transition	Purpose	Cone (V)	Collision (V)	Dwell (secs)
Cocaine	304.14 > 182.10	Quantifying ion	40	20	0.01
Cocaine	304.14 > 150.16	Qualifying ion	40	20	0.01
Cocaine-d3	307.15 > 184.96	Quantifying ion	30	20	0.01
Benzoylcegonine	290.08 > 168.24	Quantifying ion	40	20	0.01
Benzoylcegonine	290.08 > 104.78	Qualifying ion	40	40	0.01
Benzoylcegonine-d3	293.11 > 170.98	Quantifying ion	40	20	0.01

## Results and Discussion

### Equivalence

Preliminary equivalence studies indicated that calibration curves prepared in certified drug-free blood were suitable for the accurate quantification of cocaine and benzoylcegonine in the meconium matrix. The percent relative error (%RE) calculated for the 5 HQCs and 5 LQCs was less than 1.6% for cocaine and 7.2% for benzoylcegonine, indicating excellent correlation between the two calibration curves at both the high and low end of the calibration range.

### Selectivity

Blank meconium specimens were analyzed to ensure that any response generated by the matrix alone corresponded to a concentration less than the LLOQ, while spiked samples were analyzed in order to assess the ability of the methodology to accurately and precisely quantitate the analyte in the presence of possible exogenous interferents. Blank meconium specimens generated minimal detector responses which corresponded to analyte concentrations ranging from 0.3-0.5 ng/mL for cocaine and 0.9-1.9 ng/mL for benzoylcegonine which are below the LLOQ of 10 ng/mL. Analysis of spiked standards prepared at the LLOQ, indicate that the methodology is selective and specific for the analytes, even in the presence of various exogenous interferents

### Accuracy

Accuracy studies performed by analyzing five replicates at three different concentrations spanning the calibration range yielded mean values of 254.9 ng/mL, 52.7 ng/mL, and 10.5 ng/mL reflecting accuracies of 98%, 94.6%, and 95%, respectively for cocaine and 245.7 ng/mL, 50.2 ng/mL, and 10.5 ng/mL reflecting accuracies of 98.3%, 99.6%, and 95%, respectively for benzoylcegonine.

### Precision

Both intra-batch and inter-batch precision studies indicated excellent method precision over the three concentration values investigated with intra-batch % CVs ranging from 0-10.4% with the mean being 3.8% for cocaine, while % CVs for benzoylcegonine ranged from 0.4-16% with a mean value of 5.5%. Inter-batch precision was also excellent with % CV values ranging from 3.9-4.9% over the three concentration ranges for cocaine and from 4.4-8.7% over the three concentrations for benzoylcegonine.

### Recovery

Analyte recovery was investigated over three concentrations which spanned the calibration range for each analyte and was found to have a mean value of 71.7% for cocaine and 10.2% for benzoylcegonine. Mean recoveries from triplicate analysis at the three concentration ranges investigated ranged from 68.3-77% for cocaine, and 9.3-10.7% for benzoylcegonine representing good consistency and reproducibility even though the overall recovery of benzoylcegonine was poor.

### LOD

The limit of detection was determined to be 1 ng/mL for both cocaine and benzoylcegonine corresponding to a concentration of 3 ng/g in the meconium specimen prior to sonication. Unequivocal identification of analytes was not feasible at concentrations below 1 ng/mL due to significant fluctuation and inaccuracies in calculated ion ratios.

## References

1. Birchfield, M., J. Scully, and A. Handler, *Perinatal screening for illicit drugs: policies in hospitals in a large metropolitan area*. J Perinatol, 1995. 15(3): p. 208-214.

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