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A liquid chromatography tandem mass spectrometry method for the simultaneous quantification of 20 drugs of abuse and metabolites in human meconium

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Abstract

A method for the simultaneous quantification of 20 cocaine, amphetamine, opiate, and nicotine analytes in meconium, the first neonatal feces, by liquid chromatography tandem mass spectrometry was developed and validated. Specimen preparation included methanol homogenization and solid phase extraction. Two injections were required to achieve sufficient sensitivity and linear dynamic range. Linearity ranged from 0.5–25 up to 500 ng/g (250 ng/g *p*-hydroxymethamphetamine), and correlation coefficients were >0.996. Imprecision was <10.0% CV, analytical recovery 85.5–123.1%, and extraction efficiencies >46.7% at three concentrations across the linear range. Despite significant matrix effects of –305.7–40.7%, effects were similar for native and deuterated analytes. No carryover, endogenous or exogenous interferences were observed, with analyte stability at room temperature, 4 °C, and –20 °C and on the autosampler >70%, except for 6-acetylmorphine, hydrocodone, oxycodone, and morphine. Method applicability was demonstrated by analyzing meconium from drug-exposed neonates.

Keywords

Meconium; Drugs of abuse; Prenatal drug exposure; Liquid chromatography tandem mass spectrometry

Introduction

According to the 2005 and 2006 National Surveys of Drug Use and Health, 4.0% and 16.5% of pregnant respondents admitted recent illicit drug and tobacco use, respectively [1]. Adverse obstetrical and neonatal outcomes, such as fetal growth restriction, premature birth, neonatal abstinence syndrome, and neurological and behavior deficits, are associated with illicit drug and tobacco consumption during pregnancy [2, 3]. Detection of prenatal drug exposure depends upon maternal self-report or toxicological analysis of maternal and/or neonatal tissues and fluids. Maternal self-report is often inaccurate because of feelings of guilt, fear of losing custody or prosecution, or inability to precisely recall timing or magnitude of drug consumption. Interviewing methods have improved, better identifying women at high risk for substance abuse during gestation and generating more accurate responses to maternal questionnaires [4–6]; however, underreporting remains a problem. Maternal interview was the least sensitive method of identifying drug use in pregnancy

when compared to maternal hair and meconium drug testing [7], whereas for methamphetamine (MAMP), self-report identified more exposed neonates than meconium drug tests [8]. Consequently, there is a strong need for sensitive and specific bioanalytical procedures to measure biomarkers of in utero exposure in neonatal biological tissues and fluids.

Meconium offers several advantages for detecting prenatal drug exposure and has become the specimen of choice within the past 20 years. Accumulating from the 12th gestational week until birth, meconium acts as a reservoir for exogenous compounds, such as drugs and metabolites. Drugs are incorporated into meconium via fetal biliary excretion or through swallowing drug-contaminated amniotic fluid. Meconium analysis is thought to detect maternal drug use during the second and third trimesters, whereas neonatal hair and urine analyses indicate more recent exposure. Collection of meconium from diapers is easier than collection of neonatal urine; the adhesive on the urine bag may irritate newborn skin and frequently becomes dislodged, spilling the specimen. Neonatal hair is often present in insufficient quantity, and many mothers do not consent to infant hair collection for cosmetic or cultural reasons.

Meconium is usually passed within 1 to 3 days after birth but may be delayed in premature infants. Meconium's complex composition of mucopolysaccharides, water, bile salts, bile acids, epithelial cells, and other lipids makes drug isolation difficult. Illicit substances, therapeutic drugs, ethanol metabolites, pesticides, and other xenobiotics have been analyzed in meconium using commonly employed toxicological techniques, including immunoassay screening and gas and liquid chromatography/mass spectrometry (GCMS; LCMS) confirmation; reviews by Gray et al. and Lozano et al. summarize the current literature on meconium analysis [9, 10].

If maternal drug use is suspected, meconium is often assayed for cocaine (COC), amphetamine (AMP), and opiate biomarkers. Several studies identify parent COC, benzoylecgonine (BE), and *m*-hydroxybenzoylecgonine (mOHBE) as the most useful biomarkers of prenatal cocaine exposure, while cocaethylene (CE) indicates simultaneous COC and ethanol consumption [11–15]. Over the past decade, MAMP abuse rapidly increased in the USA, highlighting the need to identify neonates exposed to sympathomimetic amines. MAMP and AMP are most often tested in meconium [16], but *p*-hydroxymethamphetamine (pOHMAMP) appears to be a promising new biomarker [17, 18].

An estimated 55–94% of opiate-exposed infants develop neonatal abstinence syndrome (NAS) [19]; therefore, detecting opiates in meconium may aid in NAS diagnosis and facilitate treatment. Both illicit and licit opiates have been identified in meconium, including 6-acetylmorphine (6AM), a definitive biomarker of heroin consumption, morphine (MOR), codeine (COD), hydromorphone (HYM), hydrocodone (HYC), and oxycodone (OXY) [12, 20–24]. Opiate-dependent pregnant women often chose methadone (MTD) pharmacotherapy, while buprenorphine (BUP) is under investigation as an effective alternative within the obstetric population. These compounds and their metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and norbuprenorphine (NBUP), have been quantified in meconium [25–28]. While not part of routine testing, the nicotine metabolite cotinine (COT) has been evaluated by immunoassay in research investigations to detect prenatal tobacco exposure [29, 30]. Recently, nicotine (NIC) and *trans*-3'-hydroxycotinine (OHCOT) have emerged as important tobacco biomarkers in meconium; our laboratory found that nearly 25% of tobacco-exposed neonates' meconium contained NIC and/or OHCOT in the absence of COT when analyzed by liquid chromatography tandem mass spectrometry (LCMSMS) [31, 32].

Specimen preparation generally involves homogenization in buffer or methanol prior to analyte isolation by liquid–liquid or solid phase extraction. Analytical procedures for meconium are often time- and resource-consuming because of extensive specimen clean up. Usually, a separate 1.0 g aliquot of meconium is extracted for each drug class (i.e., opiates, cocaine, amphetamines); specimen quantity is often limited, making comprehensive screening and confirmation problematic. Previous meconium methods have obtained relatively low limits of quantification (LOQ) for amphetamines (2.5–5 ng/g) [16, 17], COC and metabolites (1–5 ng/g) [11, 23], NIC and metabolites (1.25–5 ng/g) [31], and opiates (1–5 ng/g for 6AM, MOR, COD, MTD, EDDP, HYC, HYM, and OXY and 20 ng/g BUP and NBUP) [21–23, 25, 26], but specimen quantity, time, and resource requirements can limit comprehensive drug testing.

The aim of this research was to develop an LCMSMS procedure for detecting and quantifying drugs and metabolites commonly encountered in meconium including AMP, MAMP, pOHMAMP, COC, BE, CE, mOHBE, NIC, COT, OHCOT, 6AM, MOR, COD, HYM, HYC, OXY, MTD, EDDP, BUP, and NBUP in a single 0.25-g specimen. This method will be applied in prenatal drug and tobacco exposure clinical studies. Qualitative meconium results can corroborate or refute maternal self-report and identify drug-exposed infants when maternal self-report is unavailable. Importantly, investigation of relationships between the timing, frequency, and magnitude of maternal drug consumption, the presence and concentration of drugs and metabolites in meconium, and neonatal outcomes requires a sensitive and specific analytical assay. Characterizing these relationships could identify needed medical and social resources for the child and mother, inform drug prevention and treatment efforts, guide future in utero exposure research, and shape substance abuse policies within the obstetric population.

Experimental section

Meconium

Meconium specimens screening negative by immunoassay for amphetamines, opiates, cannabinoids, cocaine, and cotinine were pooled and mixed thoroughly. Meconium pools were confirmed negative for all analytes at the method's lower LOQ prior to preparation of calibrators or quality control (QC) samples. To demonstrate method applicability, US Drug Testing Laboratory kindly donated positive meconium specimens previously analyzed by immunoassay and GCMS.

Reagents and standards

6AM, 6AM-D₆ (100 µg/mL), (±)-AMP, (±)-AMP-D₁₁, BED₈ (100 µg/mL), BUP (100 µg/mL), BUP-D₄ (100 µg/mL), CE, CE-D₃ (100 µg/mL), COC, COC-D₃ (100 µg/mL), COD, COD-D₆, (–)-COT, (±)-COT-D₃ (100 µg/mL), EDDP perchlorate, EDDP-D₃ perchlorate (100 µg/mL), HYC, HYC-D₆, HYM, HYM-D₃, (±)-MAMP, (±)-MAMP-D₁₄, mOHBE, MOR, MOR-D₃ (100 µg/mL), (±)-MTD, (±)-MTD-D₉, S(–)-NIC, (±)-NIC-D₄ (100 µg/mL), NBUP (100 µg/mL), NBUP-D₃ (100 µg/mL), OXY, and OXY-D₆ were obtained from Cerilliant (Austin, TX, USA) at 1.0 mg/mL, except where noted. OHCOT and OHCOT-D₃ were purchased as powders from Toronto Research Chemicals (North York, Ontario, Canada) and 1.0 mg/mL BE and MTD HCl from Lipomed (Cambridge, MA, USA). Powdered pOHMAMP was from the National Institute on Drug Abuse Drug Inventory (Research Triangle, NC, USA). Ammonium formate and formic acid were acquired from Sigma (St. Louis, MO, USA). Water, acetonitrile, sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous, hydrochloric acid, dichloromethane, 2-propanol, and ammonium hydroxide were purchased from J.T. Baker (Philipsburg, NJ, USA). Methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). All solvents

and reagents were high-performance liquid chromatography (HPLC) or American Chemical Society grade.

Instrumentation

The HPLC system consisted of Shimadzu LC-10ADvp pumps, CTO-10ACvp column oven, and SIL-HTc autosampler (Columbia, MD, USA). MSMS analysis was performed on a MDS Sciex API 3000 triple quadrupole mass spectrometer with an electrospray source (Applied Biosystems, Foster City, CA, USA). Analyst software version 1.4.2 was used for acquisition and data analysis. Meconium homogenization was performed with the Appropriate Technical Resources' Rotamix (Laurel, MD, USA). SPSS 16.0 for Windows (Chicago, IL, USA) was used for statistical analyses.

Preparation of standard solutions

Powdered standards were reconstituted in the manufacturer's recommended solvent. A 100,000-ng/mL stock solution (20,000 ng/mL for BUP and NBUP) was prepared in methanol and stored at -20°C . Dilutions of stock solution with methanol created 5, 50, 5,000, and 50,000 ng/mL working solutions. Appropriate volumes were added to approximately 0.25 g meconium, yielding 1, 2.5, 5, 10, 25, 100, 250, 375, and 500 ng/g calibrators.

QC solutions were prepared in a similar manner but from alternate suppliers or different manufacturer lots than for calibrators. A 100,000-ng/mL stock solution (20,000 ng/mL for BUP and NBUP) was created in methanol and stored at -20°C . Dilutions in methanol produced 1,000, 4,000 (for BUP and NBUP), and 10,000 ng/mL solutions. These solutions were combined to produce three QC working solutions that were added to approximately 0.25 g blank meconium to produce low, medium, and high QC samples as shown in Table 3.

A 500-ng/mL (1,000 ng/mL BUP- D_4 and NBUP- D_3) working internal standard solution was prepared by diluting 100 $\mu\text{g/mL}$ and 1.0 mg/mL stock solutions in methanol. The final deuterated internal standard concentration after spiking 25 μL working solution in approximately 0.25 g meconium was 50 ng/g (100 ng/g BUP- D_4 and NBUP- D_3). Deuterated analogs were available for all analytes except pOHMAMP and mOHBE (Table 1).

Procedures

Specimen preparation—Blank meconium (0.25 ± 0.005 g) was weighed into a 1.5 mL microcentrifuge tube and fortified with calibrator or QC solutions and internal standard. One milliliter of methanol with 0.01% formic acid (w/v) was added, specimens were vortexed vigorously, and mixed on a rotator for 30 min at 40 rpm. Samples were centrifuged at $14,000 \times g$ for 10 min. The supernatant was transferred to a clean glass tube and evaporated to approximately 0.25 mL under nitrogen at 40°C . The concentrated supernatant was reconstituted in 2 mL 0.1 M phosphate buffer, pH 6.0.

Solid phase extraction—CleanScreen DAU solid phase extraction columns, part ZSDAU020, were purchased from United Chemical Technologies (Bristol, PA, USA). Columns were conditioned with 3 mL methanol, 3 mL water, and 1 mL 0.1 M phosphate buffer, pH 6.0. Specimens were loaded on column with gravity flow. Columns were washed with 3 mL water, 1 mL 0.1 M aqueous acetic acid, dried 5 min, 3 mL methanol, and dried 5 min. Analytes were eluted with 3 mL dichloromethane/2-propanol/ammonium hydroxide (78:20:2, v/v). Eluates were dried under nitrogen at 40°C ; a drop of 1% hydrochloric acid in methanol (v/v) was added near the end of drying to prevent volatilization of sensitive compounds. Samples were reconstituted in 200 μL 1 mM ammonium formate pH 3.4 and transferred to glass autosampler vials.

Liquid chromatography—Two chromatographic runs were required for quantification of 20 target analytes and 18 internal standards. BUP, NBUP, and their respective deuterated internal standards could not be successfully incorporated into the main chromatographic run. Both runs were performed on the Synergi Hydro RP column (150×2.0 mm, 4 μm, Phenomenex, Torrance, CA, USA) maintained at ambient temperature and attached to a guard column of the same packing material. Mobile phase A was 1 mM ammonium formate, pH 3.4 and B, 95% acetonitrile with 5% mobile phase A. The first acquisition method (method 1) utilized a 5 μL injection volume, 0.250 mL/min flow rate, and a gradient program of 0% B, increase to 30% B over 8 min, increase to 35% B over 2 min, increase to 60% B over 2 min, hold for 1 min, decrease to 0% B over 2.5 min, and re-equilibrate at 0% B for 2.5 min. The second acquisition method (method 2) quantifying BUP and NBUP consisted of a 10 μL injection, 5 min isocratic flow set to 0.30 mL/min, and 75% mobile phase B.

Mass spectrometry—Electrospray ionization operating in positive mode was used for all analytes. Compound-specific optimization of MSMS parameters was performed via direct infusion of individual 100 ng/mL methanolic reference solutions using a syringe pump. Optimization results for the two most abundant ion transitions per analyte in multiple reaction monitoring scan mode (MRM) are given in Table 1. Source parameters were set to 14 L/min curtain gas, 10 L/min nebulizer gas, 12 L/min collision gas, 5,000 V ion source voltage, and 550 °C source temperature after flow injection analysis optimization; all gasses were 99.9% pure nitrogen. Method 1 was divided into three periods, and dwell times were set to 40 ms for each transition to increase the number of scan events over a chromatographic peak, thereby increasing accuracy and precision of the peak integration and quantification. The periods were as follows: period 1—COT, HYM, MOR, NIC, OHCOT, and pOHMAMP; period 2—6AM, AMP, COD, HYC, MAMP, mOHBE, and OXY; period 3—BE, CE, COC, EDDP, and MTD. Dwell time for method 2 was set to 150 ms, and a single period was used.

Data analysis

Calibration with deuterated internal standards and least squares regression with 1/x weighting was performed for all analytes. Deuterated analogues of mOHBE and pOHMAMP were not commercially available; therefore, COD-D₆ and COT-D₃ were used as internal standards for mOHBE and pOHMAMP, respectively, based on similar retention times, molecular weights, and matrix effects. The most abundant transition for each analyte was used for quantification, while the second transition served as a qualifier (Table 1). For OXY and OXY-D₆, the second most abundant transitions (316.1→241.0 and 322.3→262.1) were chosen for quantification because the most abundant transitions (316.1→298.1 and 322.3→304.2) correspond to a nonspecific loss of water. Less abundant transitions of COD, 6AM, mOHBE, and MAMP yielded better calibration results; therefore, these were selected for quantification.

Validation

Specificity, sensitivity, linearity, recovery, intra- and inter-day imprecision, extraction efficiency, matrix effect, carryover, dilution integrity, endogenous and exogenous interferences, and analyte stability were evaluated.

Specificity was assessed by retention time and quantifier and qualifier transition peak area ratios. Retention times for QC and authentic meconium specimens were required to be within ±0.2 min of the mean calibrator retention time. Criteria for transition peak area ratios for QC and authentic specimens were as recommended by the European Union identification criteria guidelines [33]. Permitted tolerances were based on the relative abundance of the

quantifier and qualifier transition peak area ratios; therefore, analytes with qualifier transition areas greater than 50% of the quantifier were allowed a 20% deviation from the mean calibrator transition ratio; relative abundances between 20–50% were allowed 25% deviation, between 10–20% a 30% deviation, and less than 10% a 50% deviation was permitted. Sensitivity was defined by limits of detection (LOD) and LOQ. LOD and LOQ were determined empirically with extracted meconium specimens fortified with dilutions of the 5 ng/mL working calibrator solution and defined as the concentration with acceptable peak shape, a signal to noise ratio of at least 3:1 (LOD) and 10:1 (LOQ) for all ions, transition ratios within 20% of mean calibrator ratios, retention time within ± 0.2 min of the mean calibrator retention time, and for LOQ, a calculated concentration within $\pm 20\%$ of target. Linearity, expressed as the coefficient of determination (R^2), was evaluated with a least square regression line calculated from all standard concentrations. Calibrator concentrations were required to be within $\pm 20\%$ of target when calculated against the full calibration curve.

Recovery and imprecision were assessed at three QC concentrations spanning the dynamic linear range. Inter-day recovery was defined as the relative error from the nominal target concentration of five replicates analyzed on four separate days ($N=20$). Pooled intra-day, inter-day, and total imprecision of five replicates analyzed on four separate days ($N=5$ intra-day; $N=20$ inter-day and total) were calculated according to the recommendations of Krouwer and Rabinowitz and expressed as percent coefficient of variance (% CV) [34, 35].

Extraction efficiency and matrix effects were determined using the experimental design proposed by Matuszewski et al. [36]. Set 1 was meconium fortified with QC and internal standards solutions prior to homogenization; set 2 was extracted meconium with QC and internal standard solutions added to elution solvent following solid phase extraction. Set 3 samples were QC and internal standard solutions prepared neat. Each set consisted of five replicates and the experiment was repeated at each QC level. Extraction efficiency was calculated by dividing the mean analyte peak areas from set 1 by set 2, and matrix effects were determined by dividing mean peak areas of set 2 by set 3. Variability in matrix effects among different meconium specimens was investigated by fortifying extracts from seven blank meconium pools with low QC and internal standard solutions and comparing peak areas to the mean peak area of five neat QC replicates.

Carryover was determined by injecting a negative specimen containing internal standard after a specimen containing two times the upper limit of quantification. High concentrations are often observed in meconium, making dilution necessary. Dilution integrity (1:5) was assessed with three meconium specimens fortified with high QC solution and internal standards and homogenized in acidic methanol as previously described. After centrifugation, 200 μ L supernatant was combined with 800 μ L supernatant from meconium fortified with only internal standard and procedure followed as usual.

Interference from endogenous meconium compounds was assessed by fortifying seven blank meconium specimens with low QC solution and evaluating calculated concentrations. Interferences from illicit and common therapeutic drugs, metabolites, and related compounds were evaluated by adding potential interferents into low QC samples. A compound did not interfere if the low QC quantified within 20% of target and had stable retention time and transition ratios within the permitted tolerance. High concentrations (10,000 ng/g) of cocaine metabolites, ecgonine ethyl ester, ecgonine methyl ester, and anhydroecgonine methyl ester were chosen to far exceed observed meconium concentrations [11]. For most compounds investigated, expected concentrations in meconium were unavailable; therefore, a 1,000 ng/g concentration was chosen for Δ^9 -tetrahydrocannabinol, 11-hydroxy- Δ^9 -tetrahydrocannabinol, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol, diazepam,

lorazepam, oxazepam, alprazolam, nitrazepam, flunitrazepam, temazepam, nordiazepam, bromazepam, clonazepam, flurazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, imipramine, diphenhydramine, chlorpheniramine, brompheniramine, clomipramine, paroxetine, fluoxetine, norfluoxetine, clonidine, pentazocine, ibuprofen, caffeine, acetylsalicylic acid, acetaminophen, phencyclidine, normorphine, norcodeine, noroxycodone, oxymorphone, noroxymorphone, propoxyphene, 3,4-(methylenedioxyphenyl)-2-butanamine, *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine, cathinone, ethylamphetamine, 4-bromo-2,5-dimethoxyphenethylamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyethylamphetamine, 4-hydroxy-3-methoxymethamphetamine, 4-hydroxy-3-methoxyamphetamine, phentermine, ketamine, and dextromethorphan. Minor tobacco alkaloids anabasine and anatabine concentrations in urine are 1–2 orders of magnitude below nicotine concentrations [37], therefore, 100 ng/g of anabasine, anatabine, and minor nicotine metabolites, nornicotine and norcotine, was chosen for the interference experiment.

Analyte stability in meconium was evaluated by five replicates at each QC concentration under three conditions: 24 h at room temperature, 72 h storage at 4 °C, and three freeze–thaw cycles at –20 °C. To ensure proper incorporation of the analytes, QC solution was pipetted into a 1.5-mL microcentrifuge tube containing meconium and centrifuged at 1,000 rpm for 5 min. On the day of analysis, internal standard was added before homogenization, and specimens were analyzed as usual. Calculated concentrations of stability specimens were compared to five QC specimens prepared on the day of analysis. Autosampler stability was assessed by reinjecting QC specimens after 36 h and comparing calculated concentrations to original values.

Results

Development and validation

Representative chromatograms of extracted blank meconium, LOQ, and positive authentic meconium specimens are shown in Fig. 1. Retention times were stable, and ion ratios were within the permitted tolerances for all analytes. The between-and within-day ion ratio variation for calibrators ranged from 0.99% to 17.9% over the validation four runs. MOR/HYM and COD/HYC have the same molecular weight (285 and 300 amu, respectively) and produce similar transitions, so chromatographic resolution is necessary.

Two analytical runs were required to quantify 20 analytes in meconium. It was possible to analyze BUP and NBUP in method 1; however, the LOQ for both analytes was 100 ng/g. Based on previous results in meconium, greater sensitivity is necessary, particularly for BUP [38]. Increasing the injection volume of method 1 from 5 to 10 µL decreased BUP and NBUP's LOQ, but detector saturation was observed for several of the more intense analytes, resulting in a narrower linear dynamic range. Therefore, a second injection of the same extract with a shorter run time was developed for BUP and NBUP quantification. BUP is currently under investigation as an alternative to methadone for opiate dependence treatment in pregnant women [39–44]. Also, abuse has been reported in multiple countries [45–49]. Therefore, a method for testing meconium for this important analyte in the same specimen extraction will be highly useful.

LOD and LOQ are presented in Table 2. Most analytes were detectable from 0.5 to 5 ng/g and quantifiable from 1 to 5 ng/g. BUP and NBUP do not readily fragment, resulting in lower sensitivities and higher LOQs. LOQs of 25 ng/g were achieved with a second analytical run, similar to the 20 ng/g LOQ reported by Kacinko et al. [26]. The upper LOQ was 500 ng/g for all analytes, except pOHMAMP at 250 ng/g.

Calibration with matched deuterated internal standardization was possible for all analytes except mOHBE and pOHMAMP. For these analytes, COD-D₆ and COT-D₃, respectively, were chosen as the internal standard because of similar retention times and molecular weights. A least squares regression line using a $1/x$ weighting factor was constructed for each analyte with $R^2 > 0.996$ (Table 2).

Imprecision and analytical recovery were assessed at three concentrations spanning the linear dynamic range (Table 3). Analytical recovery ranged from 85.5% to 123.1% ($N=20$). Pooled intra-day, inter-day, and total imprecision were less than 9.2%, 7.5%, and 10.0% CV. Inter-day imprecision of some analytes was set to 0 as directed by Krouwer and Rabinowitz, as the intra-day mean square (MSD) was less than the inter-day mean square (MSW), yielding a negative quantity under the square root of the inter-day imprecision equation, $((MSD-MSW)/n)^{1/2}$ [34, 35].

Extraction efficiency for native and deuterated analytes ranged from 46.7% to 98.7% (Table 3). Table 3 also displays ion suppression/enhancement caused by matrix effect for native and deuterated analytes; positive values indicate ion suppression, while negative values indicate ion enhancement. While significant matrix effects were observed for MAMP, AMP, BUP, MTD, NIC, and OHCOT, similar results were obtained for corresponding deuterated analogues, and quantification was not affected. Matrix effects varied in seven different meconium pools, but again, matrix effects of native and deuterated internal standards were similar in an individual meconium pool. Moreover, quantification of low QC samples prepared in the different meconium sources was within $\pm 20\%$ of target, and imprecision was less than 11.0% relative standard deviation. These findings highlight the importance of deuterated internal standardization in LCMSMS analyses, particularly in complex matrices. Matrix effects for pOHMAMP and mOHBE, the analytes without matching deuterated internal standards, were not significant ($<11.2\%$), and the chosen internal standards were affected similarly.

No carryover was detected in a negative specimen injected after a specimen containing two times the upper LOQ of each analyte. Dilutions (1:5) of high QC specimens were within 88.9–114.0% of target. Common therapeutic and illicit drugs, metabolites, and minor tobacco alkaloids did not interfere with analytes of interest. Analyte stability at four conditions was assessed (Table 4). Losses of less than 30.0% were detected under all conditions and concentrations, except for 6AM, HYC, OXY, and MOR. For each analyte, room temperature storage displayed the most instability. 6AM displayed the most instability of any analyte. At room temperature, 4 °C, and after three freeze–thaw cycles, up to 85.8% of 6AM was lost; whereas MOR concentrations under these conditions increased by up to 31.2%.

Application of method

Five drug-positive meconium specimens (specimens 1–5) were analyzed to demonstrate method applicability (Fig. 1); meconium containing 6AM, BUP, and NBUP were not available. Specimen 1 contained 215 ng/g MAMP, 44.9 ng/g AMP, and 11.2 ng/g pOHMAMP; specimen 2, 55.5 ng/g COC, 284 ng/g BE, 54.8 ng/g CE, and 290 ng/g mOHBE; specimen 3, 29.8 ng/g NIC, 89.7 ng/g COT, 156 ng/g OHCOT, 104 ng/g COD, and 500 ng/g OXY; specimen 4, 158 ng/g MOR, 168 ng/g HYM, and 214 ng/g HYC; and specimen 5, 387 ng/g MTD and 153 ng/g EDDP. No authentic specimen contained 6-acetylmorphine, buprenorphine, or norbuprenorphine. In addition to MTD and EDDP, 6.6 ng/g BE, 6.9 ng/g mOHBE, 3.4 ng/g MAMP, 6.9 ng/g NIC, 1.7 ng/g COT, 9.7 ng/g OHCOT, and 38.7 ng/g MOR were observed in specimen 5, indicating maternal COC, MAMP, tobacco, and opiate use. These results highlight the utility of this method for simultaneously detecting multiple drug classes.

Discussion

We present a validated analytical procedure for the quantification of amphetamines, COC, opiates, NIC, and metabolites in meconium, employing a single extraction and two acquisition methods. This novel method will be used to detect licit and illicit drug consumption within the obstetric population.

There are several advantages of this method for detecting in utero drug exposure. Meconium has a longer window of drug detection than other biological matrices, theoretically indicating second- and third-trimester use. Secondly, the described analytical procedure sensitively and selectively quantifies 20 biomarkers of prenatal drug exposure from a variety of drug classes, making it the most comprehensive test for meconium to date. Two LCMSMS acquisition methods, using the same mobile phase combination and analytical column, sufficiently resolve 20 compounds in a 25-min combined run time, saving time and resources.

Detection and quantification limits are on par with other available testing methodologies for meconium. Previous meconium methods have obtained comparable LOQs for amphetamines (2.5–5 ng/g) [16, 17], COC and metabolites (1–5 ng/g) [11, 23], NIC and metabolites (1.25–5 ng/g) [31], and opiates (1–5 ng/g for 6AM, MOR, COD, MTD, EDDP, HYC, HYM, and OXY and 20 ng/g for BUP and NBUP) [21–23, 25, 26]. However, a limitation of the current method is the relatively narrow linear range. Other published procedures for a more limited group of analytes achieved upper LOQs of 1,000 ng/g; however, we observed detector saturation with concentrations greater than 500 ng/g for the more intense analytes. Specimen dilution and reanalysis may be necessary for more abundant analytes, such as MTD, EDDP, and MAMP; however, dilution would only be required in a small number of specimens, and all specimens would have been screened for the most important in utero drug exposures.

A single extraction for all analytes, requiring only a small amount of meconium, offers substantial time and resource savings, as compared to the current procedure of extracting and analyzing one drug class at a time. Moreover, the amount of meconium is often limited, requiring laboratories to select the most important drug class for a particular case. Including multiple drug classes enables a more comprehensive screen with one extraction, while the small specimen size reduces the numbers of rejected specimens due to insufficient quantity and allows repeat analyses or additional testing.

Including recently identified meconium biomarkers of MAMP (pOHMAMP) [17, 18] and tobacco (NIC and OHCOT) [31, 32] may identify additional drug-exposed neonates than classical MAMP and NIC biomarkers alone (MAMP/AMP and COT, respectively). pOHMAMP was found in meconium positive for MAMP and AMP [18], but it is not yet known if including pOHMAMP in confirmatory analyses will improve identification of MAMP-exposed neonates. With regards to tobacco, a recent study by our laboratory revealed that 25% of positive meconium specimens contained NIC and OHCOT but not COT [32]. Typically, tobacco exposure detection is limited to immunoassays targeting COT, with little to no cross-reactivity with NIC and OHCOT. This approach may generate false negatives and underestimate the true prevalence of maternal tobacco use.

Further studies of opiate stability, particularly 6AM, are warranted, ideally in authentic meconium specimens. Fortifying meconium, a semi-solid matrix, with liquid QC solution is more challenging than fortifying a liquid matrix like urine or blood. While meconium was centrifuged after adding the methanolic QC solution, complete incorporation cannot be guaranteed. It is possible that there are differences in stability for authentic specimens as compared to fortified specimens. Based on our stability observations, 6AM may not be

detected if specimens are not frozen and transferred to the testing laboratory on ice. 6AM degrades to MOR indicating opiate exposure, but the ability to differentiate heroin from MOR consumption may be lost. Of four procedures describing 6AM quantification in meconium [12, 22–24], only one investigated analyte stability, observing <10% free thaw instability for 6AM and MOR; no other storage conditions were included [23].

Ideally, analyte quantification would employ deuterated internal standardization to compensate for any matrix effect. While this was possible for 18 analytes, deuterated analogs of pOHMAMP and mOHBE are not commercially available. Alternative internal standards were chosen based on similar retention times and molecular weights. While we attempted to minimize matrix effects through solid phase extraction, some matrix effect remained. The matrix effects for pOHMAMP, mOHBE, and their respective internal standards were not identical, but our approach is the best available at this time. Furthermore, we demonstrated that quantification was not affected by varying matrix effects by fortifying seven different meconium specimens at the low QC concentration. Despite these efforts, differential matrix effect cannot be excluded, and pOHMAMP and/or mOHBE quantification could be affected. For both pOHMAMP and mOHBE, two other biomarkers are available for confirming MAMP and COC exposure, respectively.

Meconium biomarkers provide an objective means to verify drug exposure and may correlate with observed neurobehavioral, neurophysiological, and physical effects in the drug-exposed infant. Understanding the relationships between quantitative meconium data, neonatal birth, and development parameters and the degree of maternal drug exposure would be useful in prioritizing medical and social resources for the child and mother, guiding future research of in utero drug exposure and shaping substance abuse policies within the obstetric population.

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Fig. 1.

MRM scans of **a** extracted blank meconium, **b** limit of quantification, and **c** meconium specimens from five drug-exposed neonates (specimens 1–5) demonstrating method applicability: specimen 1—215 ng/g methamphetamine (*MAMP*), 44.9 ng/g amphetamine (*AMP*), 11.2 ng/g *p*-hydroxymethamphetamine (*pOHMAMP*); specimen 2—55.5 ng/g cocaine (*COC*), 284 ng/g benzoylecgonine (*BE*), 54.8 ng/g cocaethylene (*CE*), and 290 ng/g *m*-hydroxybenzoylecgonine (*mOHBE*); specimen 3—29.8 ng/g nicotine (*NIC*), 89.7 ng/g cotinine (*COT*), 156 ng/g *trans*-3-hydroxycotinine (*OHCOT*), 104 ng/g codeine (*COD*), and 500 ng/g oxycodone (*OXY*); specimen 4—158 ng/g morphine (*MOR*), 168 ng/g hydromorphone (*HYM*), and 214 ng/g hydrocodone (*HYC*); and specimen 5—387 ng/g methadone (*MTD*), and 153 ng/g 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (*EDDP*). No authentic specimen contained 6-acetylmorphine (*6AM*), buprenorphine (*BUP*), or norbuprenorphine (*NBUP*). Quantitative and qualitative transitions are presented in *red* and *blue*, respectively. Asterisk Specimen 1 containing AMP, MAMP, and pOHMAMP was diluted (1:5) to accommodate MAMP's linear range

Table 1
Amphetamines, cocaine, nicotine, opiates, and metabolites in meconium by LCMSMS: mass spectrometer parameters

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	FP (V)	CEn (V)	CXP (V)	Internal standard	Q1 mass (amu)	Q3 mass (amu)	DP (V)	FP (V)	CEn (V)	CXP (V)
Amphetamines													
AMP	136.0	91.1	21	200	21	14	AMP-D ₁₁	147.2	130.2	26	230	15	8
	136.0	119.2	21	200	13	8		147.2	98.2	26	230	23	6
MAMP	150.1	91.2	21	200	25	14	MAMP-D ₁₄	164.2	98.2	26	210	27	16
	150.1	119.2	21	200	17	8		164.2	130.2	26	210	17	10
pOHMAMP	166.2	135.2	21	180	17	12	COT-D ₃	180.2	80.1	31	250	33	12
	166.2	107.1	21	180	29	6		180.2	101.2	31	250	31	16
Cocaine													
COC	304.2	182.1	41	350	29	12	COC-D ₃	307.1	185.3	31	270	31	28
	304.2	82.2	41	350	43	6		307.1	85.1	31	270	41	6
BE	290.2	168.3	36	270	27	12	BE-D ₈	298.2	171.2	51	370	29	14
	290.2	105.2	36	270	45	16		298.2	110.2	51	370	43	8
CE	318.1	196.1	46	370	29	12	CE-D ₃	321.2	199.3	51	370	29	12
	318.1	82.1	46	370	39	14		321.2	85.1	51	370	41	14
mOHBE	306.1	168.2	51	340	29	10	COD-D ₆	306.3	218.2	61	370	35	12
	306.1	121.2	51	340	41	8		306.3	165.2	61	370	55	10
Nicotine													
NIC	163.1	130.1	31	260	29	8	NIC-D ₄	167.2	136.3	36	260	23	10
	163.1	132.2	31	260	23	8		167.2	134.1	36	260	27	8
COT	177.0	80.1	56	370	35	14	COT-D ₃	180.2	80.1	31	250	33	12
	177.0	98.2	56	370	33	6		180.2	101.2	31	250	31	16
OHCOT	193.1	80.1	31	270	39	6	OHCOT-D ₃	196.2	80.1	31	260	35	12
	193.1	134.1	31	270	27	8		196.2	134.1	31	260	27	8
Opiates													
6AM	328.0	211.0	96	310	35	12	6AM-D ₆	334.1	165.2	101	340	53	10
	328.0	165.2	96	310	57	10		334.1	211.2	101	340	37	12
MOR	286.1	152.1	76	370	75	8	MOR-D ₃	289.0	201.0	81	370	37	79
	286.1	128.1	76	370	79	6		289.0	152.1	81	370	12	10

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	FP (V)	CE _n (V)	CXP (V)	Internal standard	Q1 mass (amu)	Q3 mass (amu)	DP (V)	FP (V)	CE _n (V)	CXP (V)
COD	300.2	198.9	101	360	39	12	COD-D ₆	306.3	218.2	61	370	35	12
	300.2	165.0	101	360	57	10		306.3	165.2	61	370	55	10
HYM	286.2	185.3	96	230	41	32	HYM-D ₃	289.1	185.0	56	370	41	12
	286.2	157.2	96	230	57	24		289.1	157.0	56	370	59	8
HYC	300.5	199.1	101	290	41	12	HYC-D ₆	306.3	202.0	91	350	41	12
	300.5	128.3	101	290	77	8		306.3	174.1	91	350	55	10
OXY	316.1	298.1	51	370	27	18	OXY-D ₆	322.3	304.2	56	350	27	20
	316.1	241.0	51	370	39	14		322.3	262.1	56	350	39	16
MTD	310.3	265.0	31	330	21	16	MTD-D ₉	319.2	268.2	31	240	21	16
	310.3	105.2	31	330	35	18		319.2	105.1	31	240	41	18
EDDP	278.3	234.1	26	260	41	16	EDDP-D ₃	281.2	234.3	56	370	43	16
	278.3	249.0	26	260	35	14		281.2	249.2	56	370	35	16
BUP	468.3	55.0	101	360	93	8	BUP-D ₄	472.2	59.0	91	360	77	10
	468.3	396.2	101	360	55	22		472.2	400.3	91	360	55	26
NBUP	414.2	83.2	101	370	73	14	NBUP-D ₃	417.1	83.2	101	360	73	6
	414.2	101.2	101	370	53	6		417.1	101.2	101	360	55	6

Bold font denotes quantifier transition

6AM 6-acetyl/morphine, AMP amphetamine, BE benzoyllecgonine, BUP buprenorphine, CE cocaethylene, CEn collision energy, COT cotinine, COD codeine, CXP cell exit potential, DP declustering potential, EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, FP focusing potential, HYC hydrocodone, HYM hydromorphone, MAMP methamphetamine, mOHBE m-hydroxybenzoyllecgonine, MOR morphine, MTD methadone, NBUP norbuprenorphine, NIC nicotine, OHCOT trans-3'-hydroxycotinine, OXY oxycodone, pOHMAMP p-hydroxymethamphetamine

Table 2

Amphetamines, cocaine, nicotine, opiates, and metabolites in meconium by LCMS/MS: limits of detection, limits of quantification, calibration results, and retention times

	LOD (ng/g)	LOQ (ng/g)	Slope $\pm$ SD (N=4)	Y-intercept $\pm$ SD (N=4)	R ² $\pm$ SD (N=4)	Linear range	Retention time (min)
Amphetamines							
AMP	1	5	0.853 $\pm$ 0.111	0.026 $\pm$ 0.015	0.999 $\pm$ 0.000	5–500	7.8
MAMP	0.5	2.5	0.464 $\pm$ 0.016	0.012 $\pm$ 0.012	0.996 $\pm$ 0.002	2.5–500	8.4
pOHMAMP	0.5	1	3.728 $\pm$ 1.545	0.027 $\pm$ 0.031	0.996 $\pm$ 0.003	1–250	5.6
Cocaine							
COC	0.5	1	1.436 $\pm$ 0.171	0.065 $\pm$ 0.016	0.999 $\pm$ 0.001	1–500	11.9
BE	1	1	0.949 $\pm$ 0.107	0.003 $\pm$ 0.006	0.999 $\pm$ 0.000	1–500	9.9
CE	0.5	1	0.801 $\pm$ 0.052	0.010 $\pm$ 0.002	0.998 $\pm$ 0.001	1–500	13.4
mOHBE	2.5	5	0.861 $\pm$ 0.773	–0.014 $\pm$ 0.013	0.999 $\pm$ 0.001	5–500	8.4
Nicotine							
NIC	2.5	2.5	1.053 $\pm$ 0.096	0.029 $\pm$ 0.022	0.998 $\pm$ 0.002	2.5–500	4.6
COT	0.5	1	1.086 $\pm$ 0.105	0.036 $\pm$ 0.016	0.998 $\pm$ 0.001	1–500	6.1
OHCOT	5	5	0.904 $\pm$ 0.162	0.047 $\pm$ 0.031	0.997 $\pm$ 0.002	5–500	4.5
Opiates							
6AM	5	5	0.883 $\pm$ 0.029	0.037 $\pm$ 0.004	0.998 $\pm$ 0.000	5–500	8.2
MOR	5	5	0.828 $\pm$ 0.118	0.010 $\pm$ 0.008	0.997 $\pm$ 0.001	5–500	5.3
COD	5	5	0.490 $\pm$ 0.155	0.003 $\pm$ 0.003	0.998 $\pm$ 0.001	5–500	7.4
HYM	5	5	0.524 $\pm$ 0.058	–0.009 $\pm$ 0.008	0.999 $\pm$ 0.002	5–500	6.2
HYC	5	5	0.979 $\pm$ 0.091	–0.009 $\pm$ 0.012	0.999 $\pm$ 0.001	5–500	8.5
OXY	2.5	5	1.134 $\pm$ 0.076	0.008 $\pm$ 0.020	0.998 $\pm$ 0.002	5–500	8.1
MTD	1	2.5	0.879 $\pm$ 0.177	0.016 $\pm$ 0.007	0.998 $\pm$ 0.001	2.5–500	16.8
EDDP	1	2.5	0.903 $\pm$ 0.136	0.021 $\pm$ 0.003	0.997 $\pm$ 0.001	2.5–500	16.3
BUP	10	25	0.846 $\pm$ 0.019	0.027 $\pm$ 0.035	0.999 $\pm$ 0.000	25–500	3.3
NBUP	25	25	1.190 $\pm$ 0.090	0.028 $\pm$ 0.035	0.998 $\pm$ 0.002	25–500	2.5

6AM 6-acetylmorphine, AMP amphetamine, BE benzoylcegonine, BUP buprenorphine, CE cocaethylene, COC cocaine, COT cotinine, COD codeine, EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HYC hydrocodone, HYM hydromorphone, MAMP methamphetamine, mOHBE *m*-hydroxybenzoylcegonine, MOR morphine, MTD methadone, NBUP norbuprenorphine, NIC nicotine, OHCOT *trans*-3'-hydroxycotinine, OXY oxycodone, pOHMAMP *p*-hydroxymethamphetamine

Table 3
Amphetamines, cocaine, nicotine, opiates, and metabolites in meconium by LCMS/MS: imprecision, recovery, extraction efficiency, and matrix effects

Analyte	Concentration (ng/g)	Observed range (ng/g)	Imprecision (% CV)		Inter-day recovery (N=20, %)	Extraction efficiency (N=5, %)	Matrix effect ^a (N=5, %)	Matrix effect, pools ^a (N=7, %)	Internal standard extraction efficiency (N=5, %)	Internal standard matrix effect ^a (N=5, %)	Internal standard matrix effect, pools ^a (N=7, %)			
			Pooled intra-day (N=5)	Total (N=20)										
Amphetamines														
AMP	Low	12	10.0–12.6	6.5	1.4	6.7	91.4	73.4	–5.6	6.0–16.0	AMP-D ₁₁	69.7	–7.1	4.7–17.2
	Medium	60	57.2–69.5	3.9	4.0	5.5	106.4	61.9	–15.0			64.4	–13.5	
	High	300	268–340	6.3	0.0	6.3	101.6	73.4	–48.7			67.6	–51.5	
MAMP	Low	6	5.1–6.3	4.2	3.8	5.7	92.7	74.3	3.0	1.0–20.1	MAMP-D ₁₄	69.6	1.4	4.3–14.1
	Medium	60	58.8–72.3	3.3	5.5	6.4	112.4	59.9	–8.1			57.8	–3.1	
	High	300	277–332	4.7	1.5	4.9	103.3	74.4	–33.3			62.7	–52.4	
pOHMAMP	Low	3	2.4–2.7	3.3	2.5	4.1	85.5	60.2	7.3	4.5–32.5	COT-D ₃	89.6	18.3	5.3–27.0
	Medium	60	51.5–65.3	5.9	0.0	5.9	96.4	50.2	6.1			79.8	13.9	
	High	150	124–161	5.0	4.9	7.0	90.8	61.1	–3.1			85.6	–4.0	
Cocaine														
COC	Low	3	2.7–3.4	6.4	3.3	7.3	99.5	73.9	19.5	–24.5–16.2	COC-D ₃	85.8	26.9	–1.5–18.1
	Medium	60	58.6–67.3	3.0	2.6	4.0	103.9	77.2	22.8			80.8	25.0	
	High	300	280–343	5.6	0.0	5.6	105.8	82.6	13.8			79.5	10.1	
BE	Low	3	2.4–3.2	4.9	6.1	7.8	92.7	57.2	11.5	–25.0–12.6	BE-D ₈	55.4	–5.4	–12.2–1.6
	Medium	60	50.6–66.2	5.0	3.6	6.1	100.5	53.2	6.1			51.1	–3.1	
	High	300	252–346	7.8	1.8	8.0	101.9	60.8	–5.6			64.8	0.2	
CE	Low	3	2.4–3.2	4.9	6.6	8.2	91.8	75.3	–1.0	0.1–18.7	CE-D ₃	78.8	–1.4	–4.7–20.8
	Medium	60	61.1–69.4	3.5	0.3	3.6	109.4	74.8	2.4			81.8	0.4	
	High	300	297–360	4.0	3.2	5.1	109.0	83.0	–2.9			87.0	–0.7	
mOHBE	Low	12	10.2–13.8	6.1	2.2	6.5	96.9	51.4	0.8	–16.3–4.5	COD-D ₆	77.6	1.6	–6.2–18.9
	Medium	60	49.2–62.5	5.1	6.2	8.0	92.9	46.7	10.5			77.6	1.5	
	High	300	250–357	6.6	7.5	10.0	98.9	54.7	–11.2			81.5	–4.9	
Nicotine														
NIC	Low	6	5.6–7.2	7.2	4.2	8.3	104.6	79.6	–41.6	–118.7–34.9	NIC-D ₄	76.1	–46.5	–104.1–7.1

Analyte	Concentration (ng/g)	Observed. range (ng/g)	Imprecision (% CV)		Inter-day recovery (N=20, %)	Extraction efficiency (N=5, %)	Matrix effect ^a (N=5, %)	Matrix effect, different pools ^a (N=7, %)	Internal standard	Internal standard extraction efficiency (N=5, %)	Internal standard matrix effect ^a (N=5, %)	Internal standard matrix effect, different pools ^a (N=7, %)
			Pooled intra-day (N=5)									
			Inter-day (N=20)	Total (N=20)								
COT	Medium	60 51.2–65.5	6.4	1.8	6.7	75.9	−76.7			67.8	−95.2	
	High	300 253–346	8.6	0.0	8.6	71.0	−305.7			77.9	−282.9	
	Low	3 2.7–3.6	6.1	6.9	9.2	94.1	10.5	−9.7–35.1	COT-D ₃	89.6	18.3	5.3–27.0
	Medium	60 51.9–71.6	5.2	7.2	8.9	79.6	16.4			79.8	13.9	
	High	300 285–349	4.6	3.7	5.9	82.4	−1.9			85.6	−4.0	
OHCOT	Low	12 10.5–13.4	6.7	0.0	6.7	71.8	40.4	−7.6–24.5	OHCOT-D ₃	64.3	33.6	−7.9–19.6
	Medium	60 52.2–70.8	6.6	3.3	7.4	54.7	24.0			56.7	27.8	
	High	300 277–337	6.2	0.0	6.2	48.1	10.3			56.0	14.4	
Opiates												
6AM	Low	12 10.1–13.9	9.1	4.2	10.0	91.4	12.3	4.4–15.1	6AM-D ₆	76.8	−1.7	0.3–18.1
	Medium	60 57.2–71.1	5.3	5.4	7.5	82.6	−13.7			79.3	6.3	
MOR	High	300 250–356	7.9	2.7	8.4	81.4	1.3			76.6	−7.3	
	Low	12 9.7–12.1	7.1	4.7	8.5	78.8	11.2	−10.6–34.7	MOR-D ₃	76.8	2.5	−4.6–19.6
	Medium	60 50.5–69.0	6.1	5.6	8.3	79.6	5.5			83.7	0.2	
COD	High	300 248–350	9.2	0.0	9.2	78.0	−3.5			85.4	−7.6	
	Low	12 10.2–13.4	7.8	1.6	7.9	85.6	7.0	−15.1–13.4	COD-D ₆	77.6	1.6	−6.2–18.9
	Medium	60 56.6–71.1	5.9	4.7	7.5	80.3	0.7			77.6	1.5	
HYM	High	300 252–343	7.1	4.3	8.3	90.3	−2.6			81.5	−4.9	
	Low	12 10.0–13.4	7.4	4.9	8.9	63.9	23.3	−3.9–40.2	HYM-D ₃	71.0	18.4	7.7–48.0
	Medium	60 48.0–63.9	4.5	5.5	7.1	69.6	16.4			65.2	13.1	
HYC	High	300 245–338	6.2	5.6	8.4	59.3	−6.1			62.9	4.4	
	Low	12 9.7–12.9	5.5	5.5	7.7	72.6	−0.9	−1.3–10.5	HYC-D ₆	77.5	−1.0	−6.7–16.2
	Medium	60 48.8–64.5	5.5	6.3	8.4	71.3	12.0			82.5	4.2	
OXY	High	300 260–333	6.0	2.3	6.4	77.3	−6.1			81.3	1.2	
	Low	12 10.0–12.7	5.4	2.2	5.8	76.6	24.5	−8.8–25.1	OXY-D ₆	69.7	5.0	−6.9–12.6
	Medium	60 50.6–70.4	6.7	6.0	9.0	73.1	3.1			82.1	−2.2	
MTD	High	300 268–332	2.9	5.8	6.4	69.0	−5.2			69.2	−6.4	
	Low	6 5.2–6.1	3.7	3.8	5.3	79.5	−38.0	−40.6 to −18.3	MTD-D ₉	88.0	−24.6	−40.7 to −18.6

Analyte	Concentration (ng/g)	Observed range (ng/g)	Imprecision (% CV)		Inter-day recovery (N=20, %)	Extraction efficiency (N=5, %)	Matrix effect ^a (N=5, %)	Matrix effect, different pools ^a (N=7, %)	Internal standard extraction efficiency (N=5, %)	Internal standard matrix effect ^a (N=5, %)	Internal standard matrix effect, different pools ^a (N=7, %)
			Pooled intra-day (N=5)	Inter-day (N=20)							
EDDP	Medium	60	51.6–66.8	3.0	7.3	88.1	–12.1		86.0	–17.5	
	High	300	257–323	5.3	1.1	95.7	–5.3		98.7	–10.4	
	Low	6	5.0–6.3	4.5	4.4	52.1	–13.6	–7.7–18.5	53.8	–18.0	–3.4–10.5
BUP	Medium	60	60.3–71.9	3.6	3.4	49.7	–3.3		49.4	–5.1	
	High	300	272–336	4.6	2.3	66.7	–14.9		64.1	–22.1	
	Low	48	44.4–54.2	4.4	3.4	102.2	–32.1	–38.3 to –23.7	98.2	–32.5	–39.4 to –22.0
NBUP	Medium	120	116–130	2.9	1.2	91.9	–28.7		93.4	–30.6	
	High	300	350–386	2.3	4.6	123.1	–16.6		93.1	–14.6	
	Low	48	40.0–53.2	5.6	6.6	79.2	–12.1	–0.1–20.5	84.3	–14.0	–3.2–34.0
	Medium	120	112–137	4.5	1.2	100.1	–17.3		78.9	–21.8	
	High	300	340–385	3.1	4.4	80.0	–16.4		76.8	–16.5	

6AM 6-acetylmorphine, AMP amphetamine, BE benzoylcegonine, BUP buprenorphine, CE cocaethylene, COT cocaine, COD codeine, CV coefficient of variation, EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, HYC hydrocodone, HYM hydromorphone, MAMP methamphetamine, mOHBE m-hydroxybenoylcegonine, MOR morphine, MTD methadone, NBUP norbuprenorphine, NIC nicotine, OHCOT trans-3'-hydroxycotinine, OXY oxycodone, pOHMAMP p-hydroxymethamphetamine

^aPositive values indicate matrix suppression

Table 4
Amphetamines, cocaine, nicotine, opiates, and metabolites in meconium by LCMS/MS: analyte stability

	Concentration (ng/g)	36h autosampler (% lost)	72h 4°C (% lost)	24 h RT (% lost)	3 Freeze/thaw cycles (% lost)
<i>Amphetamines</i>					
AMP	Low	12	0.8	19.7	24.8
	Medium	60	0.3	10.6	20.2
	High	300	5.5	19.9	24.7
MAMP	Low	6	-0.7	19.0	19.2
	Medium	60	2.5	5.2	19.9
	High	300	1.7	10.2	25.5
pOHMAMP	Low	3	5.2	16.5	28.7
	Medium	60	7.2	8.1	23.2
	High	150	-4.8	15.4	22.8
<i>Cocaine</i>					
COC	Low	3	-12.3	22.5	11.5
	Medium	60	-2.9	12.5	21.4
	High	300	-1.2	12.5	27.5
BE	Low	3	-16.4	17.5	23.3
	Medium	60	-3.9	9.5	16.3
	High	300	-4.9	12.8	22.6
CE	Low	3	0.6	4.4	28.1
	Medium	60	5.7	4.0	18.5
	High	300	7.5	7.2	28.0
mOHBE	Low	12	13.5	0.9	23.6
	Medium	60	3.4	10.1	16.4
	High	300	10.4	12.1	29.3
<i>Nicotine</i>					
NIC	Low	6	8.1	6.0	8.7
	Medium	60	6.7	1.7	10.8
	High	300	-3.5	19.1	27.3
COT	Low	3	14.9	-2.1	19.9
	Medium	60	10.2	9.2	12.2
					2.7

	Concentration (ng/g)	36h autosampler (% lost)	72h 4°C (% lost)	24 h RT (% lost)	3 Freeze/thaw cycles (% lost)
OHCOT	High	300	3.3	13.5	21.9
	Low	12	5.9	22.2	24.2
	Medium	60	-13.0	12.4	18.4
	High	300	-5.0	13.5	15.8
<i>Opiates</i>					
6AM	Low	12	28.8	60.7	85.8
	Medium	60	-0.3	51.5	67.5
MOR	High	300	10.5	51.6	71.6
	Low	12	7.4	-10.3	-17.1
	Medium	60	10.3	-24.6	-31.2
	High	300	-17.5	-22.4	-12.7
COD	Low	12	18.3	15.7	23.7
	Medium	60	-8.0	15.8	28.7
HYM	High	300	0.6	5.5	31.2
	Low	12	-11.8	18.3	15.5
	Medium	60	9.9	14.3	12.9
	High	300	5.2	13.9	24.3
HYC	Low	12	-7.4	8.4	23.0
	Medium	60	-12.4	20.3	10.4
	High	300	7.8	15.1	30.0
	Low	12	-6.5	8.1	29.4
OXY	Medium	60	5.8	6.8	23.9
	High	300	9.5	13.6	32.2
	Low	6	-0.7	-1.2	18.9
	Medium	60	3.0	-0.1	7.0
MTD	High	300	1.4	5.6	19.2
	Low	6	-0.2	3.1	20.9
	Medium	60	4.8	2.2	7.9
	High	300	-1.3	8.7	14.8
EDDP	Low	6	0.6	8.2	10.9
	Medium	60	1.5	2.3	5.9
	High	300	-1.3	8.7	14.8
	Low	48	0.6	8.2	10.9
BUP	Medium	120	1.5	2.3	5.9
	High	300	-1.3	8.7	14.8
BUP	Low	48	0.6	8.2	10.9
	Medium	120	1.5	2.3	5.9
	High	300	-1.3	8.7	14.8
	Low	48	0.6	8.2	10.9

	Concentration (ng/g)	36h autosampler (% lost)	72h 4°C (% lost)	24 h RT (% lost)	3 Freeze/thaw cycles (% lost)
NBUP	High	300	0.1	-2.3	8.6
	Low	48	-0.3	11.2	13.3
	Medium	120	1.5	6.3	13.8
	High	300	-1.5	6.9	16.2
					1.4
					11.5
					5.2
					8.6