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Determination of amphetamine and methamphetamine in umbilical cord using liquid chromatography–tandem mass spectrometry

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ABSTRACT

The use of meconium as a drug-screening matrix for newborns has been the gold standard of care for the past two decades. A recent study using matched pairs of meconium and umbilical cord demonstrated a high degree of agreement. The use of liquid chromatography–tandem mass spectrometry as a means to confirm amphetamines presumptive positive umbilical cord specimens for amphetamine and methamphetamine is described here for the first time. The limit of detection for both compounds was 0.2 ng/g. The limit of quantitation for both compounds was 0.6 ng/g. The assay was linear for both compounds up to 100 ng/g.

1. Introduction

A liquid chromatography–tandem mass spectrometry (LCMSMS) method for the detection of amphetamine (AMP) and methamphetamine (MAMP) in umbilical cord (UC) is described for the first time. AMP and MAMP are central nervous system stimulants. Use of methamphetamine by pregnant mothers increases the risk of premature delivery and placental abruption [1].

Because of its lengthy window of detection and relative ease of collection, meconium, the first fecal material passed by a newborn, has been the testing matrix of choice for identifying newborns that have been exposed to drugs and alcohol *in utero* for the past two decades [2–5]. Meconium testing has two distinct disadvantages. First, some newborns may not pass their meconium for several days, therefore increasing undesirable turn-around-time and cost. Secondly, 10–15% of newborns pass their meconium in utero because of fetal stress [6]. As one cause of fetal stress is exposure to drugs and alcohol in the womb, identification of drug and alcohol exposure for this group is of great importance [7,8].

UC, formed from fetal origins during the first 5 weeks of gestation, is a tether protecting the vessels that connect the fetus to the placenta [9–11]. UC has several distinct advantages over meconium as a specimen for drug testing newborns [3,12]. UC is available for testing immediately after birth. The specimen is in route to the lab while the newborn is passing meconium. All newborns have sufficient UC for testing, while the most prevalent reason for meconium specimen rejection is due to insufficient quantity of specimen. UC collection has a single step procedure, whereas meconium collection may have up to 6–7 cumulative collections by multiple collectors and with multiple donors in the near vicinity. Because the UC collection procedure has a single donor and a single collector present the integrity of the specimen's chain of custody is greatly improved.

The detection of cocaine and metabolites in UC has been previously described in the literature [13,14]. In 2003, the detection of drug metabolite in UC was used to provide evidence of a mother's drug history during pregnancy [15]. The interpretation that the detection of benzoylecgonine in UC was proof of cocaine use by the mother during pregnancy was upheld on appeal to the South Carolina Supreme Court [16]. The detection of buprenorphine and metabolites was recently reported in UC [12]. A recently published study indicated that amphetamines immunoassay testing performed on UC demonstrated excellent agreement with matched meconium pairs [17]. The study demonstrated a 96.6% agreement between UC and meconium for the amphetamines drug class.

A positive UC test may ultimately lead to intervention by social services, which could include litigation, forced rehabilitation and/or loss of parental rights. Due to the severity of the consequences, a reliable confirmation method that exhibits a high degree of specificity, such as LCMSMS, is required for UC to be considered as an adequate alternative matrix for newborn drug screening.

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2. Experimental

2.1. Chemicals and materials

AMP, MAMP, AMP-d_{11}, MAMP-d_{14} and analytes for the interference study were purchased from Cerilliant (Austin, TX, USA) as 1.0 mg/mL ampules. Stock standards (100 μg/mL) were prepared by appropriate dilution with methanol. All solvents were HPLC grade and reagents were ACS grade from Fisher (Hanover Park, IL, USA). Clean Screen ZSDAU020, 10 mL, 200 mg bed, mixed mode, solid phase extraction columns were purchased from United Chemical Technologies (Bristol, PA, USA).

2.2. Calibrator, control and internal standard spiking solutions

The calibrator spiking solution (20 ng/mL, AMP and MAMP) was prepared by appropriate dilution of AMP and MAMP stock standards with methanol. Using different lots of AMP and MAMP stock standards, the control spike solution (20 ng/mL, AMP and MAMP) was prepared by the appropriate dilution of AMP and MAMP stock standards with methanol. The Internal Standard spiking solution was prepared at 20 ng/mL by the appropriate dilution with methanol of the AMP-d_{11} and MAMP-d_{14} stock standards.

2.3. Specimens

Over a 15-month period (August 2006 through October 2007), 707 UC were collected at McKay-Dee Hospital Center (Ogden, UT, USA), Logan Regional Hospital (Logan, UT, USA), LDS Hospital (Salt Lake City, UT, USA) and the University Hospital, University of Medicine and Dentistry of New Jersey (Newark, NJ, USA). The respective hospital's institutional review board approved the collection protocol and Western Institutional Review Board (WIRB) approved the study protocol. The UC were de-identified and shipped to the laboratory for analysis.

2.4. Equipment

Chromatography was performed using an Agilent 1100 high pressure liquid chromatography (HPLC) system comprised of a G1312A binary pump, a G1310A isocratic pump, a G1322 online vacuum degasser, a G1329A autosampler, a G1330B autosampler thermostat and a G1316A heated column compartment (Wilmington, DE, USA). The detector for the system was an Applied Biosystems MDS Sciex 3200 Q-Trap LC/MS/MS System with an electro-spray ionization (ESI) source (Toronto, ON, Canada). Homogenates were prepared using a ProScientic Pro250 homogenizer fitted with a 20-mm probe (Oxford, CT, USA). Specimens were centrifuged using a Fisher Scientific Centrific 225 fitted with a 4-position swinging bucket rotor (Hanover Park, IL, USA). Solid phase extractions were performed using a 20 place Varian VacElute Extraction Manifold (Harbor City, CA, USA). Extracts were evaporated under a stream of nitrogen using a Zymark TurboVap LV II (Hopkinton, MA, USA).

2.5. Calibrator and control preparation

The single point calibrator (1.0 ng/g), was prepared by the addition of 50 μL of calibrator spiking solution to a 1.0 g aliquot of the certified negative pool in a 50-mL screw topped polypropylene conical tube. Four controls were prepared by adding 0 μL (negative), 25 μL (0.5 ng/g), 50 μL (1.0 ng/g), and 500 μL (10 ng/g) to four 1.0 g aliquots of the certified negative pool in 50-mL screw topped polypropylene conical tubes. To each calibrator and control, 50 μL (1.0 ng/g) of internal standard solution and 5.5 mL of acetonitrile was added. Each sample was homogenized until uniform and centrifuged at 580 × *g* for approximately 5 min. For each calibrator and control, the supernatant was decanted into a 13 × 100-culture tube, 50 μL of 10% succinic acid dissolved in acetone was added and the supernatant was evaporated to dryness under a stream of nitrogen at 40 °C in the TurboVap LV II. To each residue, 3 mL of 0.1 M phosphate buffer (pH 6) was added and subjected to the solid phase procedure.

2.6. Sample preparation

Using umbilical scissors and tweezers, 0.1–1.0 g of UC was accurately weighed and placed in a 50-mL screw topped polypropylene conical tube. Between each specimen the scissors and tweezers were rinsed in deionized water and isopropanol to prevent carryover. To each specimen, 50 μL of internal standard solution (1.0 ng/g) and 5.5 mL of acetonitrile was added. Each sample was homogenized until uniform and centrifuged at 580 × *g* for approximately 5 min. For each specimen, the supernatant was decanted into a 13 × 100-culture tube, 50 μL of 10% succinic acid dissolved in acetone was added and was evaporated to dryness under a stream of nitrogen at 40 °C in the TurboVap LV II. To each residue, 3 mL of 0.1 M phosphate buffer (pH 6) was added and subjected to solid phase extraction.

2.7. Solid phase extraction (SPE)

The SPE columns were conditioned on the VacElut by passing through each column 3 mL of methanol, 3 mL of deionized water and 3 mL of 0.1 M phosphate buffer (pH 6) without allowing the column bed to go dry between each step. The calibrator, controls and specimens were loaded into the columns and allowed to flow freely under the force of gravity. The columns were rinsed with 3 mL of deionized water, 1 mL of 1.0 M acetic acid and 3 mL of methanol. The columns were allowed to dry for 5 min while drawing air through the manifold using high vacuum. The analytes were eluted into labeled 13 × 100-culture tubes by passing 3 mL of methylene chloride/isopropanol/ammonium hydroxide (78/20/2) through the extraction columns. The extracts were evaporated under a stream of nitrogen at 40 °C in the TurboVap LV II after adding 50 μL of 10% succinic acid dissolved in acetone to each tube. The residue was reconstituted in 50 μL of 10 mM ammonium acetate/0.1% formic acid, vortexed, and transferred into a 2-mL vial fitted with a 300 μL conical glass insert.

2.8. High pressure liquid chromatography (HPLC)

Separation was achieved using a Phenomenex Synergi Hydro-RP (50 mm × 2.0 mm, 2.0 μm particle size) polar end capped C-18 column (Torrance, CA, USA) held at 40 °C. The solvent system was isocratic and consisted of 88% of A (10 mM ammonium acetate and 0.1% formic acid) and 12% of B (acetonitrile and 0.1% formic acid). The flow was 0.6 mL/min for 3.0 min.

2.9. Mass spectrometry

Electro-spray ionization (ESI) in the positive mode was used. The curtain and collision gas was nitrogen. The curtain gas was set to 40 psi, source temperature at 500 °C and the ion spray set at 4000 V. The parameters for each analyte were determined by the infusion of methanolic solutions using the onboard infusion pump. The determined mass transitions and voltage settings are listed in Table 1.

Table 1
LCMSMS detection settings for AMP and MAMP.

Analyte	Q1 → Q3 ions	Voltage settings					
		DP	FP	EP	CEP	CE	CXP
AMP-d_{11}	147 → 98	30	400	11	15	30	0.5
AMP[a]	136 → 91	40	400	11	5	25	0.5
AMP	136 → 65	20	400	11	5	50	0.5
MAMP-d_{14}	164 → 98	40	400	11	15	30	0.5
MAMP[a]	150 → 91	30	400	11	10	30	0.5
MAMP	150 → 65	25	400	11	55	60	6.0

DP = declustering potential; FP = focusing potential; EP = entrance potential; CEP = collision cell entrance potential; CE = collision cell energy; CXP = collision cell exit potential.
[a] Quantification ion.

2.10. Validation

The following parameters were evaluated: selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision, extraction efficiency, matrix effect, carryover potential, stability of extracts on the autosampler and stability of specimens during freeze–thaw conditions [18–20].

The effects of interfering compounds and the selectivity of the method was determined analyzing negative controls and controls at the LOQ spiked with a cocktail of 48 potentially interfering compounds. Six negative controls and six LOQ controls were spiked with cocktail of ephedrine, pseudoephedrine, phenylpropanolamine, phentermine, dihydrocodeine, ibuprofen, naproxen, ketoprofen, lidocaine, dextromethorphan, cocaine, cocaethylene, benzoylecgonine, norcocaine, codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, mono-acetylmorphone, phencyclidine, Δ9-tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC, amobarbital, butalbital, pentobarbital, secobarbital, phenobarbital, diazepam, nordiazepam, oxazepam, temazepam, α-hydroxyalprazolam, alprazolam, midazolam, methadone, EDDP, meperidine, normeperidine, tramadol, fentanyl, norfentanyl, sufentanil, norsufentanil, alfentanil, ketamine and norketamine to yield a theoretical concentration of 500 ng/g of potentially interfering compounds. The effects of interfering compounds was considered to be acceptable if the blanks quantitated less than the LOD and the selectivity of the assay was considered acceptable if the fortified LOQ controls were properly identified and quantitated within 20% of the theoretical concentration [18].

The LOD and LOQ were determined by analyzing a series of fortified controls in triplicate with decreasing concentrations. The concentrations assessed were 1.0, 0.6, 0.4, and 0.2 ng/g. The LOQ was considered the lowest consecutive concentration where all three replicates met all identification requirements, the quantitation was within 20% of the theoretical concentration and the precision of the replicates was less than 20% [18]. The LOD was the lowest concentration that met all identification requirements [18].

Linearity was evaluated by analyzing a series of fortified negative UC aliquots in triplicate. The means of the triplicates were calculated, a least-squares fit determined and the r^2 calculated. The concentrations evaluated were 0.4, 1.0, 2.0, 10.0, 20.0, 40.0 and 100.0 ng/g. The linearity was considered to be acceptable within a range where the coefficient of determination (r^2) was greater than 0.998 and each mean was within 15% of the theoretical concentration with the exception of the LOQ, which was allowed to be within 20% of target concentration [18].

The accuracy and precision was determined by analyzing a series of fortified negative UC aliquots, replicates of five, over three concentrations on 4 different days. The means, standard deviations, coefficient of variations (%CV) and percent of target concentration (Target %) were calculated for each run and over all four batches. The concentrations evaluated were 1.0, 10.0 and 40.0 ng/g. The intra-assay accuracy and inter-assay accuracy for each concentration is the Target % for each batch and over the four batches, respectively. Accuracy determinations between 85% and 115% were considered acceptable [18]. The intra-assay precision and inter-assay precision for each concentration is the %CV for each batch and over the four batches, respectively. Precision determinations less than 15% were considered acceptable [18].

The extraction efficiency and matrix effect were evaluated for each analyte using a procedure defined by Matuszewski et al. [19]. Three sets of controls were prepared over four concentrations with five replicates each. The first set was unextracted controls reconstituted in mobile phase A. The second set was negative UC extracts fortified with calibrator spiking solution after being subjected to the extraction procedure. The third set was negative UC controls fortified with calibrator spiking solution that were subjected to the extraction procedure. The extraction efficiency for each analyte is expressed as the ratio of the average peak area in set 3 to set 2. The matrix effect for each analyte is defined as the ratio of the mean peak area of set 2 to set 1.

The potential for carryover was evaluated by analyzing a negative UC fortified with internal standard immediately after a UC control fortified at 500 ng/g AMP and MAMP. The potential for carryover at 500 ng/g was considered negligible if the negative UC quantitated less than the LOQ [20].

The stability of the method was evaluated for extracts on the autosampler and specimens under freeze–thaw conditions. To examine the stability of the extracts on the autosampler, a set of controls was re-injected after remaining on the autosampler for 48 h at 15 °C. The result was reported as the ratio of the stability challenge injections to the original result for each analyte. Freeze–thaw cycle stability was evaluated by preparing a set of five replicates of low (0.5 ng/g) and high controls (10.0 ng/g). The set of controls was kept in a freezer at −20 °C for 16 h and then allowed to thaw at room temperature for 8 h. After the third freeze–thaw cycle, a fresh set of low and high controls was prepared and both sets were subjected to the method. The stability was reported as a ratio of the mean of the freeze–thaw results to the mean of the fresh preparation results for each analyte [18].

2.11. Identification criteria

The identification criteria used for this procedure included four components: retention time, signal to noise, baseline resolution and relative ion intensity. The retention time of each analyte was required to be within 0.2 min of the calibrator. A signal to noise of greater than 3:1 was required of each ion chromatogram. A minimum of 90% return to baseline was required to consider a peak to be adequately resolved from a co-eluting peak. The relative ion intensity of the product ions for each analyte (mass ratio) was required to be within 20% of the corresponding relative ion intensity of the calibrator.

2.12. Application to real specimens

The method was applied to 707 authentic UC specimens received from 3 hospitals in Utah and 1 hospital in New Jersey. The specimens were also subjected to a previously established immunoassay screening procedure utilizing a cutoff of 5.0 ng/g [21]. A comparison of the two methods was achieved by calculating the sensitivity, specificity and negative predictive value.

3. Results and discussion

The parameters and transitions determined for the mass spectrometry were consistent with previously published studies

Table 2
Intra- and inter-assay accuracy and precision of AMP and MAMP.

Compound	Target concentration (ng/g)	Intra-assay ($n=5$)		Inter-assay ($n=20$)	
		Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
AMP	1	92.4–104.4	4.0–6.8	97.6	6.4
	10	97.5–108.2	2.3–4.1	102.7	5.6
	40	95.5–109.6	1.2–4.5	101.4	6.1
MAMP	1	95.4–106.4	3.8–7.2	100.7	6.2
	10	88.8–96.1	1.0–4.4	91.6	4.1
	40	90.0–94.7	1.4–2.6	92.3	2.6

[22–28]. The precursor ion for each compound was the protonated molecular weight ion 136, 147, 150 and 164 m/z for AMP, AMP-d_{11}, MAMP and MAMP-d_{14}, respectively. Both analytes formed tropylium cations (91 m/z), which are very stable due to resonance stabilization. The second most abundant product ion observed was the 2° carbocation, 1-phenylpropan-2-ylium (119 m/z) [22,29]. The mass transitions selected proved clean and stable during the duration of the validation.

The LOD and LOQ for both analytes were 0.2 and 0.6 ng/g, respectively. The method allowed for the proper identification of AMP and MAMP at the 0.2 and 0.4 ng/g concentration but the quantitations were outside the required 20% range. AMP and MAMP passed identification criteria and quantitation criteria at the 0.6 ng/g concentration with mean concentrations of 0.51 and 0.53 ng/g and %CV's of 1.1% and 6.0%, respectively.

Triplicate analysis of negative UC fortified at 0.6, 1.0, 2.0, 10.0, 20.0, 40.0, and 100.0 ng/g yielded acceptable linearity using a least-squares fit. The determination coefficients (r^2) were 0.9999 and 0.9996 for AMP and MAMP, respectively. The mean of each triplicate was within 15% of target value except for the LOQ (0.6 ng/g), which was within 20% of target value.

The accuracy and precision of the method proved to be acceptable. The results are listed in Table 2. All intra- and inter-assay accuracy determinations were within 11.2% of target concentration. All intra- and inter-assay precision calculations were less than 7.2%.

Negative controls spiked with 48 potentially interfering compounds did not exhibit any detectable AMP or MAMP at or above the reported LOD. The selectivity of the method proved to be adequate by successful analysis of six LOQ controls prepared from negative UC that were spiked with 48 potentially interfering compounds.

AMP and MAMP were not detected in a negative control analyzed immediately following a control fortified with 500 ng/g of AMP and MAMP. The potential for carryover at 500 ng/g of AMP and MAMP was acceptable.

Re-injection of low and high controls after incubating 48 h at 15 °C on the autosampler did not demonstrate any obvious degradation. All quantitations were within 15% of original analysis. Excessive degradation was not observed in the freeze–thaw experiment. The freeze–thaw stability challenge yielded ratios for amphetamine of 103.1% and 108.6% for the low and high controls, respectively. The freeze–thaw study yielded ratios for methamphetamine of 88.0% and 102.8% for the low and high controls, respectively.

Table 3
Matrix effect of umbilical cord in amphetamines detection.

Analyte concentration (ng/g)	AMP (%)	MAMP (%)	AMP-d_{11} (%)	MAMP-d_{14} (%)
1	73.4	58.3	74.0	37.0
2	72.8	55.3	72.2	36.1
10	83.3	64.8	84.6	40.5
40	83.0	73.1	83.2	40.2

Table 4
Extraction efficiency of amphetamines in umbilical cord.

Analyte concentration (ng/g)	AMP (%)	MAMP (%)	AMP-d_{11} (%)	MAMP-d_{14} (%)
1	63.6	63.6	64.2	65.1
2	55.4	59.0	57.3	61.9
10	84.7	82.0	87.6	87.8
40	59.3	66.3	56.3	64.6

The extraction efficiency and matrix effect was determined over 4 concentrations using replicates of 5. Extraction efficiencies ranged from 55.4% to 87.8%. Significant matrix effect was observed with ranges of 72.2–84.6% for AMP/AMP-d_{11} and 36.1–73.1% for MAMP/MAMP-d_{14}. However, the matrix effect for each analyte was consistent over the 4 concentrations tested. The results are listed in Tables 3 and 4.

Table 5
LCMSMS results for AMP and/or MAMP positive umbilical cord specimens.

Subject	AMP (ng/g)	MAMP (ng/g)
A	150.82	755.74
B	49.62	564.15
C	45.30	443.94
D	76.18	425.45
E	118.33	416.67
F	63.81	402.38
G	43.00	385.00
H	87.00	342.00
HI	38.00	280.00
J	93.10	274.14
K	42.00	272.00
L	48.89	253.70
M	60.39	236.36
N	34.00	210.00
O	59.39	207.58
P	97.17	149.57
Q	36.00	139.00
R	12.54	124.07
S	42.86	121.07
T	32.60	113.40
U	37.83	84.13
V	31.18	64.56
W	15.72	63.96
X	20.00	50.18
Y	20.42	43.94
Z	16.00	36.00
AA	9.44	34.92
BB	9.00	26.00
CC	34.00	21.00
DD	28.97	18.97
EE	11.48	9.61
FF	Detected	8.48
GG	11.70	8.46
HH	7.16	8.02
II	7.07	7.72
JJ	2.92	5.03
KK	2.24	4.59
LL	29.84	2.14

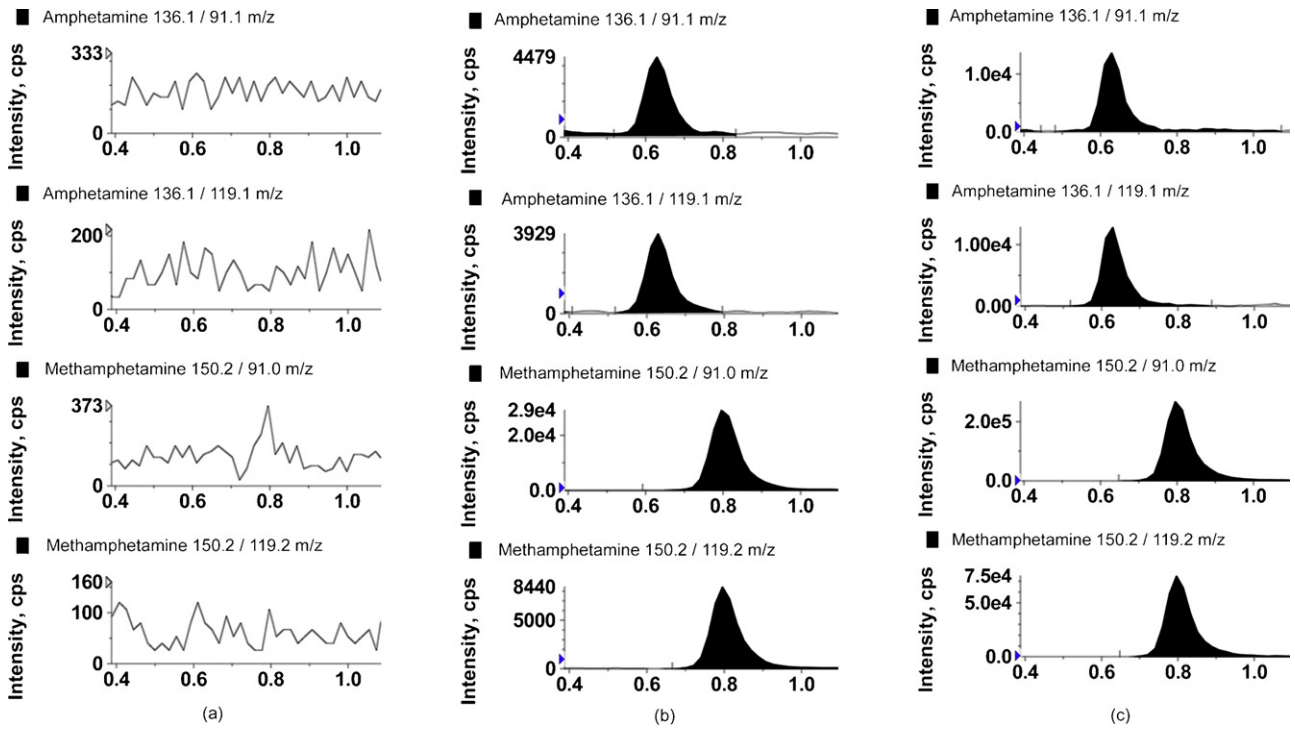

Fig. 1. Extraction ion chromatograms of (a) a negative umbilical cord specimen, (b) a negative umbilical cord specimen fortified with 1.0 ng/g of AMP and MAMP, and (c) an authentic positive umbilical cord specimen containing 48 ng/g AMP and 253 ng/g MAMP.

3.1. Application to real specimens

Using the LOQ as the cutoff for this method and the previously established cutoff of 5.0 ng/g for the immunoassay screening procedure, 38 specimens were positive by both methods and 647 were negative by both methods. This method found seven specimens that contained detectable AMP and/or MAMP but were under the immunoassay cutoff. Fifteen specimens were above the immunoassay cutoff but did not contain detectable AMP or MAMP. The calculated sensitivity was 84.4% and specificity was 97.7%. The negative predictive value was 98.9%. The confirmed positive results are listed in Table 5. Extracted ion chromatograms for a negative UC, a low control and an authentic positive specimen are given in Fig. 1.

4. Conclusion

A simple, sensitive and specific method was validated for the simultaneous quantitation of AMP and MAMP in human UC. This method was applied to 707 authentic specimens with excellent sensitivity and specificity. This method will be used to confirm immunoassay presumptive positive UC.

The use of UC to detect newborn drug exposure to AMP and MAMP is described here for the first time. UC is the superior matrix for the purpose of newborn drug screening with inherent improvements in turn-around-time, chain of custody integrity and specimen availability. The UC is available for collection immediately after birth, therefore eliminating the frequent long delay waiting for a sufficient amount of specimen to void. When the UC is collected, there is only one donor and one collector in the vicinity performing a single collection under chain of custody, therefore vastly reducing the possibility of specimen switching. UC is available in sufficient quantity for each and every birth, eliminating unfortunate situations of having no or too little specimen to analyze. UC provides all of the previous advantages while maintaining a high degree of agreement with matched meconium paired specimens.

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