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Novel biomarkers of prenatal methamphetamine exposure in human meconium

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Abstract

Meconium analysis can detect fetal exposure to drugs taken by the mother during pregnancy. Methamphetamine and amphetamine have previously been observed in meconium of methamphetamine-exposed neonates; the presence of other metabolites has not been investigated. Detection of such analytes may lead to more sensitive identification and, thus improved medical treatment of affected infants.

Methods and Materials—Forty-three methamphetamine-positive meconium specimens were analyzed for newly identified methamphetamine biomarkers, *p*-hydroxymethamphetamine, *p*-hydroxyamphetamine, and norephedrine. Due to methamphetamine adulteration in illicit ecstasy and to simultaneously monitor 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine prenatal exposure, MDMA, its metabolites and related sympathomimetic amines were assayed.

Results—Methamphetamine, amphetamine and unconjugated *p*-hydroxymethamphetamine were the most prevalent and abundant analytes present in meconium; however, unconjugated *p*-hydroxyamphetamine and norephedrine also were identified.

Discussion—It is possible that one of these additional analytes could be important for predicting toxicity or maternal or neonatal outcome measures in fetuses exposed to methamphetamine at specific gestational ages or with different metabolic capabilities. Although these new biomarkers were present in lower concentrations than methamphetamine and amphetamine in the meconium of previously

confirmed specimens, additional research will determine if inclusion of these analytes can increase identification of methamphetamine-exposed neonates.

Conclusion—Novel methamphetamine biomarker concentrations were characterized in meconium of infants exposed *in utero* to methamphetamine.

Keywords

methamphetamine; meconium; *in utero*

Identifying methamphetamine (MAMP)-exposed neonates is critical to ascertain potential maternal and neonatal toxicities, to develop prevention strategies, and to establish medical and social interventions for children and their mothers. In communities with high MAMP use, over 5% of pregnant women reported using the drug at some point during pregnancy.¹ To date, few studies have investigated human maternal MAMP use on neonatal outcomes, and the effect of MAMP on the developing fetus is unclear. The largest study of prenatal MAMP use, the Infant Development, Environment, and Lifestyle (IDEAL) study found that exposed neonates were more likely to be small for gestational age and have lower birth weights than non-MAMP-exposed children.² After adjusting for covariates, exposure to MAMP was associated with increased physiological stress.³ Other studies reported MAMP-induced growth restriction as evidenced by smaller head circumference, lower birth weight, and decreased gestational age.^{4, 5}

MAMP, a sympathomimetic amine, stimulates the release and blocks the reuptake of dopamine, norepinephrine, and serotonin in the neuronal synapse, resulting in feelings of pleasure and euphoria. MAMP may be ingested orally, snorted, injected or smoked; each route of exposure has high bioavailability. Figure 1 summarizes the metabolic pathway of MAMP. In adults, MAMP is primarily *N*-demethylated by CYP2D6 to amphetamine (AMP).⁶ Minor metabolic reactions include CYP2D6-catalyzed hydroxylation to *p*-hydroxymethamphetamine (pOHMAMP) and *p*-hydroxyamphetamine (pOHAMP)⁶ and oxidation to norephedrine (NOREPH).⁷ Additionally, pOHMAMP and pOHAMP may undergo glucuronidation or sulfation prior to terminal excretion.⁸ CYP2D6 activity increases during pregnancy, particularly in the third trimester, but is not present in human placental tissue.^{9,10} The ability of the fetus to metabolize MAMP and metabolites specifically is unknown, however, fetal liver expresses CYP2D6¹¹ and is capable of Phase II conjugation, with sulfation being better developed than glucuronidation.¹²

Prenatal drug exposure is often determined by maternal self-report or toxicological analysis of maternal and/or neonatal biological fluids and tissues. Meconium, the first fecal material passed by the neonate, is frequently assayed to determine gestational drug exposure because of its sensitivity and long window of drug detection. Compared to neonatal hair testing and maternal interview, meconium analysis is more sensitive for detecting *in utero* exposure to drugs of abuse.^{13, 14} Meconium formation begins around the twelfth week¹⁵, accumulating sloughed cells, swallowed amniotic fluid, bile salts, lipids, other endogenous compounds and xenobiotics until birth; therefore, positive meconium results theoretically indicate exposure during the second and third trimesters.

Typically, meconium is initially screened for MAMP and/or AMP with an immunoassay technique for qualitative detection. Immunoassay testing is quick and economical, but susceptible to cross-reactivity with compounds structurally similar to the target compound.¹⁶⁻¹⁹ For this reason, the identities of compounds triggering positive immunoassay results must be confirmed by a more specific technique, most often gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). Several studies have observed low confirmation rates for presumptive MAMP-positive meconium specimens,

with values ranging from 0% to 40.7%.²⁰⁻²³ It is possible that minor MAMP and/or AMP metabolites, such as hydroxylated metabolites pOHMAMP and pOHAMP, or other sympathomimetic amines present in meconium trigger positive immunoassay responses in the absence of MAMP and AMP and are not detected by current chromatographic methodologies. For the highest sensitivity in identifying *in utero* cocaine and cannabinoid exposure, hydroxylated metabolites, *m*-hydroxybenzoylecgonine, 11-hydroxy- Δ^9 -tetrahydrocannabinol (11-OH-THC) and 8 β ,11-dihydroxy- Δ^9 -tetrahydrocannabinol (8 β ,11-diOH-THC) should be included in the confirmation method.²⁴⁻²⁶ ElSohly et al. increased cannabinoid confirmation rates from 26% to 100% by including the hydroxylated metabolites.²⁶

GC/MS and LC/MS methods for the detection of sympathomimetic amines in meconium have primarily focused on AMP, MAMP and more recently, MDMA, MDEA, MDA, 4-hydroxy-3-methoxymethamphetamine (HMMA) and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB).^{27, 28} Our laboratory developed a liquid chromatography tandem mass spectrometry (LC/MS/MS) method for the simultaneous quantification of MAMP, AMP, and metabolites, NOREPH, pOHMAMP, and pOHAMP, and methylenedioxy-derivatives MDMA, MDEA and metabolites 4-hydroxy-3-methoxyamphetamine (HMA), HMMA, and MDA.²⁹ This was the first time pOHMAMP, pOHAMP, and NOREPH were identified in meconium, but these analytes' distributions are unknown. The primary purpose of this study was to characterize the disposition of MAMP-related analytes in meconium by analyzing 43 MAMP-positive meconium specimens with this new analytical method. The ultimate goal was to determine if metabolites pOHMAMP, pOHAMP and NOREPH were useful biomarkers for detecting prenatal MAMP or AMP exposure. Identifying additional biomarkers of MAMP exposure might explain the low confirmation rates observed in meconium with current methods focused on MAMP and AMP, and could provide new biomarkers for MAMP exposure in fetuses exposed during different stages of gestation or with different metabolic capabilities.

Materials and Methods

Meconium

Drug-free meconium was purchased from ElSohly Laboratories (Oxford, MS, USA) and further analyzed by LC/MS/MS to ensure absence of potential interferences. Authentic meconium specimens screened and confirmed positive for MAMP and/or AMP by EMIT and GC/MS, respectively, were kindly provided by the United States Drug Testing Laboratory (Des Plaines, IL, USA). These MAMP-positive meconium specimens were submitted to the laboratory for screening and confirmation of a panel of illicit and licit drugs. Meconium specimens were stored frozen for one year after confirmation. After the required storage, all identification was removed from the specimens and they were sent to the author's laboratory for research purposes. Results of the IDEAL study suggested the possibility of additional novel biomarkers of MAMP exposure other than MAMP and AMP; therefore, it was appropriate to first test known MAMP-positive meconium specimens to determine if these analytes could be identified in meconium of infants with proven *in utero* MAMP exposure. Furthermore, little meconium from IDEAL participants remained after the original analyses, making it imperative to determine if novel biomarkers were indeed present in meconium before reanalyzing these precious specimens.

Analysis

All meconium specimens were analyzed using a validated LC/MS/MS method for the identification of ten AMP-related compounds.²⁹ Specimen preparation included methanol homogenization and solid phase extraction. Limits of quantification (LOQ) were 1.25 ng/g for MDEA, 2.5 ng/g for HMMA, 8 ng/g for pOHMAMP, 12.5 ng/g for pOHAMP, NOREPH, AMP, MAMP, MDA, and MDMA, and 40 ng/g for HMA. Two calibration curves were

constructed to extend the method's linear dynamic range; curve A, using a 10 μ L injection, ranged from the analytes' LOQ – 2,500 ng/g, while curve B, using a 1 μ L injection, ranged from 125 – 10,000 ng/g (except for HMA whose lowest calibrator was 250 ng/g). The two curve system allowed simultaneous quantification of high parent drug concentrations and low metabolite concentrations from the same meconium extract (where necessary). Inter- and intra-assay precision were less than 9.7% relative standard deviation, and accuracy was between 79 – 116% for all analytes at three quality control concentrations. Extraction efficiency and matrix effect ranged from 60.4 – 86.8% and 85.2 – 149.4% for analytes and internal standards, respectively.

Data acquisition and analysis was performed by Analyst software, version 1.4.1 (Applied Biosystems, Foster City, CA, USA). SPSS software version 13.0 for Windows was used for all statistical analyses.

Results

All samples (N=43) were positive for MAMP and AMP as expected. Unconjugated pOHMAMP, previously uncharacterized in meconium, was quantifiable in 37 specimens (range 17.8 – 412.9 ng/g). Free pOHAMP was identified in four specimens with high MAMP concentrations (1,107, 3,254, 5,643, and 8,259 ng/g), but was present at concentrations less than the method's LOQ (12.5 ng/g). NOREPH was found in low concentrations in 11 specimens (range 15.9 – 38.7 ng/g); 26 additional specimens were found to contain NOREPH at concentrations below the method's LOQ (12.5 ng/g). No specimens contained methylenedioxy-derivatives, MDMA, MDEA, MDA, or HMA, although HMMA was detected in eight samples at concentrations lower than 6 ng/g. Table 1 displays the median and range of concentrations and the number of specimens positive for each analyte.

Ratios of metabolites to MAMP are shown in Table 1. MAMP was always present at the highest concentration, although there was significant variation in the ratios of metabolites to MAMP. On average, MAMP concentrations were approximately 4 and 12 times greater than concentrations of AMP and free pOHMAMP, respectively. AMP was the second-most abundant analyte, followed by unconjugated pOHMAMP and NOREPH. MAMP concentrations were significantly correlated with AMP ($r = 0.807$, $p < 0.0001$) and unconjugated pOHMAMP ($r = 0.635$, $p < 0.0001$) concentrations; however, there was no significant relationship between NOREPH and MAMP concentrations (Figure 2).

Seventeen MAMP-positive and 19 AMP-positive meconium specimens had concentrations between 125 and 2,500 ng/g, and therefore, were quantifiable from both calibration curves A and B. The mean $\pm$ SD percent difference between concentrations calculated from the two curves was $8.2 \pm 4.5\%$ (range 0.0 – 15.6%) for MAMP and $3.2 \pm 3.9\%$ (range 0.0 – 16.6%) for AMP.

Discussion

The detection of MAMP and AMP in meconium as primary biomarkers of MAMP exposure is consistent with adult urinary excretion patterns. However, the relative proportion of AMP in meconium is higher than observed in adult urine, with AMP:MAMP ratios ranging from 2.4 – 67.2%. After controlled smoking and intravenous administration of *S*-(+)-MAMP hydrochloride, nearly half of the dose was excreted in urine as parent drug and approximately 7% was excreted as AMP.³⁰ Another study investigating excretion of MAMP and AMP after oral MAMP administration reported AMP to MAMP ratios ranging from 1 to 18%.³¹ In addition to MAMP and AMP, we described the distribution of minor MAMP metabolites, pOHMAMP, pOHAMP, and NOREPH in meconium. Of these newly identified analytes,

unconjugated pOHMAMP was most prevalent and abundant. Over 85% of specimens contained unconjugated pOHMAMP, ranging from 17.8 – 412.9 ng/g. On average, free pOHMAMP concentrations were 8.5% of MAMP concentrations and are consistent with adult urinary excretion results. In urine of individuals using unknown quantities of MAMP via several routes of administration, free pOHMAMP concentrations were less than 10% of MAMP concentrations.^{8, 32-34}

This is the first study investigating the distribution of minor MAMP metabolites, pOHMAMP, pOHAMP, and NOREPH in human meconium. It is possible that the metabolites investigated in this study, particularly the hydroxylated compounds, trigger positive responses in AMP/MAMP immunoassays, analogous to hydroxylated cocaine and cannabis metabolites. From the current data, we have determined that pOHMAMP, pOHAMP and NOREPH are present in meconium, but cannot conclude that inclusion of these analytes will increase confirmation rates for amphetamines immunoassay-positive specimens. However, it is possible that one or more of these minor metabolites might reflect MAMP exposure at a particular gestational age, predict poorer infant outcomes and/or child development, or are produced by different fetal metabolism. Further research is necessary to confirm if pOHMAMP, pOHAMP and/or NOREPH could identify additional MAMP-exposed neonates, perhaps those with amphetamines negative immunoassay tests.

Only one report of MAMP and AMP concentrations in human meconium is available. Pichini et al. analyzed eight previously identified MAMP-positive meconium specimens donated by the United States Drug Testing Laboratory to demonstrate the applicability of a validated LC/MS method.²⁸ Mean $\pm$ SD MAMP and AMP concentrations were 924 ± 317 ng/g (range 226 – 1,157 ng/g) and 538 ± 366 ng/g (range 38 – 1,019 ng/g), respectively. The results from our study encompass this range of concentrations, but one-quarter of our specimens contained greater than 2,000 ng/g MAMP. The high concentrations observed in our samples could be attributable to differences in maternal routes of administration and/or frequency and duration of MAMP use during pregnancy. Maternal drug use history was not available in either study to compare use between groups or correlate meconium concentrations to self-reported MAMP exposure.

The MDMA metabolite, HMMA, had not been identified previously in meconium. The “Meconium Project” in Europe reported one meconium specimen containing MDMA without other metabolites.³⁵ No HMMA-positive meconium specimen in this study also was MDMA-positive; however, all specimens but one had MAMP concentrations greater than 1,000 ng/g (range 136.4 – 8,258.7 ng/g). These data could indicate that the mother used both MAMP and MDMA during gestation or that HMMA is a metabolite of MAMP. Kraemer et al suggest HMMA as a possible metabolite of MAMP³⁶, though, to our knowledge, HMMA has not been detected in urine after controlled MAMP administration. Also, HMMA was reported as a metabolite of p-methoxymethamphetamine (PMMA) after administration to rats³⁷; again, no data exists demonstrating PMMA metabolism to HMMA in humans.

Maternal MAMP, pholedrine or PMMA exposure may produce pOHMAMP in meconium. Pholedrine is a therapeutic medication identified in blood, urine and liver of a fatal intoxication case.³⁸ Additionally, pOHMAMP was identified as a PMMA metabolite in rat urine³⁷, again with no available human data. Examining the entire metabolic profile in meconium may be helpful in determining exposure to a specific drug, in light of shared metabolites of several AMP-related precursors. Moreover, co-ingestion of precursors cannot be excluded, as multiple AMP-related compounds are frequently found in illicit drug preparations. Unfortunately, maternal self-reported exposure histories are not available to provide additional information.

It should be noted that the present study may underestimate total pOHMAMP and pOHAMP deposited in meconium, as only unbound concentrations were determined. Metabolite profiles in urine of MAMP abusers indicate extensive pOHMAMP and pOHAMP glucuronidation and pOHMAMP sulfation.^{8, 32} Additionally, pOHAMP glucuronide is the primary metabolite of AMP in rat bile.³⁹ Xenobiotic compounds and metabolites accumulate in meconium through bile excretion following fetal liver metabolism and by swallowing drug-contaminated amniotic fluid, which in later stages of pregnancy is mostly fetal urine.⁴⁰ Since hydrolysis was not performed during specimen preparation, pOHMAMP and pOHAMP concentrations reflect only the presence of unbound analytes. Possibly, hydrolysis of meconium samples with an enzyme capable of glucuronide and sulfate cleavage would result in increased pOHMAMP and pOHAMP concentrations and improved detection of pOHAMP.

Another limitation of the current study is the potential influence of long-term frozen storage on quantitative results. Meconium specimens were stored for at least one year, in accordance with United States Drug Testing Laboratory's policies, before transfer and analysis by our laboratory. MAMP and AMP demonstrate excellent stability at frozen conditions after 1 year²⁹, however, the long-term stability of pOHMAMP, pOHAMP, and NOREPH is unknown. All analytes showed good stability after 3 freeze/thaw cycles during method development with values within 14% of freshly extracted samples.²⁹ *Ex vivo* addition or removal of a hydroxyl group would not be expected in meconium during storage, but additional testing should be conducted to assess long-term stability.

These new meconium biomarkers of methamphetamine exposure provide additional tools for studying the *in utero* effects of methamphetamine exposure and might improve identification of affected neonates. Further research is required to determine if concentrations of sympathomimetic amines in meconium correlate with the degree of MAMP or AMP exposure, and more importantly, if these concentrations predict maternal and/or infant outcome measures. Research with specimens collected during the IDEAL study will focus on elucidating whether relationships exist between meconium concentrations and the frequency, amount, duration and route of maternal MAMP use. Since many of the women self-reporting MAMP use also consumed other drugs, including tobacco, alcohol and cannabis,³ the effect of polydrug exposure on neonatal outcomes will be examined. Understanding these relationships will be useful in prioritizing medical resources for the child and mother, guiding future research of *in utero* MAMP exposure, and shaping substance abuse policies within the obstetric population.

Conclusion

We present disposition data for MAMP and 4 metabolites in human meconium. MAMP, AMP, and pOHMAMP are good biomarkers of *in utero* MAMP exposure in meconium, being both highly abundant and prevalent. pOHAMP and NOREPH were present in lower concentrations in few specimens. Low concentration minor metabolites and high concentration parent compounds were simultaneously quantified in 1 g meconium specimens from two calibration curves generated from different extract injection volumes. Further research is necessary to determine if pOHMAMP, pOHAMP and NOREPH can improve identification of MAMP-exposed neonates, and whether these analytes predict neonatal outcomes or exposure during specific gestational stages.

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Figure 1. Metabolic pathways of methamphetamine; Abbreviations: AMP – amphetamine; MAMP – methamphetamine; NOREPH – norephedrine; pOHMAMP – *p*-hydroxymethamphetamine; pOHAMP – *p*-hydroxyamphetamine

Figure 2. Concentration correlations between a) methamphetamine and amphetamine ($r = 0.807$, $p < 0.0001$), b) methamphetamine and unconjugated *p*-hydroxymethamphetamine ($r = 0.635$, $p < 0.0001$), c) and methamphetamine and norephedrine ($r = -0.020$, $p = 0.954$).

Table 1

Disposition of methamphetamine, metabolites and related sympathomimetic amines and metabolite ratios in human meconium following prenatal methamphetamine exposure. Abbreviations: AMP – amphetamine; HMMA – 4-hydroxy-3-methoxymethamphetamine; LOQ – limit of quantification; MAMP – methamphetamine; NOREPH – norephedrine; pOHMAMP – *p*-hydroxymethamphetamine

	N > LOQ	%	Median	Range
MAMP	43	100.0	1,341 ng/g	141.3 – 8,259 ng/g
AMP	43	100.0	222.0 ng/g	12.6 – 1,837 ng/g
pOHMAMP (free drug)	37	86.0	76.8 ng/g	17.8 - 412.9 ng/g
NOREPH	11	25.6	24.7 ng/g	15.9 - 38.7 ng/g
HMMA	8	18.6	4.2 ng/g	3.2 – 5.1 ng/g
AMP : MAMP	43	-	0.226	0.024 – 0.672
pOHMAMP : MAMP	37	-	0.050	0.006 – 0.549
NOREPH : MAMP	11	-	0.011	0.003 – 0.180