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Development and validation of a liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry method for simultaneous analysis of 10 amphetamine-, methamphetamine- and 3,4-methylenedioxymethamphetamine-related (MDMA) analytes in human meconium

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ABSTRACT

A liquid chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (LC–APCI-MS/MS) method for quantification of 10 amphetamine-related analytes in 1 g meconium is presented. Specimen preparation included homogenization and solid-phase extraction. Two multiple reaction monitoring transitions were monitored per analyte. Ten and 1 μL injection volumes permitted quantification up to 10,000 ng/g, with sufficient sensitivity to quantify minor metabolites. Lower limits of quantification ranged from 1.25 to 40 ng/g. Precision was less than 14.2%, with accuracy between 79 and 115%. Meconium from a methamphetamine-exposed neonate was analyzed. Metabolites *p*-hydroxymethamphetamine, norephedrine and 4-hydroxy-3-methoxymethamphetamine were identified in meconium for the first time.

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1. Introduction

According to the 2005 National Survey on Drug Use and Health National Findings, 3.9% of pregnant women, ages 15–44 years, reported illicit drug use within the previous month [1]. Drug exposure to the developing fetus has been associated with reduced birth weight, small head circumference, premature birth, fetal distress, potential birth defects, and perinatal complications [2]. Limited studies have considered specific adverse outcomes of human *in utero* methamphetamine (MAMP) or amphetamine (AMP) exposure, either alone or in combination with other drugs such as cocaine [3–7]. Human neonatal outcomes after *in utero* exposure to methylenedioxy-derivatives of AMP, such as 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) are not well understood. Exposed infants may be identified by maternal self-report or chromatographic confirmation of MAMP, AMP, MDMA and/or metabolites in meconium. Due to potential social and medical implications of *in utero* drug exposure, exposed neonates need to be accurately identified, so that appropriate resources and follow-up are available for the child throughout development, and drug treatment, child care training and other support also are available for the mother.

Meconium is the first neonatal bowel movement, typically excreted within a few days of birth. It is a complex matrix consisting of water, epithelial cells, lanugo, bile acids and salts, enzymes, lipids, sugars, proteins, and swallowed amniotic fluid [8,9]. Meconium formation begins around the 12th week of gestation, offering a wider window of detection than other neonatal matrices [10]. Meconium analysis can detect maternal drug use over the second and third trimesters, but specimen weight is frequently quite limited, while positive results in hair or urine only indicate use during the third trimester or the 1–3 days immediately preceding delivery, respectively. Obtaining meconium from diapers also is easier than obtaining neonatal urine with a special collection device that frequently falls off or irritates newborn skin. Hair is often present in quantities insufficient for drug analysis; when ample hair is available, many mothers are reluctant to consent to hair collection for cosmetic or cultural reasons. Analysis of meconium requires extensive sample preparation due to its complexity and heterogeneity, with homogenization commonly employed prior to liquid–liquid and/or solid–phase extraction (SPE). Cocaine and metabolites, cannabinoids, amphetamines, opiates, phencyclidine,

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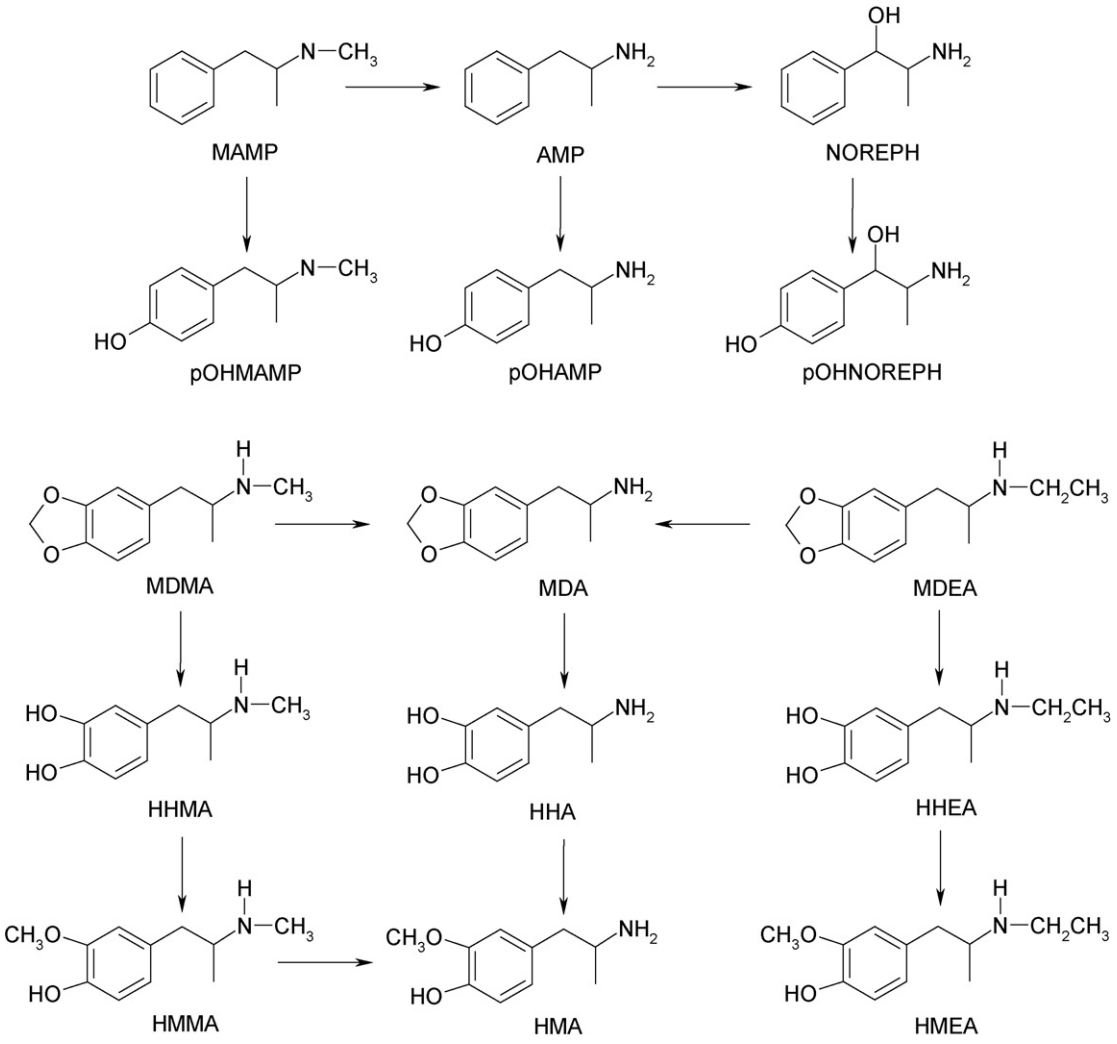

Fig. 1. Metabolic pathways of methamphetamine (MAMP), amphetamine (AMP), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA). *Abbreviations*: HHA, 3,4-dihydroxyamphetamine; HHEA, 3,4-dihydroxyethylamphetamine; HHMA, 3,4-dihydroxymethamphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMEA, 4-hydroxy-3-methoxyethylamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHNOREPH, *p*-hydroxynorephedrine; pOHMAMP, *p*-hydroxymethamphetamine.

methadone, nicotine, non-steroidal anti-inflammatory drugs, and ethanol metabolites, fatty acid ethyl esters have previously been analyzed in meconium [11–31]. Gareri et al. [8], Moore et al. [9], and Gray et al. [32] have reviewed a number of these methods, which have primarily involved immunoassays, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS).

Primary metabolic pathways of MAMP include *N*-demethylation to AMP and aromatic hydroxylation to *p*-hydroxymethamphetamine (pOHMAMP, pholedrine); additional minor metabolites include *p*-hydroxyamphetamine (pOHAMP), norephedrine (NOREPH) and *p*-hydroxynorephedrine (pOHNOREPH) [33,34]. The main metabolic pathways of MDMA involve *N*-dealkylation to MDA, followed by *O*-demethylation to form intermediates of 3,4-dihydroxyamphetamine (HHA) and 3,4-dihydroxymethamphetamine (HHMA). HHA and HHMA undergo *O*-methylation, forming 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA). 3,4-Methylenedioxyethylamphetamine (MDEA) is metabolized via *N*-deethylation to MDA and 4-hydroxy-3-methoxyethylamphetamine (HMEA), via the formation of 3,4-dihydroxyethylamphetamine (HHEA) following cleavage of the methylenedioxy-group [34–37]. HMA, HMMA, HMEA, HHA, HHMA, HHEA may also undergo conjugation to form glucuronide and/or sulfate metabolites [34,37,38]. Fig. 1 summarizes the metabolic pathways of MAMP, AMP, MDMA and MDEA.

Meconium specimens screening positive for amphetamines by immunoassay sometimes cannot be confirmed for AMP, MAMP, MDMA, MDA and MDEA by GC–MS; between 60% and 100% of meconium specimens positive by AMP immunoassays are negative for AMP or MAMP after GC–MS reanalysis [15,39–41]. This raises the question of whether MAMP and AMP are the most appropriate biomarkers in meconium for the detection of *in utero* AMP exposure. Possibly, unidentified MAMP or AMP metabolites, other sympathomimetic amines, or endogenous meconium compounds cross-react with the immunoassay, triggering positive responses. The presence of MAMP and AMP metabolites in meconium cannot always be detected with current confirmatory analytical methods that focus on parent compounds, AMP, MAMP, MDMA, MDA, MDEA and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) [15,22,23]; therefore MAMP-exposed neonates could be misidentified and

denied interventional measures. Additionally, identification and quantification of other MAMP and AMP metabolites in meconium may provide valuable data regarding the maternal/fetal transfer of AMP-related drugs.

The aim of this study was to develop and validate a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of 10 AMP-related analytes including four compounds never before investigated in meconium. AMP and MAMP concentrations exceeding 1000 ng/g have been previously identified in meconium [23]; therefore, analysis of AMP-related analytes requires simultaneous quantification of high-parent compound concentrations (that is, greater than 1000 ng/g) and low-minor metabolite concentrations in quantity-limited meconium specimens. To accommodate the large analytical range, two calibration curves were constructed using different injection volumes. The method will be employed to characterize AMP and related compound disposition in infant meconium.

2. Experimental

2.1. Chemicals and reagents

All drug standards were obtained as racemic mixtures. Methanolic solutions (1.0 mg/mL) of AMP, AMP-d_{11}, MAMP, MAMP-d_{14}, phenylpropanolamine hydrochloride (also known as NOREPH), NOREPH-d_3 hydrochloride, MDA, MDA-d_5, MDMA, MDMA-d_5, MDEA, MDEA-d_5, and HMMA were purchased from Cerilliant (Austin, TX, USA). A 1.0 mg/mL methanolic standard HMA hydrochloride was obtained from Lipomed (Cambridge, MA, USA). pOHAMP hydrobromide and pOHMAMP hydrochloride were kindly provided by the National Institute on Drug Abuse (NIDA) Drug Inventory (maintained by Research Triangle Institute, Research Triangle Park, NC, USA), and pOHNOREPH hydrochloride was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Morphine-6-β-D-glucuronide·H_2O solution (1.0 mg base/mL dimethyl sulfoxide) was sourced from Lipomed (Cambridge, MA, USA). *m*-Hydroxycocaine, *p*-hydroxycocaine, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine were obtained from Research Biochemicals International (Natick, MA, USA). (±)-Norcotinine and *trans*-3′-hydroxycotinine were purchased as powders from Toronto Research Chemicals (North York, Ontario, Canada). Acetylsalicylic acid, acetaminophen, brompheniramine maleate, caffeine, chlorpheniramine maleate, and ibuprofen were obtained as powders from Sigma–Aldrich (St. Louis, MO, USA). All other potential interference standards were obtained from Cerilliant (Austin, TX, USA) as 100 μg/mL or 1.0 mg/mL racemic solutions (where applicable) in methanol or acetonitrile (Table 1).

All solvents were HPLC grade and obtained from the following suppliers: methanol (Fisher Scientific, Pittsburgh, PA, USA) and acetonitrile and water (JT Baker, Phillipsburg, NJ, USA). All reagents were analytical reagent grade or higher. Ammonium acetate was obtained from Sigma Chemicals (St. Louis, MO, USA); ammonium hydroxide, hydrochloric acid and phosphoric acid were purchased from JT Baker (Phillipsburg, NJ, USA). Formic acid was sourced from Merck KGaA (Darmstadt, Germany).

2.2. Specimens

Pools of meconium screening negative for amphetamines, cannabinoids, cocaine and opiates were obtained from ElSohly Laboratories (Oxford, MS, USA); 1 g aliquots of each blank pool were analyzed to verify the absence of potential interferences prior to use as a matrix base for calibrators and quality control (QC) samples.

Authentic MAMP and/or AMP positive meconium specimens were obtained from United States Drug Testing Laboratory (USDTL, Des Plaines, IL, USA) to demonstrate the applicability of the validated method. Specimens were stored at −20 °C at USDTL and transferred frozen to NIDA with additional storage at −80 °C until analysis.

2.3. Preparation of calibrators and QC samples

Stock solutions (1.0 mg base/mL) of pOHAMP, pOHMAMP and pOHNOREPH were prepared in methanol. Methanolic standards (1.0 mg/mL) of the 10 analytes were combined and diluted in methanol to prepare a 50,000 ng/mL mixed calibrator stock solution. Working solutions were prepared at concentrations of 25, 50, 250, 500, 2500, 5000, 10,000, 25,000 and 50,000 ng/mL. Fifty, 100 or 200 μL aliquots of working solutions were added to 1.0 ± 0.1 g blank meconium to yield final concentrations of 1.25–10,000 ng/g. Two calibration curves, A and B, were generated to extend the dynamic analytical range and allow quantification of minor metabolites, as well as high concentrations of AMP and MAMP from a single meconium extract. Calibration curve A was generated using 10 μL injections of 1.25–2500 ng/g calibrators to quantify analytes from their lower limit of quantification to 2500 ng/g; calibration curve B was constructed with 1 μL injections of 125–10,000 ng/g calibrators for quantification of concentrations greater than 2500 ng/g. Calibrator concentrations shared by both curves were injected from the same extracted sample. Presently, there are no estimates of minor metabolite concentrations in meco-

Table 1
Compounds evaluated as potential interferences with amphetamine-related compounds in meconium

	Compounds
Opiates	Buprenorphine, norbuprenorphine, morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide, normorphine, 6-acetylmorphine, codeine, norcodeine, 6-acetylcodeine, hydromorphone, hydrocodone, oxymorphone, noroxymorphone, oxycodone, noroxycodone, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, 2-ethyl-5-methyl-3,3-diphenylpyrroline, methadol
Cocaine	Cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, ecgonine ethyl ester, ecgonine methyl ester, anhydroecgonine methyl ester, ecgonine, cocaethylene, norcocaethylene, *m*-hydroxycocaine, *p*-hydroxycocaine, *m*-hydroxybenzoylecgonine, *p*-hydroxybenzoylecgonine
Cannabinoids	Δ^9-Tetrahydrocannabinol, 11-nor-9-carboxyl-Δ^9-tetrahydrocannabinol, 11-hydroxy-Δ^9-tetrahydrocannabinol
Amphetamine	*N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine, 3,4-methylenedioxyphenyl-2-butanamine, cathinone, *N*-ethylamphetamine, 4-bromo-2,5-dimethoxyphenethylamine, ephedrine, fenfluramine, pseudoephedrine, norpseudoephedrine, *p*-methoxyamphetamine, *p*-methoxymethamphetamine, phentermine
Benzodiazepines	Alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, bromazepam, clonazepam, diazepam, flunitrazepam, flurazepam, lorazepam, nitrazepam, nordiazepam, oxazepam, temazepam
Nicotine	Nicotine, cotinine, norcotinine, *trans*-3′-hydroxycotinine
Antidepressants	Clomipramine, fluoxetine, imipramine, norfluoxetine, paroxetine
Antihistamines	Brompheniramine, chlorpheniramine, diphenhydramine
Miscellaneous	Acetylsalicylic acid, acetaminophen, ibuprofen, pentazocine, propoxyphene, caffeine, clonidine, phencyclidine

Low-quality control samples were fortified with 2500 ng/g potential interferent to determine if accurate quantification (±20% of target) could be obtained.

nium. In the event that minor metabolite concentrations exceed 2500 ng/g in authentic meconium specimens, the method was validated at higher concentrations with the 1 μL injection curve for minor metabolites, as well as parent metabolites.

QC working solutions were prepared from different ampules of 1.0 mg/mL methanolic standards than were used to prepare the calibrator solutions; when available, different manufacturer lot numbers were employed. Blank meconium samples were fortified with 160, 800, 8000 and 50,000 ng/mL working QC solutions yielding low-, medium- and high-QC samples for each analyte. For the 10 μL injection curve, low-, medium- and high-QC concentrations were 8 ng/g for HMMA and MDEA or 40 ng/g for pOHAMP, pOHMAMP, NOREPH, HMA, AMP, MAMP, MDA and MDMA, 400 and 2500 ng/g, respectively. The 1 μL injection curve low-, medium- and high-QC concentrations were 400, 2500, and 10,000 ng/g, respectively. The same 400 or 2500 ng/g extract was injected at 1 and 10 μL, serving as QC samples for both calibration curves.

Individual 1.0 mg/mL methanolic standards of six internal standards were combined and diluted in methanol to prepare a 100,000 ng/mL mixed internal standard stock solution. A 4000 ng/mL working internal standard solution was prepared in methanol. Twenty-five microliters of the working solution was added to each calibrator, QC and authentic specimen, yielding internal standard concentrations of 100 ng/g. When available, the corresponding deuterated analogue was utilized as an internal standard; the internal standard for pOHAMP, pOHMAMP, HMA and HMMA was NOREPH-d_3, the closest eluting deuterated internal standard.

All stock solutions were stored at −80 °C prior to use; working solutions were prepared daily.

2.4. Instrumentation

Experiments were performed on a Shimadzu HPLC system, consisting of two LC-10ADvp pumps, a CTO-10ACvp column heater and a SIL-HTC autosampler (Columbia, MD, USA). The HPLC was interfaced with an Applied Biosystems MDS Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA), equipped with an atmospheric pressure chemical ionization (APCI) source. All data were acquired and quantified using Analyst software, Version 1.4.

A Tekmar ultrasonic disrupter (Cincinnati, OH, USA), Branson 3510 ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT, USA) and Eppendorf Refrigerated 5804R centrifuge (Brinkmann, Westbury, NY, USA) were utilized during specimen preparation. Samples were concentrated with a Zymark TurboVap LV evaporator (Hopkinton, MA, USA).

Strata XC 33 μm cation mixed-mode polymer (100 mg/6 mL) SPE cartridges, Synergi Polar RP 4 μm, 150 mm × 2.0 mm analytical columns and Synergi Polar RP 4.0 mm × 2.0 mm guard columns were obtained from Phenomenex (Torrance, CA, USA).

2.5. Specimen preparation

Twenty-five microliters of the 4000 ng/mL working internal standard solution and 50, 100 or 200 μL of working calibrator or QC solution (when appropriate) were added to 1.0 ± 0.1 g of meconium. Specimens were centrifuged at 290 × *g* at room temperature for 5 min to drive meconium to the bottom of the 15 mL polypropylene centrifuge tube prior to adding 3 mL 17 mM methanolic hydrochloric acid. After vortex mixing, ultrasonic homogenization using 60 A, output 7–10 W was performed for 1 min in an ice-water bath, followed by sonication in an ultrasonic bath for 30 min, and centrifugation at 8230 × *g* at room temperature for 10 min. The supernatant was decanted into a clean glass centrifuge tube and evaporated under nitrogen at 37 °C to approximately 0.5 mL. Prior to SPE, 3 mL 2% phosphoric acid were added to each sample, and vortexed.

Strata XC SPE cartridges were conditioned with 3 mL methanol, followed by 3 mL water. After acidified samples were applied, cartridges were washed with 3 mL 0.1% phosphoric acid and allowed to dry under approximately 10 mm Hg vacuum for 3 min. After a 3 mL methanol wash, analytes were eluted with freshly prepared 3 mL methanol:ammonium hydroxide (95:5, v/v). Twenty-five microliters 1% HCl were added to each extract prior to evaporation to dryness under nitrogen at 37 °C and reconstitution in 200 μL of 10 mM ammonium acetate with 0.01% (v/v) formic acid.

2.6. Liquid chromatography–mass spectrometry

Synergi Polar RP 4 μm, 150 mm × 2.0 mm analytical and 4.0 mm × 2.0 mm guard columns adequately separated the 10 analytes. Gradient elution with a binary mobile phase system of (A) 10 mM ammonium acetate containing 0.01% (v/v) formic acid and (B) acetonitrile was performed with a 30 °C column temperature and 200 μL/min flow rate. The gradient profile was 10% B for 1 min, linear increase to 50% B over 13 min, hold at 50% B for 2 min, ramp to 90% B over 2 min, hold at 90% B for 2 min, and decrease to 10% B over 2 min. The column was re-equilibrated for 18 min at 10% B after each injection. Injection volume was either 10 μL (Curve A) or 1 μL (Curve B). The autosampler was maintained at 15 °C.

MS/MS parameters were optimized with acidified aqueous standard solutions of each analyte infused at 10 μL/min into the MS/MS via a Hamilton syringe infusion pump. Nebulizing (50 psi) and curtain gases (30 psi) were nitrogen. The corona discharge current was 3.0 μA and the source temperature was 375 °C.

Multiple reaction monitoring (MRM) scans in positive ion mode of the molecular ion and the two most predominant fragments for each analyte were utilized. Selected MRM transitions for each analyte and internal standard, with corresponding collision energies, are given in Table 2. The 20 min analysis time was divided into two periods. Dwell times for all analyte and internal standard transitions was 200 ms. Quadrupoles operated at unit resolution for all experiments.

2.7. Validation

Following bioanalytical validation guidelines [42–44], the method was fully validated in 4 days for the following parameters: selectivity, linearity, including limit of detection (LoD) and lower limit of quantification (LLoQ), accuracy and precision, extraction efficiency, matrix effect, and stability, including autosampler stability and freeze/thaw stability at −80 °C.

Selectivity was established by analysis of eight different lots of blank meconium and through evaluation of potential interferences (Table 1). Duplicate low-QC samples were fortified with 2500 ng/g potential interferences. A lack of interference was documented if analytes of interest quantified within 20% of target, and qualifier/quantification transition ratios were within permitted tolerances.

LoD and LLoQ were evaluated using triplicates of samples at or below the lowest calibrator for each analyte. LoD was defined as the concentration producing a Gaussian-shaped peak eluting within ±2% of mean calibrator retention time, a minimum signal to noise (S/N) ratio of 3:1, and qualifier/quantification transition ratio within the maximum tolerance of mean calibrator qualifier/quantification transition ratio. LLoQ was defined as the concentration that met LoD criteria and had a minimum S/N ratio of 10:1 and QC precision and accuracy within ±20%.

Table 2
Precursor and product ions with corresponding collision energies for each analyte and internal standard

Analyte/internal standard	Precursor ion (*m*/*z*)	Quantification transition product ion (*m*/*z*)	Collision energy (V)	Qualifier transition product ion (*m*/*z*)	Collision energy (V)
Period 1					
pOHAMP	152	107	24	77	45
pOHMAMP	166	135	17	107	28
NOREPH	152	117	24	134	14
NOREPH-d_3	155	119	26	137	15
HMA	182	105	31	165	14
HMMA	196	165	18	105	32
Period 2					
AMP	136	91	24	119	12
AMP-d_{11}	147	98	23	130	14
MAMP	150	119	17	91	22
MAMP-d_{14}	164	98	30	130	17
MDA	180	105	32	163	11
MDA-d_5	185	110	32	168	15
MDMA	194	163	17	105	35
MDMA-d_5	199	165	18	107	37
MDEA	208	163	19	105	36
MDEA-d_5	213	163	20	105	40

AMP, amphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHMAMP, *p*-hydroxymethamphetamine.

To assess linearity, calibration curves were obtained by analyzing drug-free meconium fortified with working calibrator solutions yielding 1.25–2500 ng/g final concentrations for Curve A (10 μL injection) or 125–10,000 ng/g for Curve B (1 μL injection). Calibration curves were constructed using linear regression with $1/x$ weighting based on a minimum of six calibrator peak area ratios, excluding the blank. Calibrators and duplicate QC samples at low, medium and high concentrations were analyzed daily in each set of specimens.

Accuracy and precision were evaluated with five replicates at low, medium and high concentrations. Intra-assay ($n = 5$) precision and accuracy were assessed within the same validation batch on four separate days, while inter-assay precision and accuracy were evaluated over four validation batches ($n = 20$). Accuracy is the percentage of calculated to target concentration, while precision, expressed as % coefficient of variation (% CV), is the standard deviation of the concentrations divided by the mean concentration of 5 or 20 replicates.

Extraction efficiency and matrix effect for each analyte and internal standard were assessed through the preparation of three sets of samples, as outlined by Matuszewski et al. [42]: set 1 included unextracted standards reconstituted in mobile phase A; set 2 was extracted negative meconium samples with QC and internal standard solutions added *after* extraction during reconstitution; set 3 was fortified meconium samples with QC and internal standard solutions added *before* extraction prior to homogenization. Each set had five replicates at four QC concentration levels: low, medium, and highs for the 10 and 1 μL injection methods. Extraction efficiency was calculated as the ratio of the average peak area in sets 3 to 2, expressed as a percentage. Matrix effect was defined as the percentage ratio of the average peak areas for sets 2 and 1.

Stability of extracted QC samples under 15 °C autosampler storage was evaluated at low- and high-QC concentrations for each injection method. Each QC sample was injected every 3 h, up to 78 h. The % CV of the peak area of each analyte and internal standard was calculated for each QC sample. Analyte stability in unprocessed meconium samples was also evaluated after three freeze–thaw cycles with five replicates at low- and high-QC concentrations using the 10 μL injection method. Each cycle consisted of freezing at −80 °C for a minimum of 24 h, then thawing unassisted at room temperature. The freeze–thaw stability was calculated as the ratio of the average concentration of freeze–thawed QC samples and freshly prepared QC samples, expressed as a percentage.

3. Results and discussion

While electrospray and APCI ionization were evaluated for each analyte, APCI was selected on the basis of less matrix effect. Monitoring two MRM transitions per analyte adheres to the Commission of European Communities (EC) identification criteria requiring a minimum of three identification points (IP). Under these guidelines, the LC–MS^n precursor ion has a value of 1.0 IP and each LC–MS^n transition product earns 1.5 IP, resulting in a total of 4.0 IP [45]. Mass spectrometric conditions for each MRM transition, such as collision energy, were individually optimized. Selection of the quantification and qualifier transitions was based on transitions from the molecular ion to the most and second-most predominant fragment ions, respectively. For NOREPH, HMA and MDA, selection of the quantification and qualifier transitions was reversed; the second-most predominant fragment ion was chosen for quantification since the most predominant fragment corresponded to the loss of water. The MAMP quantification transition also was based on the second-most abundant fragment ion, as this transition had a lower level of background noise in blank meconium samples.

Currently, there is no consensus on acceptable relative abundance variability of LC–MS/MS MRM transition ratios, as several regulatory bodies have proposed differing guidelines [45–47]. In the current method, the EC guidelines for maximal permitted tolerances were followed, where permitted tolerances of relative ion intensities for LC–MS^n techniques are dependent on relative transition intensities [45]. Mean relative abundance ratios of each analytes' two MRM transitions ranged from 0.17 to 0.95 in calibrators, with % CVs of less than 12% observed over four days for both the 10 and 1 μL injection methods. Mean transition abundance ratios and permitted tolerances under EC guidelines are given in Table 3. Using the 10 and 1 μL injection methods, all analytes had acceptable MRM transition ratios within these permitted tolerances for all QC samples.

Representative extracted ion chromatograms for blank meconium and a low-QC sample, and authentic meconium obtained from a neonate following *in utero* MAMP exposure are given in Figs. 2 and 3, respectively. All analytes eluted within 15 min; each

Table 3
Mean relative abundance ratios of two MRM transitions monitored for amphetamine-related analytes in meconium

Analyte	10 μL injection		1 μL injection		Permitted tolerance (%)[a]
	Relative abundance[b]	Inter-day % CV (n = 4)	Relative abundance[b]	Inter-day % CV (n = 4)	
pOHAMP	0.166	3.8	0.170	1.4	30
pOHMAMP	0.898	5.5	0.946	1.2	20
NOREPH	0.338	1.5	0.331	2.2	25
HMA	0.330	2.9	0.291	12.2	25
HMMA	0.323	3.3	0.309	3.9	25
AMP	0.787	3.0	0.786	2.1	20
MAMP	0.656	0.8	0.660	1.3	20
MDA	0.420	3.9	0.416	3.0	25
MDMA	0.436	8.2	0.382	1.3	25
MDEA	0.409	2.7	0.410	2.4	25

AMP, amphetamine; CV, coefficient of variation; HMA, 4-hydroxy-3-methoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHMAMP, *p*-hydroxymethamphetamine.

[a] As specified by Commission of European Communities Council Directive 96/23/EC [45]

[b] Ratio of qualifier to quantification transition peak areas, except for NOREPH, HMA, MAMP, and MDA which are the ratio of quantification to qualifier transition.

analytical run was less than 33 min. The column was allowed to re-equilibrate for 18 min after the final step of the gradient program and the beginning of the next injection.

Blank meconium specimens from eight different sources had minimal interferences with analytes of interest. Seventy-three common licit and illicit compounds were investigated as potential interferences with analytes of interest (Table 1). Duplicate low-QC samples were fortified with 2500 ng/g potential interferences. A concentration of 2500 ng/g was chosen for the interference study because for most analytes evaluated, 2500 ng/g exceeds the highest observed concentrations in human meconium reported in the literature. In only a small percentage of cocaine-positive

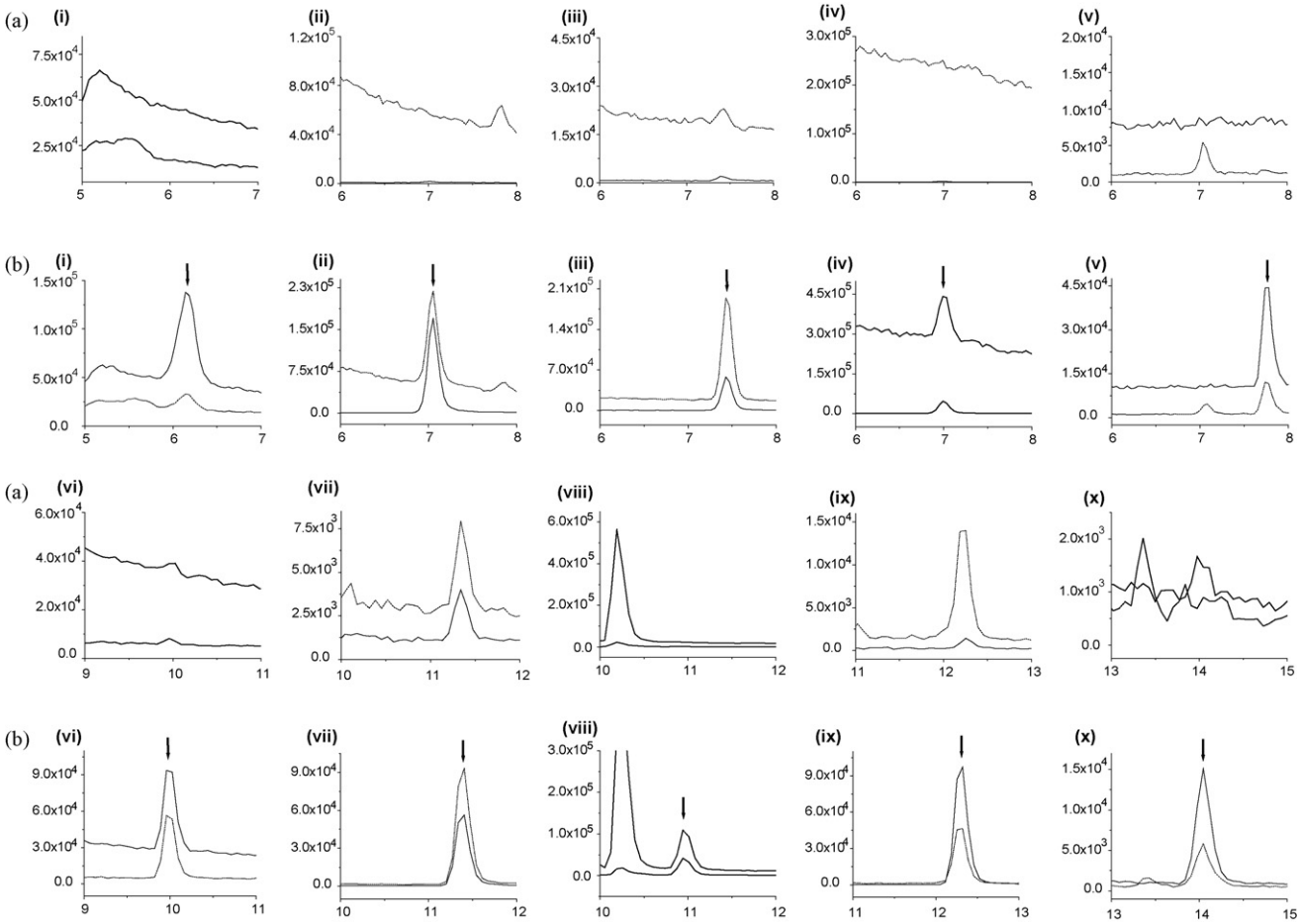

Fig. 2. Extracted ion chromatograms of (a) a blank meconium sample and (b) a low-quality control sample. Quantification and qualifier transitions are denoted by solid and dashed lines, respectively; analyte retention time indicated by arrow: (i) *p*-hydroxyamphetamine (pOHAMP), (ii) *p*-hydroxymethamphetamine (pOHMAMP), (iii) norephedrine (NOREPH), (iv) 4-hydroxy-3-methoxyamphetamine (HMA), (v) 4-hydroxy-3-methoxymethamphetamine (HMMA), (vi) amphetamine (AMP), (vii) methamphetamine (MAMP), (viii) 3,4-methylenedioxyamphetamine (MDA), (ix) 3,4-methylenedioxymethamphetamine (MDMA), and (x) 3,4-methylenedioxyethylamphetamine (MDEA). QC concentrations: 8 ng/g for HMMA and MDEA; 40 ng/g for all other analytes.

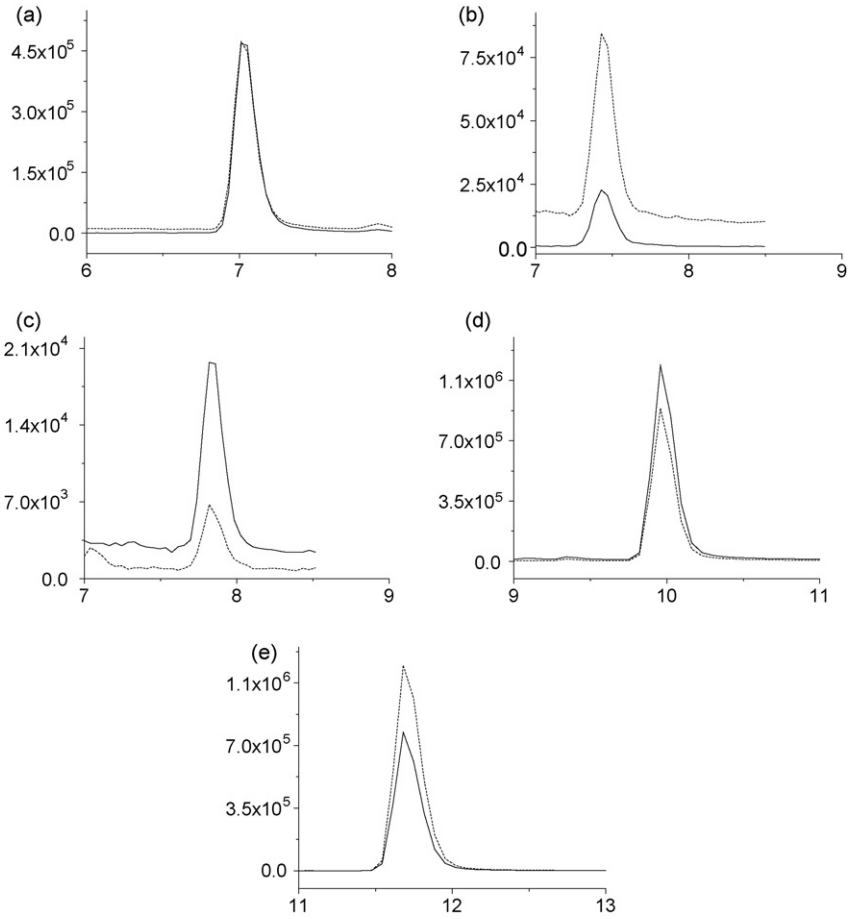

Fig. 3. Extracted ion chromatograms of an authentic meconium specimen collected from a neonate following *in utero* drug exposure. Quantification and qualifier transitions are denoted by solid and dashed lines, respectively. Injection volume of 10 μL, except for methamphetamine (1 μL). Specimen contains: (a) 129.6 ng/g *p*-hydroxymethamphetamine, (b) 19.3 ng/g norephedrine, (c) 4.2 ng/g 4-hydroxy-3-methoxymethamphetamine, (d) 639.2 ng/g amphetamine, and (e) 4638.8 ng/g methamphetamine.

specimens, concentrations of cocaine and some of its metabolites may be greater than 2500 ng/g. Anhydroecgonine methyl ester (AEME), a pyrolytic biomarker of cocaine base, coelutes with HMA's quantification transition, m/z 182 → 105; however, the qualifier transition is unaffected. AEME's presence results in a false negative HMA result as the qualifier/quantification transition ratio would be decreased. If HMA interference was suspected, AEME can be confirmed by monitoring AEME-specific transitions, m/z 182 → 91 and m/z 182 → 122. Norpseudoephedrine, a stereoisomer of NOREPH, gave a response for both NOREPH transitions at a retention time immediately after NOREPH, leading to a split peak. However, the two QC samples containing 40 ng/g of NOREPH and 2500 ng/g norpseudoephedrine were 77% accurate. MAMP qualifier/quantification transition peak area ratio was considerably affected by coelution of phentermine, a structural isomer, with the MAMP qualifier m/z 150 → 91 transition. The average qualifier/quantification transition peak area ratio of two low-QC samples increased from 1.5 to 32.3 in the presence of phentermine; therefore, MAMP false negatives due to the presence of phentermine could be identified by a dramatic increase in the qualifier/quantification transition ratio.

LoDs and LLoQs ranged from 0.8–5 and 1.25–40 ng/g, respectively for the 10 μL injection curve; LLoQs for the 1 μL injection curve were 125 ng/g for all analytes, except HMA at 250 ng/g (Table 4). Linear dynamic ranges for the 10 and 1 μL injection methods were LLoQ –2500 ng/g and 125 (or 250)–10,000 ng/g, respectively. A previous LC–MS method employing electrospray ionization and selective ion monitoring achieved LLoQs of 4 ng/g for MDA, MDEA and MDMA and 5 ng/g AMP, MAMP and HMMA; upper limits of quantification (ULoQ) were 1000 ng/g [23]. The LLoQs observed in this method using the 10 μL injection were lower than those for MDEA and HMMA, but higher for AMP, MAMP, MDMA and MDA. The higher LLoQs for these analytes were associated with low-level interferences present within blank meconium. Additional analytes included in this method that were not considered by the previous method included pOHMAMP, NOREPH, pOHAMP and HMA with LLoQs of 8, 12.5, 12.5 and 40 ng/g, respectively. The elevated LLoQ for HMA was due to background interference from blank meconium with the qualifier transition. The ULoQ of the 10 μL injection method was higher than the previously reported method and inclusion of the 1 μL injection method further increased the dynamic range to 10,000 ng/g for all analytes. Extending the ULoQ of the 10 μL injection method beyond 2500 ng/g was not possible, as detector saturation was observed; therefore, the two injection volume system was employed to allow quantification up to 10,000 ng/g from a single extract. The 1 μL injection method need only be employed for specimens containing analyte concentrations greater than the ULoQ with the 10 μL injection method (2500 ng/g); this is often the case with MAMP. Limited specimen amount is a significant concern in meconium analysis; extending the linear dynamic range reduces the need for dilution and reanalysis, and preserves the specimen for other testing, if necessary.

Mean (±S.D.) calibration curve slope and intercept and mean (±S.D.) retention time for each analyte for the 10 and 1 μL injec-

Table 4
Mean retention times, limits of detection (LoDs), lower limits of quantification (LLoQs) and calibration slope and intercept ($n = 4$) for amphetamine-related analytes in meconium using 10 and 1 μL injection methods

Analyte	Retention time (±S.D.)	10 μL injection				1 μL injection		
		LoD (ng/g)	LLoQ (ng/g)	Slope (±S.D.)	Intercept (±S.D.)	LLoQ (ng/g)	Slope (±S.D.)	Intercept (±S.D.)
pOHAMP	5.94 (0.05)	4	12.5	4.220 (0.786)	−0.109 (0.154)	125	5.313 (0.977)	−2.204 (1.222)
pOHMAMP	6.81 (0.05)	2.5	8	4.485 (0.659)	−0.042 (0.088)	125	5.745 (1.088)	−1.978 (1.419)
NOREPH	7.21 (0.05)	2.5	12.5	1.650 (0.226)	−0.044 (0.036)	125	1.925 (0.321)	−0.694 (0.287)
HMA	6.79 (0.05)	25	40	1.403 (0.117)	−0.130 (0.060)	250	1.568 (0.054)	−1.422 (0.382)
HMMA	7.55 (0.04)	1.25	2.5	4.665 (0.475)	−0.025 (0.040)	125	5.365 (0.604)	−1.208 (1.451)
AMP	9.69 (0.06)	4	12.5	0.623 (0.055)	0.018 (0.010)	125	0.725 (0.089)	−0.0514 (0.180)
MAMP	10.98 (0.09)	2.5	12.5	0.702 (0.033)	0.001 (0.016)	125	0.878 (0.022)	−0.192 (0.183)
MDA	10.64 (0.07)	4	12.5	2.198 (0.477)	−0.074 (0.064)	125	2.470 (0.524)	−1.063 (0.448)
MDMA	11.87 (0.08)	8	12.5	1.465 (0.245)	−0.016 (0.029)	125	1.755 (0.265)	−0.306 (0.459)
MDEA	13.54 (0.10)	0.8	1.25	0.794 (0.049)	−0.001 (0.005)	125	0.918 (0.045)	−0.206 (0.200)

AMP, amphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHMAMP, *p*-hydroxymethamphetamine.

tion curves over four batches are given in Table 4. Coefficients of determination (R^2) were greater than 0.993 and 0.979 for all analytes for the 10 and 1 μL injection curves, respectively, using least squares regression and $1/x$ weighting. All calibrators used in each calibration were calculated to be within ±20% of their target concentrations.

Accuracy and precision were evaluated with five replicates of low, medium and high concentrations for each analyte. Intra-assay ($n = 5$) and inter-assay ($n = 20$) accuracy and precision data for the 10 and 1 μL injection curves are shown in Table 5. Intra-assay ($n = 5$) precision was less than 6.4% and accuracy 79–115% for 10 μL injection QC samples. Inter-assay ($n = 20$) precision was

Table 5
Intra- and inter-assay precision and accuracy of amphetamine-related analytes in meconium for the 10 and 1 μL injection curves

Analyte	10 μL injection					1 μL injection				
	Concentration (ng/g)	Intra-assay ($n = 5$)		Inter-assay ($n = 20$)		Concentration (ng/g)	Intra-assay ($n = 5$)		Inter-assay ($n = 20$)	
		Accuracy (%)	Precision (% CV)	Accuracy (%)	Precision (% CV)		Accuracy (%)	Precision (% CV)	Accuracy (%)	Precision (% CV)
pOHAMP	40	83–92	2.0–4.5	87	4.7	400	83–103	3.1–5.5	92	9.5
	400	94–107	2.0–5.9	101	7.2	2,500	93–109	3.7–6.8	100	8.7
	2500	90–101	2.1–5.0	96	5.3	10,000	88–97	4.0–6.3	93	6.1
pOHMAMP	40	81–99	1.7–5.7	91	7.9	400	84–108	3.6–5.7	95	10.6
	400	96–112	2.5–4.0	102	6.8	2,500	91–108	4.6–6.6	99	9.1
	2500	92–99	1.8–3.5	96	3.8	10,000	89–97	4.0–5.4	94	5.6
NOREPH	40	83–92	1.6–4.7	89	4.9	400	87–102	3.4–4.5	92	7.6
	400	88–104	2.9–4.3	96	7.7	2,500	87–102	4.8–6.2	94	8.3
	2500	92–98	1.8–4.3	96	3.8	10,000	90–96	4.3–6.3	93	5.5
HMA	40	89–108	1.5–2.8	99	8.8	400	94–111	2.5–4.0	99	8.1
	400	85–100	2.5–4.6	90	7.7	2,500	85–97	4.9–6.6	90	7.1
	2500	91–98	1.8–4.1	94	3.7	10,000	90–96	3.9–8.3	94	6.3
HMMA	8	79–91	3.2–5.9	84	7.7	400	88–99	2.3–5.8	92	6.2
	400	96–111	2.7–5.4	104	8.0	2,500	92–108	4.5–6.4	100	7.9
	2500	84–92	1.5–4.4	89	4.6	10,000	89–94	3.7–7.5	92	6.0
AMP	40	84–98	1.7–4.9	93	7.2	400	88–102	1.9–4.7	93	7.1
	400	99–114	2.6–5.1	103	7.2	2,500	91–111	1.0–6.2	100	8.2
	2500	87–96	1.8–3.5	92	4.7	10,000	89–92	0.9–4.1	90	2.7
MAMP	40	82–104	1.4–3.7	93	9.1	400	90–103	3.1–4.3	94	6.6
	400	93–115	3.6–5.8	101	9.5	2,500	90–105	2.6–4.7	97	6.8
	2500	85–97	1.6–2.7	92	5.6	10,000	91–95	2.3–4.9	92	3.9
MDA	40	86–98	2.5–6.1	93	6.4	400	88–100	2.8–6.2	94	7.7
	400	87–105	3.6–6.4	97	8.1	2,500	84–114	4.1–9.7	96	14.2
	2500	89–109	2.1–6.2	101	9.1	10,000	84–107	2.8–5.4	95	10.3
MDMA	40	84–97	2.4–7.3	93	7.3	400	90–106	3.0–7.1	96	7.7
	400	96–113	3.0–4.8	104	8.5	2,500	89–116	2.7–5.3	101	11.5
	2500	92–96	1.4–4.7	94	3.6	10,000	86–97	1.3–4.2	92	5.8
MDEA	8	81–101	3.4–6.0	87	11.0	400	87–96	2.6–5.1	90	5.1
	400	90–107	2.6–5.4	97	8.0	2,500	87–105	2.2–5.5	97	7.6
	2500	89–98	2.9–4.7	93	4.9	10,000	91–93	1.9–5.0	92	3.2

AMP, amphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHMAMP, *p*-hydroxymethamphetamine.

Table 6
Extraction efficiency and matrix effect of amphetamine-related analytes and internal standards in meconium

Analyte/internal standard	Concentration (ng/g)		Extraction efficiency (%) ($n = 5$)	Matrix effect (%) ($n = 5$)
pOHAMP	Low	40	71.9	117.7
	Medium	400	73.9	109.7
	High (10 μL)	2,500	85.4	111.0
	High (1 μL)	10,000	80.4	113.2
pOHMAMP	Low	40	76.3	117.6
	Medium	400	77.2	108.4
	High (10 μL)	2,500	86.8	104.2
	High (1 μL)	10,000	81.9	109.8
NOREPH	Low	40	72.6	108.9
	Medium	400	72.8	104.3
	High (10 μL)	2,500	82.4	98.5
	High (1 μL)	10,000	78.2	108.0
HMA	Low	40	74.2	144.0
	Medium	400	75.4	123.9
	High (10 μL)	2,500	83.4	107.5
	High (1 μL)	10,000	74.8	115.5
HMMA	Low	40	75.0	149.4
	Medium	400	75.8	132.3
	High (10 μL)	2,500	83.3	114.9
	High (1 μL)	10,000	79.5	114.4
AMP	Low	40	72.4	92.5
	Medium	400	73.3	85.2
	High (10 μL)	2,500	81.0	86.7
	High (1 μL)	10,000	80.4	97.0
MAMP	Low	40	69.1	101.8
	Medium	400	71.3	91.3
	High (10 μL)	2,500	79.6	90.0
	High (1 μL)	10,000	77.9	97.9
MDA	Low	40	70.5	100.5
	Medium	400	70.4	96.0
	High (10 μL)	2,500	80.2	94.5
	High (1 μL)	10,000	75.6	106.0
MDMA	Low	40	68.8	107.0
	Medium	400	71.2	99.3
	High (10 μL)	2,500	79.8	98.6
	High (1 μL	10,000	77.5	105.0
MDEA	Low	40	70.1	106.0
	Medium	400	73.9	99.1
	High (10 μL)	2,500	82.5	96.8
	High (1 μL	10,000	79.2	103.8
NOREPH-d_3	Low	100	68.6	119.3
	Medium	100	67.0	114.5
	High (10 μL)	100	70.2	107.1
	High (1 μL)	100	71.8	109.9
AMP-d_{11}	Low	100	66.0	100.1
	Medium	100	65.7	95.9
	High (10 μL)	100	69.2	89.3
	High (1 μL)	100	72.9	102.5
MAMP-d_{14}	Low	100	62.8	122.8
	Medium	100	63.9	114.1
	High (10 μL)	100	66.5	103.7
	High (1 μL)	100	71.9	102.1
MDA-d_5	Low	100	64.0	119.5
	Medium	100	64.1	114.0
	High (10 μL)	100	66.1	107.1
	High (1 μL)	100	67.3	112.2
MDMA-d_5	Low	100	60.4	131.9
	Medium	100	62.9	120.4
	High (10 μL)	100	63.2	117.4
	High (1 μL)	100	66.6	112.7
MDEA-d_5	Low	100	71.2	110.7
	Medium	100	66.0	118.0
	High (10 μL)	100	68.9	110.8
	High (1 μL)	100	72.1	109.8

AMP, amphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MAMP, methamphetamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; NOREPH, norephedrine; pOHAMP, *p*-hydroxyamphetamine; pOHMAMP, *p*-hydroxymethamphetamine.

less than 11.0% and accuracy 84–104%. One microliter injection QC samples demonstrated intra-assay ($n = 5$) precision and accuracy of less than 9.7% and 83–116%, respectively; with inter-assay ($n = 20$) precision and accuracy of 2.7–14.2% and 90–101%, respectively.

Extraction efficiency and matrix effect for each analyte and internal standard were assessed at four concentrations, low, medium, and highs for the 10 and 1 μL injection methods (Table 6). Extraction efficiencies ranged from 74.2%–0.6% for all analytes and 63.3–69.6% for internal standards. Matrix effect was observed in most analytes and internal standards at all concentrations, with values ranging from 85.2%–49.4%.

Stability studies suggest no relevant degradation of extracts under autosampler storage conditions. There was less than 10% change in absolute peak area over 78 h at low- and high-QC concentrations for both injection methods. The meconium specimen demonstrating method applicability was initially analyzed only for MAMP and AMP at USDTL, where specimens were stored at −20 °C prior to and following analysis. Specimens were shipped on dry ice and stored at −80 °C at NIDA. USDTL validated their analytical method including stability at −20 °C; analyte stability greater than 97% and 83% for 6 months and 1 year after one freeze/thaw cycle, respectively was observed. Stability after three freeze–thaw cycles at −80 °C was demonstrated at two QC concentrations for the current method. Mean concentration of the five freeze–thawed replicates was within 14% of the mean concentration of freshly prepared QC samples.

3.1. Application of method

A meconium specimen previously identified as MAMP- and AMP-positive by GC–MS was generously donated by USDTL to demonstrate method applicability. The specimen contained 4638.8 ng/g MAMP, 639.2 ng/g AMP, 129.6 ng/g pOHMAMP, 19.3 ng/g NOREPH, and 4.2 ng/g HMMA; extracted ion chromatographs from this specimen are presented in Fig. 3. The high concentration (>2500 ng/g) of MAMP demonstrates the importance of the 1 μL injection method for accurate quantification. pOHMAMP, NOREPH, and HMMA were detected for the first time in an authentic meconium specimen.

A low-HMMA meconium concentration was found in the absence of MDMA. This is in contrast to one reported MDMA-positive meconium specimen that did not contain other methylenedioxy-derivatives (MDA or MDEA) or the HMMA metabolite [48]. We are unaware of any clinical data demonstrating HMMA after controlled MAMP administration, although it has been suggested as a possible MAMP metabolite [37]. Also, HMMA was reported as a metabolite of *p*-methoxymethamphetamine (PMMA) after administration to rats, although there are no supportive human data [49]. The presence of pOHMAMP in meconium may indicate maternal MAMP, pholedrine or PMMA exposure. Pholedrine (pOHMAMP) is a therapeutic medication identified in blood, urine and liver of a fatal intoxication case [50]. Additionally, pOHMAMP was identified as a PMMA metabolite in rat urine [49], again with no available human data. Since pOHMAMP and HMMA can be products of several AMP-related precursors, examining the entire metabolic profile in meconium may be helpful to determine exposure to a specific drug. Also, co-ingestion of precursors cannot be excluded, since multiple AMP-related compounds are frequently found in illicit drugs. It is possible that in our case the fetus was exposed to MAMP and MDMA, pholedrine and/or PMMA. Unfortunately, no maternal self-report data are available for this specimen to provide additional information regarding exposure history.

While Pichini et al. applied their developed method to approximately 830 meconium specimens collected as part of the 'Meconium Project' by Hospital del Mar, Spain, none were AMP or MAMP positive, and only 1 MDMA positive was identified [23,48]. Additional meconium from neonates of self-reported heavy MAMP abusing mothers were analyzed by Pichini et al. using a reduced specimen aliquot, 0.3–0.5 g rather than 1 g [23]. High concentrations of AMP (38–1019 ng/g) and MAMP (226–1157 ng/g) were identified in these specimens, but no methylenedioxy-derivatives were detected, with the exception of one specimen with 12 ng/g MDMA. The advantage of the current method is that analyte concentrations up to 10,000 ng/g can be quantified simultaneously with lower concentrations of minor metabolites. This is accomplished by injecting 10 and 1 μL (if necessary) from a single 1 g aliquot extract and identifying and quantifying analyte concentrations from the appropriate concentration curve.

4. Conclusion

A validated LC–APCI-MS/MS method for the simultaneous quantification of 10 AMP-related analytes in meconium was reported. Two MRM transitions were selected for each analyte for improved selectivity. Injection volumes of 1 and 10 μL allowed quantification of concentrations of parent analytes up to 10,000 ng/g, while sufficient sensitivity was available to quantify low-minor metabolite concentrations. The validated method was applied to a meconium specimen obtained from a neonate following *in utero* exposure to AMP-related compounds. Three new biomarkers, pOHMAMP, NOREPH, and HMMA, were quantified in meconium for the first time. The validated method will be used to assess *in utero* drug exposure to amphetamine-related compounds and characterize disposition of these analytes in meconium.

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