

# Simultaneous Quantification of Amphetamine and Methamphetamine in Meconium Using ISOLUTE<sup>®</sup> HM-N Supported Liquid Extraction Columns and UPLC/MS/MS



Joshua A Gunn, B.S (Hons)\*; Scott Kriger, PhD; Andrea R Terrell, PhD  
AIT Laboratories, 2265 Executive Drive, Indianapolis, IN 46241

## Abstract

A procedure for the rapid extraction and quantification of amphetamine and methamphetamine from meconium using ISOLUTE HM-N supported liquid extraction columns and ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) is described. Due to the matrix complexity of meconium samples, extraction and sample preparation prior to instrumental analysis can prove difficult and time consuming. The present study introduces a novel sample preparation technique for the simultaneous quantification of amphetamine and methamphetamine in meconium using UPLC/MS/MS. 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 ISOLUTE HM-N supported liquid extraction columns containing a modified form of diatomaceous earth. Subsequent separation and quantification using UPLC/MS/MS was achieved in less than 3 minutes. Limits of detection for amphetamine and methamphetamine were 3 ng/g and 750 pg/g respectively. The lower limit of quantitation (LLOQ) was 15 ng/g. Linearity was achieved over the range 15 ng/g to 1500 ng/g. The methodology showed excellent intra run precision with %CV values ranging from 1-9% for amphetamine and 1-6% for methamphetamine. Inter run precision experiments produced %CV values ranging from 3-7% for amphetamine and 1-6% for methamphetamine. The reported methodology proved suitable for the accurate quantification of amphetamine and methamphetamine in meconium samples and greatly reduced sample preparation time normally required for traditional solid phase extraction. Development and validation of such analytical methodologies will prove beneficial for the identification of prenatal substance abuse which is an ongoing concern across socioeconomic lines.

**Keywords:** Meconium; UPLC/MS/MS; supported liquid extraction

## Experimental

### Chemicals and reagents

Amphetamine, methamphetamine, amphetamine-d<sub>6</sub>, and methamphetamine-d<sub>9</sub> standards (1mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). ISOLUTE<sup>®</sup> 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 amphetamines using a 50 ng/g cutoff at AIT laboratories (Indianapolis, IN) were collected and spiked with both amphetamine and methamphetamine to give concentrations of 10 ng/mL (n=5) and 500 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 amphetamine and methamphetamine calibration curves.

Standard Concentration (ng/mL)	Volume of Working Standard (µL)	Volume of Negative Blood (µL)
500	1000 (Std 1)	0
250	500 (Std 1)	500
100	200 (Std 1)	800
50	1000 (Std 2)	0
25	500 (Std 2)	500
10	200 (Std 2)	800
5	100 (Std 2)	900
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. Amphetamine and methamphetamine standards and deuterated internal standards were added to samples which were then block vortexed for 5 minutes. Samples were centrifuged for 5 minutes at 3500 rpm after which the supernatants were transferred to appropriately labeled culture tubes (12x75). Sample recovery was optimized by adding 500 µL of 2.0M NaOH to ensure that the analyte was present in its basic state. Samples were then diluted with 2mL of deionized water and vortexed for 10-15 seconds. Samples were loaded into 5mL ISOLUTE HM-N supported liquid extraction columns and left to sit for 10 minutes. Analytes were initially eluted with 5 mL of ethyl acetate and after a 3 minute waiting period a second elution step was performed with 3 mL of ethyl acetate. 200 µL of 1% HCl was added to all eluates to ensure formation of the hydrochloride salts in order to reduce the possibility of analyte loss during evaporation steps. Samples were dried down under a gentle stream of nitrogen and reconstituted in 200 µL of DI water. Samples were transferred to UPLC vials and injected.

## Liquid Chromatography

Liquid chromatographic separations were performed on a Waters ACQUITY<sup>™</sup> ultra performance liquid chromatograph (UPLC) (Waters Corp., Milford, MA, USA). Separations were achieved on an ACQUITY UPLC<sup>®</sup> phenyl column (2.1x 50mm) packed with 1.7µm 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 99:1 (H<sub>2</sub>O:ACN). The composition of solvent B was increased to 2% over the first 0.10 mins after which time it was linearly increased to 10% over 2.9 mins followed by an increase to 100% over 0.50 mins, finally conditions were returned to their initial composition of 99:1 (H<sub>2</sub>O:ACN) over 0.50 mins and held for 1 min to equilibrate the column before the next injection in the sequence. The total run time was 5 mins. Samples were maintained at 10°C in the sample organizer and sample injection volumes were 1µL for all analyses. Flow rates remained constant at 0.5 mL/min and 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 0.60 kV, cone voltage 22 V, extractor voltage 3.1 V, RF lens voltage 0.1 V. The source temperature was 150°C while the desolvation temperature was set at 350°C. Cone gas was set at a flow of 50 L/hr while the desolvation gas flow was 900 L/hr. The collision gas flow was set to 0.18 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)
Amphetamine	135.97 > 90.90	Quantifying ion	20	14	0.02
Amphetamine	135.97 > 119.0	Qualifying ion	20	10	0.02
Amphetamine-d6	141.94 > 93.00	Quantifying ion	20	16	0.02
Methamphetamine	149.97 > 90.90	Quantifying ion	25	16	0.02
Methamphetamine	149.97 > 119.0	Qualifying ion	25	12	0.02
Methamphetamine-d9	159.03 > 92.90	Quantifying ion	25	18	0.02

Figure 1 illustrates the fragmentation pathways for amphetamine and methamphetamine under ESI conditions and the resulting product ions for analyte detection in MRM experiments.

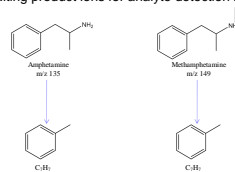


Figure 1: Fragmentation pathways for amphetamine and methamphetamine during tandem mass spectrometry experiments

## Results and Discussion

### Equivalence

Equivalence studies were performed to investigate the accuracy of employing calibration curves constructed in certified drug-free blood for the purpose of quantifying analytes in the meconium matrix. Five LQC and five HQC standards were prepared and quantified using a calibration curve constructed in negative whole blood, and a calibration curve constructed in meconium. The relative error of the two quantitative values was calculated for each QC and used to determine the level of agreement between the two calibration curves. Relative error did not exceed 2% indicating excellent correlation between calibration curves.

### Selectivity

The analytical methodology was deemed selective following the analysis of five meconium blanks and five QC standards prepared at the LLOQ which had been spiked with various exogenous interferences commonly encountered in forensic specimens. Blank meconium specimens were analyzed in order to ensure minimal analyte response was generated from any endogenous matrix interference.

Analyte responses during the analysis of blank samples did not exceed 0.2 ng/mL for either analyte indicating that false positives arising from endogenous matrix interferences are unlikely. Analysis of QC standards prepared at the LLOQ, which had been spiked with various exogenous interferences, indicated that accurate and selective identification of amphetamine and methamphetamine was possible even in the presence of various other xenobiotics.

### Accuracy

The accuracy of the analytical method was investigated by analyzing five replicate QC standards over three different concentrations spanning the calibration range. Accuracy was assessed by calculating the closeness of the mean test results to the known standard concentration. Mean values were determined using five replicates prepared at concentrations of 500, 50, and 5 ng/mL. Mean values of 491.1 ng/mL, 50.8 ng/mL, and 4.8 ng/mL were obtained from replicate analysis of amphetamine standards at concentrations of 500, 50, and 5 ng/mL respectively, representing accuracies of 98.2%, 98.4%, and 96%. Replicate analysis of methamphetamine QCs prepared at concentrations of 500, 50, and 5 ng/mL produced mean test values of 530.9 ng/mL, 53.2 ng/mL, and 4.7 ng/mL respectively, representing accuracies of 93.8%, 93.6%, and 94%.

### Precision

Both intra- and inter-batch studies indicated excellent method precision with %CV values falling well within established acceptance criteria. Precision of the analytical methodology was investigated by analyzing five replicate QC standards at concentrations of 500, 50, and 5 ng/mL over four consecutive days. Intra-batch precision studies yielded %CVs ranging from 1.4%-8.5% for amphetamine and 0.7%-5.4% for methamphetamine indicating excellent intra-batch precision over the entire calibration range. Inter-batch precision studies were also promising with four-day %CVs of 3.9%, 2.0%, and 6.2% for amphetamine QCs prepared at concentrations of 500, 50, and 5 ng/mL, while analysis of methamphetamine standards over the four day time period produced %CVs of 1.9%, 3.7%, and 5.3% for the 500, 50, and 5 ng/mL QCs respectively.

### Recovery

Recovery of amphetamine and methamphetamine from the meconium matrix using the HM-N supported-liquid extraction technique was investigated in order to determine the efficiency of the extraction. Extraction efficiency was assessed by comparing the detector response for unextracted standards prepared at concentrations of 500, 50, and 5 ng/mL with the detector response for extracted standards prepared at the same concentrations. Standards were prepared in triplicate and peak area responses for unextracted standards represented 100% recovery. Peak area responses for extracted standards were then used to calculate recovery. Mean analyte recoveries over the three concentrations investigated were 52% for amphetamine and 52.7% for methamphetamine. Mean analyte recoveries for triplicate standards at each of the three concentrations ranged from 49-57% for amphetamine and 47-58% for methamphetamine.

### LOD

The limit of detection was 1.0 ng/mL for amphetamine and 0.250 ng/mL for methamphetamine corresponding to concentrations of 3.0 ng/g and 0.750 ng/g in the meconium specimen prior to sonication. Limits of detection were calculated based on acceptance criteria for retention times and ion ratios. At concentrations below 1 ng/mL for amphetamine and 0.250 ng/mL for methamphetamine, unequivocal identification was not possible due to inaccurate ion ratios.

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