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Development and validation of a liquid chromatography mass spectrometry assay for the simultaneous quantification of methadone, cocaine, opiates and metabolites in human umbilical cord

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ABSTRACT

A liquid chromatography mass spectrometric selected reaction monitoring mode (SRM) method for methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), cocaine, benzoylecgonine (BE), 6-acetylmorphine, morphine and codeine quantification in human umbilical cord was developed and fully validated. Analytes were extracted from homogenized tissue (1 g) by solid phase extraction. Linearity was 2.5–500 ng/g, except for methadone (10–2000 ng/g). Method imprecision was <12.7%CV with analytical recovery 85.9–112.7%, extraction efficiency >59.2%, matrix effect 4.5–39.5%, process efficiency 48.6–92.6% and stability >84.6%. Analysis of an umbilical cord following controlled methadone administration and illicit drug use contained in ng/g, 40.3 morphine, 3.6 codeine, 442 BE, 186 methadone and 45.9 EDDP.

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1. Introduction

Illicit drug use during pregnancy is a major public health concern. In the 2007 National Survey on Drug Use and Health, an average of 5.2% of pregnant women 15–44 years old reported illicit drug use in the prior month [1].

Pregnant opiate-exposed women are more likely to experience serious obstetric complications, including spontaneous abortion, premature labor, abruption placenta, placenta insufficiency, preeclampsia or gestational diabetes [2,3]. *In utero* drug exposure also may impact fetal and neonatal development including decreased birth weight, body length and head circumference, fetal distress or neurodevelopment complications [4–7]. There also may be long term consequences for the child [3,8].

Methadone is the only pharmacotherapy approved for the treatment of opioid-dependent pregnant women in the US, and is associated with decrease in illicit drug use, better prenatal care and improved birth outcomes [9–14]. However, methadone pharmacotherapy is not without controversy because of the risk of neonatal abstinence syndrome (NAS) [15–17].

Detecting drug exposure during pregnancy is the most effective way to improve pre- and post-natal care in the mother and newborn, as it permits implementation of adequate treatment and follow-up. However, many pregnant women avoid prenatal care due to feelings of guilt about illicit drug use, and fear of legal consequences if their drug use is identified. Furthermore, although self-report surveys on drug consumption during pregnancy have been improved with new interviewing techniques [18], underreporting is still a concern [19]. Maternal or neonatal urine screening has traditionally been the primary analytical technique for detecting drug use during pregnancy [20,21] because of ease of specimen collection, reduced cost, and lack of sensitive and accurate analytical methodologies for alternative specimens [22]. The main disadvantage of urine testing is the short window of detection [18,23], identifying drug exposure only a few days before delivery.

Meconium, the first neonatal fecal matter, begins to form between the 12th and 16th weeks of gestation, offering the possibility of detection of *in utero* drug exposure from as early as the second trimester of pregnancy [24–26]. For this reason, meconium is currently considered the specimen of choice for detecting drug exposure in neonates. However, recent evidence from our laboratory monitoring pregnant opioid and cocaine dependent women suggests that *in utero* drug exposure is identified much more readily in the third as compared to second trimester of gestation [27,28]. Additional limitations associated with this matrix include delays of up to 5 days in meconium expulsion, especially in premature

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neonates, and in cases of fetal distress, meconium may be passed before birth, making collection impossible.

Umbilical cord is abundant, considered a waste product after delivery, and available at the time of birth. Umbilical cord also is more suitable for anonymous epidemiological studies. Montgomery et al. suggested umbilical cord as an alternative to meconium for detecting fetal drug exposure after demonstrating that comparable results were obtained in these two alternative matrices [29]. This research was later extended to screening and GCMS confirmation of 500 umbilical cords from pregnant women suspected of illicit drug consumption. Negative and positive predictive values were all >98% and >70%, respectively, for methamphetamine, cocaine, opiates, cannabinoids and phencyclidine, supporting the efficacy of this alternative specimen for a rapid diagnosis of fetal drug exposure [30]. Despite these promising results, the window of drug detection in umbilical cord is unknown, demonstrating the need for additional research.

From a quantitative point of view, only Winecker et al. described a validated GCMS method for the determination of cocaine and its main metabolites in umbilical cord tissue and amniotic fluid [31]. This method was applied to authentic specimens from subjects admitting cocaine consumption during pregnancy; however, it was not clearly indicated whether the specimens were matched from the same woman. Also, the time of last drug consumption was unknown, making it impossible to establish the window of drug detection in either matrix, although detection is likely to be shorter in umbilical cord than amniotic fluid. Moore et al. also reported the determination of benzoylecgonine (BE) and cocaine in umbilical cord from an authentic premature delivery case, although no validation data for the method were described [32].

The aim of the present paper was development of a liquid chromatography mass spectrometry (LCMS) analytical method for the quantification of methadone, cocaine, opiates, and metabolites in umbilical cord tissue. The method is needed to support our research on opioid and cocaine-dependent pregnant women enrolled in methadone maintenance treatment programs.

2. Experimental

2.1. Reagents and standards

Methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), BE, morphine, codeine (1 mg/mL in methanol), cocaine and 6-acetylmorphine (6AM) (1 mg/mL in acetonitrile) standards, and methadone-d_9, EDDP-d_3, cocaine-d_3, BE-d_8, morphine-d_6, codeine-d_6 and 6AM-d_6 internal standards (IStd) (0.1 mg/mL, in the same solvent as non-deuterated analogues) were obtained from Cerilliant™ (Round Rock, TX, USA). Methadone, cocaine and BE for quality control samples (QC) were purchased from Lipomed (Cambridge, MA, USA); morphine, codeine, and 6AM QC samples were different lot numbers from Cerilliant, and for EDDP, a different vial from the same Cerilliant™ lot number was employed. Reagent grade formic and perchloric acids were from Sigma-Chemicals (St. Louis, MO, USA). Dichloromethane, acetonitrile, ammonium hydroxide were obtained from J.T. Baker (Phillipsburg, NJ, USA). Strata™ XC cartridges 3 mL 60 mg were purchased from Phenomenex (Torrance, CA, USA). Anonymous blank umbilical cord specimens were kindly supplied by the Department of Pathology, Johns Hopkins Bayview Medical Center. Blank specimens were tested to confirm the absence of analytes prior to preparation of calibrators and QC.

2.2. Instrumentation

Mass spectrometric analyses were performed on a Thermo Finnigan LCQ Deca XP ion trap mass spectrometer with an electrospray ionization source (ESI) interfaced with a Surveyor autosampler and liquid chromatographic (LC) pump (Thermo Electron, San Jose, CA). A Tissue Tearor™ homogenizer (BioSpec Products Inc., Bartlesville, OK) was employed for umbilical cord homogenization. Solvent evaporation was carried out on a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA).

2.3. Preparation of standard solutions

To generate a seven-point calibration curve, mixed working solutions at 0.2, 0.4, 0.8, 4, 10 and 20 μg/mL for methadone and 0.05, 0.1, 0.2, 1, 2.5 and 5 μg/mL for the other analytes were prepared by dilution of individual vials (1 mg/mL) in methanol. For preparing the three QC concentrations, separate working solutions were generated at 0.4, 4 and 20 μg/mL for methadone, and at 7.5, 75 and 375 μg/mL for the other analytes. Deuterated analogues of IStd for each compound were combined and diluted with methanol to a final concentration of 2 μg/mL for methadone-d_9, and 0.5 μg/mL for other analytes.

2.4. Procedures

2.4.1. Specimen preparation

1 ± 0.025 g umbilical cord was placed in a 15 mL polypropylene centrifuge tube and homogenized with 5 mL 0.1% perchloric acid for 1–2 min. Blank homogenate was fortified with appropriate volumes of calibrator or QC solution and mixed. 50 μL IStd mixture was added to each tube and vortexed. Specimens were centrifuged at 4000 rpm for 15 min and supernatant was subjected to solid phase extraction.

2.4.2. Solid phase extraction

Mixed mode cartridges (reversed phase and cation exchange mechanisms) were conditioned with 2 mL each methanol, water and 0.1%, v/v perchloric acid. Samples were applied to cartridges, and two washing steps were performed with 2 mL 0.1% perchloric acid and 2 mL methanol. After drying cartridges under vacuum for 15 min, samples were eluted with a mixture of dichloromethane:acetonitrile:ammonium hydroxide (45:50:5, v/v/v). Eluates were evaporated under nitrogen until dry, reconstituted in 100 μL 0.1% formic acid and 20 μL injected onto the LCMS.

2.4.3. Liquid chromatography

Separation of analytes was achieved with a Synergi Polar-RP column (75 mm × 2 mm, 4 μm, Phenomenex®, Torrance, CA, USA) attached to a guard column of the same packing material. Column and autosampler temperature were maintained at 30 and 10 °C, respectively. Gradient elution with 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.2 mL/min was as follows: 0% B for 0.5 min; increased to 85% over 9.5 min; decreased to 0% B over 1 min and re-equilibrated for 3.5 min. A divert valve directed the LC flow initially to waste for 0.2 min, subsequently to the mass spectrometer for 11.8 min, and then back to waste for the remaining chromatographic run.

2.4.4. Mass spectrometry

Mass spectral data were obtained in positive electrospray mode (ESI+), with the following parameters: spray voltage, 4 kV; sheath gas flow rate setting, 50; auxiliary gas flow rate setting, 10; and transfer capillary temperature, 300 °C. Ion transitions for each analyte were optimized by direct infusion of individual solutions (0.1 μg/mL in methanol) into the mass spectrometer. Detection was performed by selected reaction monitoring (SRM) of three MS^2 or MS^3 transitions for each analyte, except for EDDP, for which only two transitions were available.

2.5. Validation

Selectivity, sensitivity, linearity, intra- and inter-assay imprecision and analytical recovery, extraction efficiency, matrix effect, process efficiency, drug carry over, hydrolysis, dilution integrity and analyte stability were evaluated.

Selectivity was assessed by measuring endogenous and exogenous interferences. Potential endogenous interferences were determined by the analysis of blank umbilical cord specimens from 10 different sources. For assessment of exogenous interferences, licit and illicit compounds were added at a concentration of 1 μg/mL to blank specimens fortified at the low QC. The following drugs and metabolites were tested: buprenorphine, norbuprenorphine, THC, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, chlorpheniramine, brompheniramine, salicylic acid, acetaminophen, PCP, nicotine, diazepam, lorazepam, oxazepam, alprazolam, bromazepam, clonazepam, flurazepam, nitrazepam, flunitrazepam, temazepam, nordiazepam, imipramine, clomipramine, fluoxetine, norfluoxetine, paroxetine, 7-aminoclonazepam, 7-aminoflunitrazepam, and 7-aminonitrazepam. Umbilical cord specimens from five opioid-dependent pregnant women on buprenorphine treatment also were analyzed.

Sensitivity of the method was examined by establishing limits of detection (LOD) and limits of quantification (LOQ). LOD was defined as the lowest concentration with retention times within ±0.2 min from the average of all calibrator concentrations, appropriate chromatography, and a signal-to-noise ratio of at least three for all selected transitions. LOQ was the lowest concentration that could be quantified with acceptable imprecision (%CV ≤20%) and analytical recovery (%target concentration ±20%). LOD and LOQ were determined empirically by analyzing umbilical cord samples fortified at decreasing concentrations.

Linearity was determined using least-squared regression with 1/× weighting to compensate for heteroscedasticity. Calibration curves were generated with calibrators at 10, 20, 40, 200, 500, 1000 and 2000 ng/g for methadone, and at 2.5, 5, 10, 50, 125, 250 and 500 ng/g for the other analytes. Low, medium and high QC concentrations were prepared at 30, 300 and 1500 ng/g for methadone, and 7.5, 75 and 375 ng/g for other analytes.

Intra-assay imprecision and analytical recoveries were assessed with five replicates at low, medium and high QC concentrations in the same batch. Inter-assay imprecision and analytical recoveries were examined with 20 replicates at three concentrations on 4 different days. Imprecision was determined by calculating the coefficient of variation (CV) using one-way analysis of variance following Krouwer and Rabinowitz recommendations [33,34]. Analytical recovery was expressed as a percentage of target concentration.

Extraction efficiency, matrix effect and process efficiency were calculated at three QC concentrations. Five blank samples were fortified with IStd and the appropriate QC solution before and another five blank samples fortified after extraction. Extraction efficiency was calculated by dividing mean analyte peak area of blank samples fortified before extraction by mean analyte peak area of samples fortified after extraction, and expressed as a percentage. Absolute matrix effect compared analyte peak areas of samples fortified after SPE with peak areas of analytes in neat mobile phase. Ten unique blank umbilical cords were fortified at each QC concentration after SPE. Mean peak analyte areas after SPE were compared to mean peak areas of the analyte in 0.1% formic acid ($n = 10$). Relative matrix effect is the different matrix effects observed in multiple specimens, and was determined by calculating %CV of the analyte peak areas in 10 different umbilical cords tested. Process efficiency examines overall effect of extraction recovery and matrix effect, and was calculated by dividing mean analyte peak areas of samples fortified prior to extraction

Table 1
Selected mass spectrometric quantifier and qualifier transitions, retention times and imprecision (%CV) after 40 injections for morphine, codeine, 6-acetylmorphine (6AM), benzoylecgonine (BE), cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and methadone.

Compound	Quantifier transition (CE)[a]	Qualifier transitions (CE)[a]	Rt[b] (min)	CV Rt[b] (%, $n = 40$)
Morphine	286.2 > 201.1 (37)	286.2 > 229.0 (37) 286.2 > 268.1 (37)	4.54	0.51
Morphine-d6	292.3 > 201.1 (37)	292.3 > 229.0 (37)	4.52	0.47
Codeine	300.2 > 215.1 (37)	300.2 > 243.0 (37) 300.2 > 282.2 (37)	5.40	0.30
Codeine-d6	306.2 > 218.2 (37)	306.2 > 246.0 (37)	5.38	0.28
6AM	328.2 > 211.1 (38)	328.2 > 268.1 (38) 328.2 > 193.1 (38)	5.68	0.30
6AM-d6	334.1 > 211.1 (38)	334.1 > 271.2 (38)	5.67	0.34
BE	290.1 > 168.1 (29)	290.1 > 168.1 > 150.0 (32) 290.1 > 168.1 > 82.1 (32)	6.11	0.24
BE-d8	298.2 > 171.1 (29)	298.2 > 171.1 > 153.1 (33)	6.09	0.22
Cocaine	304.1 > 182.1 (30)	304.1 > 182.1 > 150.1 (32) 304.1 > 182.1 > 82.1 (32)	6.91	0.30
Cocaine-d3	307.1 > 185.1 (30)	307.1 > 185.1 > 153.1 (35)	6.90	0.32
EDDP	278.2 > 249.1 (40)	278.2 > 249.1 > 234.2 (34)	8.55	0.78
EDDP-d3	281.3 > 249.1 (40)	281.3 > 249.1 > 234.2 (35)	8.54	0.78
Methadone	310.1 > 265.0 (27)	310.1 > 265.0 > 247.1 (31) 310.1 > 265.0 > 219.1 (31)	8.67	0.69
Methadone-d9	319.2 > 268.1 (27)	319.2 > 268.1 > 250.1 (31)	8.63	0.74

[a] CE: collision energy (V).
[b] Rt: retention time.

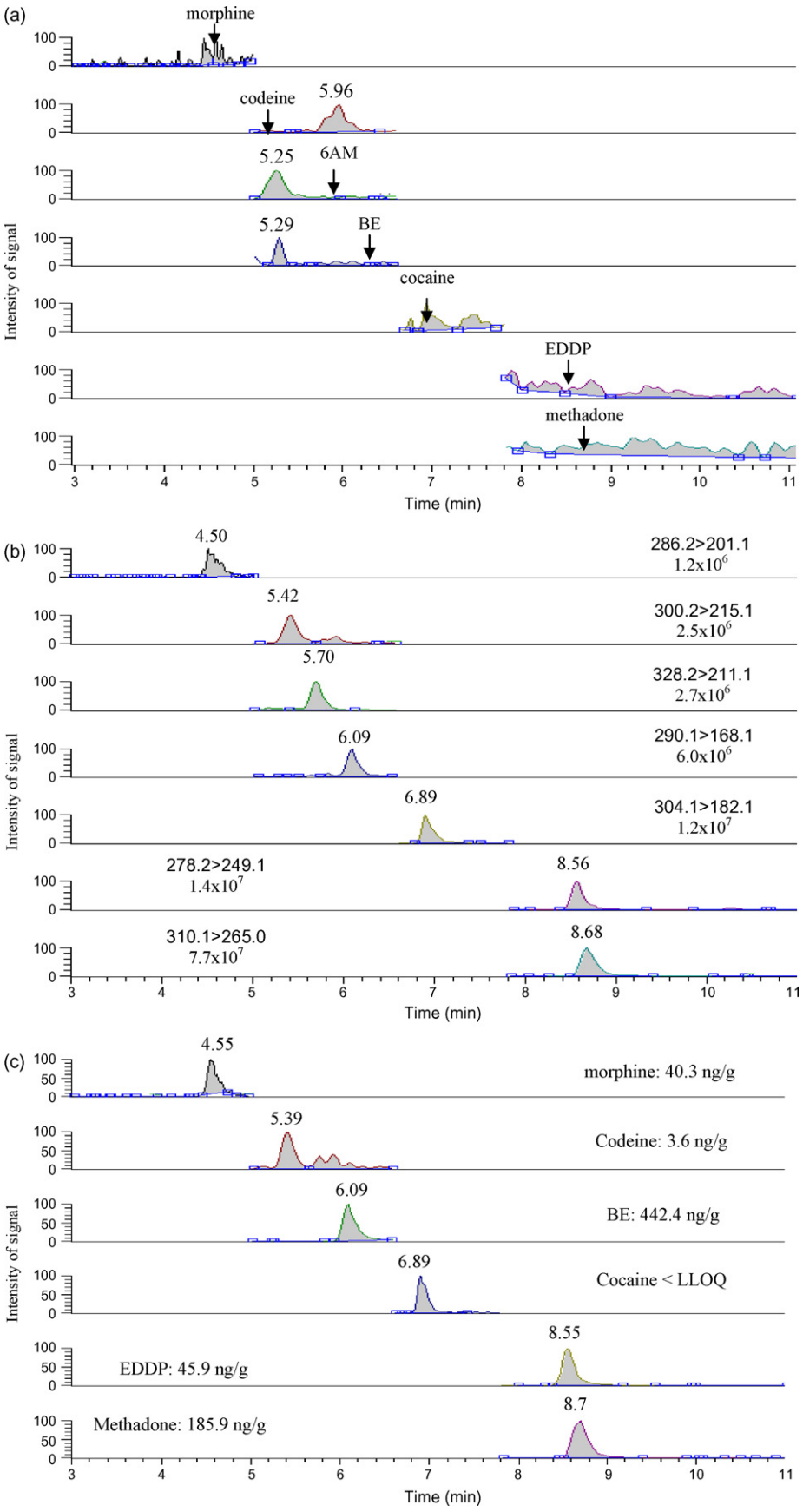

Fig. 1. Selected reaction monitoring chromatograms of the quantifier transitions for morphine, codeine, 6-acetylmorphine (6AM), benzoylecgonine (BE), cocaine, methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) of (a) extracted blank umbilical cord, (b) umbilical cord sample fortified with all analytes at the limits of quantification (10 ng/g for methadone and 2.5 ng/g for other analytes), and (c) an authentic umbilical cord specimen containing in ng/g morphine 40.3, codeine 3.6, BE 442.4,

Table 2
Imprecision, analytical recovery and calibration curve parameters for morphine, codeine, 6-acetylmorphine (6AM), benzoylecgonine (BE), cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and methadone.

Compound	QC (ng/g)	Imprecision[a]			Analytical recovery[b]	
		Intra-assay ($n=5$)	Inter-assay ($n=20$)	Total	Intra-assay ($n=5$)	Inter-assay ($n=20$)
	7.5	10.7	0.0	10.7	92.2	97.2
Morphine	75	7.0	0.0	7.0	94.7	95.9
	375	6.2	5.1	8.0	91.2	95.1
Calibration curve ($n=4$): slope = 0.0525 ± 0.0016; intercept = 0.0878 ± 0.0859; $r^2 = 0.9971 \pm 0.0021$						
	7.5	11.4	5.4	12.7	109.6	99.1
Codeine	75	10.1	0.0	7.0	94.6	97.9
	375	11.1	0.0	11.1	94.9	100.2
Calibration curve ($n=4$): slope = 0.0222 ± 0.0005; intercept = -0.0112 ± 0.0080; $r^2 = 0.9933 \pm 0.0015$						
	7.5	10.8	5.8	12.3	110.5	99.5
6AM	75	9.9	0.0	9.9	91.6	94.8
	375	8.7	4.8	9.9	87.7	94.7
Calibration curve ($n=4$): slope = 0.0223 ± 0.0003; intercept = 0.0013 ± 0.0023; $r^2 = 0.9973 \pm 0.0004$						
	7.5	9.7	0.0	9.7	89.3	92.4
BE	75	6.8	7.8	10.4	85.9	94.2
	375	6.1	9.0	10.9	86.4	94.2
Calibration curve ($n=4$): slope = 0.0225 ± 0.0016; intercept = 0.0019 ± 0.0107; $r^2 = 0.9959 \pm 0.0027$						
	7.5	4.2	0.0	4.2	109.3	107.0
Cocaine	75	6.4	3.1	7.1	108.9	104.7
	375	4.0	8.6	9.5	109.5	104.6
Calibration curve ($n=4$): slope = 0.0201 ± 0.0008; intercept = -0.0001 ± 0.0054; $r^2 = 0.9966 \pm 0.0025$						
	7.5	9.1	0.0	9.1	94.5	98.4
EDDP	75	5.2	2.3	5.6	112.7	109.4
	375	9.2	4.8	10.3	108.1	103.2
Calibration curve ($n=4$): slope = 0.0198 ± 0.0007; intercept = 0.0392 ± 0.0125; $r^2 = 0.9962 \pm 0.0023$						
	30	6.8	2.6	7.3	109.7	106.9
Methadone	750	9.7	7.5	12.3	89.2	100.5
	1500	6.9	9.1	11.4	88.7	97.9
Calibration curve ($n=4$): slope = 0.0204 ± 0.0011; intercept = -0.0005 ± 0.0078; $r^2 = 0.9965 \pm 0.0019$						

[a] Imprecision is expressed as %CV.
[b] Analytical recovery is expressed as % of target concentration.

($n=5$) by mean peak areas of analytes prepared in 0.1% formic acid ($n=5$).

The presence of carry over was assessed by analysis of a blank umbilical cord sample fortified only with the IStd after the injection of a sample at a concentration two times the upper LOQ.

Hydrolysis of cocaine and 6AM during sample preparation was examined by analysis of blank umbilical cord samples fortified with only these two analytes at the high QC ($n=3$), and calculating the percentage of BE and morphine formed.

For evaluation of dilution integrity, umbilical cord samples were fortified at a concentration two times the upper LOQ ($n=3$), and subsequently diluted with blank matrix using a 1:4 dilution factor. The concentrations of diluted analytes $\times 4$ should be within $\pm 20\%$ of target to demonstrate dilution integrity.

Analyte stability was investigated at the three QC levels under a variety of conditions. Prepared specimen stability was evaluated by re-injecting QC samples ($n=5$) after 24 and 72 h of autosampler storage at 10 °C, and comparing calculated concentrations to original values. For stability after 24 h room temperature storage, 72 h at 4 °C, and after three freeze/thaw cycles, IStd was added to fortified umbilical cord samples ($n=5$) after being subjected to the described conditions. Stability samples were analyzed with freshly prepared calibrators and QC samples.

2.6. Method application

The method was applied to the analysis of an umbilical cord specimen from an opioid-dependent pregnant woman enrolled in methadone maintenance treatment. The specimen was collected as part of an Institutional Review Board approved protocol, and the participant provided written informed consent.

3. Results and discussion

We present the first validated LCMS method for the simultaneous quantification of methadone, EDDP, cocaine, BE, 6AM, morphine and codeine in human umbilical cord.

Umbilical cord analysis required a homogenization step to disrupt the tissue, followed by an efficient extraction methodology. A rapid homogenization of 1 g umbilical cord for 1–2 min was performed in 5 mL 0.1% perchloric acid. Following centrifugation, a clear supernatant was achieved that did not obstruct the solid phase extraction cartridge. Analytes eluted from the chromatographic column within 10 min, with a total run time of 15 min. CV in retention times after 40 consecutive injections was <0.78%.

Quantification was based on SRM of the most prominent MS^2 fragment for all analytes. Two additional MS^2 or MS^3 fragments were monitored for identification of each analyte, except for EDDP, for which only one MS^3 fragment was available. However, in all cases the number of identification points was >5, fulfilling the acceptance criteria of at least three identification points [35]. Table 1 shows selected MS transitions for identifying and quantifying analytes, retention times and CV of retention times for each analyte and deuterated analogs.

No quantifiable peaks were found in umbilical cord specimens from 10 different women, documenting specificity from endoge-

EDDP 45.9, and methadone 185.9. The umbilical cord was obtained from a methadone-maintained pregnant woman at delivery who received 75 mg daily methadone. Arrows in (a) indicate the actual retention time of analytes of interest to distinguish from potential interferences in the blank specimen.

Table 3
Extraction efficiency ($n = 5$), matrix effect ($n = 10$) and process efficiency ($n = 5$) in umbilical cord for morphine, codeine, 6-acetylmorphine (6AM), benzoylecgonine (BE), cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and methadone. %CV in matrix effect in 10 different umbilical cords is also shown.

Compound	QC (ng/g)	Matrix effect ($n = 10$)		Extraction efficiency	Process efficiency	Compound	Matrix effect ($n = 10$)		Extraction efficiency	Process efficiency
		Absolute	Relative				Absolute	Relative		
	7.5	−14.4	8.3	111.0	88.6		−13.5	9.3	78.7	68.3
Morphine	75	−14.3	6.0	78.0	64.5	Morphine-d6	−4.6	9.7	75.3	69.9
	375	−5.2	6.2	76.2	73.1		−4.2	7.5	72.9	70.4
	7.5	−22.8	17.6	84.5	64.4		−16.7	10.0	80.9	68.4
Codeine	75	−9.1	9.6	71.3	60.7	Codeine-d6	−13.5	12.3	74.3	63.8
	375	−9.6	11.9	80.3	63.5		−16.7	9.3	75.2	62.4
	7.5	−28.5	15.8	75.5	52.6		−26.7	11.8	74.8	55.8
6AM	75	−18.2	16.1	62.4	48.6	6AM-d6	−18.4	11.7	65.0	51.9
	375	−24.9	22.6	76.4	55.6		−23.3	11.3	80.5	57.1
	7.5	−24.7	9.4	82.0	60.9		−22.6	8.6	79.2	55.7
BE	75	−17.5	11.1	93.0	71.2	BE-d8	−19.4	9.0	81.8	62.9
	375	−13.9	10.7	85.3	72.7		−5.2	7.9	77.9	74.8
	7.5	−18.6	6.9	79.7	61.5		−17.4	5.2	76.6	60.0
Cocaine	75	−11.1	5.6	80.1	70.9	Cocaine-d3	−9.3	9.8	82.5	70.1
	375	−11.8	4.5	87.8	76.1		−10.2	6.0	87.7	80.1
	7.5	39.5	8.5	60.9	85.2		29.5	14.4	58.4	82.0
EDDP	75	15.3	9.5	59.2	70.8	EDDP-d3	10.0	8.9	57.6	60.8
	375	13.1	8.9	66.9	78.7		10.8	13.9	69.7	71.8
	30	4.5	10.2	85.0	82.7		8.2	9.6	79.9	86.7
Methadone	750	−4.9	8.8	83.1	79.0	Methadone-d9	7.7	10.9	78.7	85.5
	1500	3.8	6.2	90.8	92.6		9.4	8.2	94.8	97.5

nous matrix components. In addition, specimens fortified with analytes of interest at low QC concentrations and common licit and illicit drugs at 1000 ng/g, quantified within 85–115% of target with a CV <15% for all analytes. Furthermore, there was no interference from buprenorphine or metabolites, as specimens from opioid-dependent women receiving buprenorphine pharmacological treatment were negative for analytes of interest. Fig. 1a represents the extracted ion chromatograms obtained after the injection of a blank specimen.

Linear calibration curves were obtained with mean coefficients of determination $r^2 > 0.99$ ($n = 4$). Calibrators residuals were <15%, except for LOQ, for which residuals <20% were acceptable. Table 2 includes calibration parameters for all analytes. LOD's were 0.25 ng/g for EDDP, 0.5 ng/g for cocaine and BE, 1 ng/g for 6AM, 2 ng/g for methadone and 2.5 ng/g for morphine and codeine. LOD and LOQ were the same concentration for morphine and codeine, based on stated acceptance criteria. Acceptable peak shape was obtained for all analytes at the LOQ, as shown in Fig. 1b.

Imprecision was calculated following Krouwer and Rabinowitz recommendations [33,34]. These authors differentiate three kinds of imprecision: intra-day, inter-day and total. It is necessary to subtract intra-assay imprecision from the standard deviation of the

Table 4
Stability of morphine, codeine, 6-acetylmorphine (6AM), benzoylecgonine (BE), cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methadone in umbilical cord after storage on the autosampler (10 °C) for 24 and 72 h, at room temperature for 24 h, 4 °C for 72 h and three freeze/thaw cycles, expressed as % loss compared to fresh controls.

Compound	QC (ng/g)	Autosampler (10 °C)		Room temperature 24 h	Fridge 72 h (4 °C)	Three freeze/thaw cycles
		24 h	72 h			
	7.5	5.4	0.5	−2.7	7.7	5.6
Morphine	75	4.4	7.1	−6.5	−3.1	5.9
	375	−7.3	−2.6	−2.7	−3.8	10.4
	7.5	0.6	−1.8	10.4	−1.2	−3.0
Codeine	75	10.9	1.2	−1.5	11.4	0.0
	375	−3.7	−11.4	2.9	−0.9	−1.9
	7.5	11.9	11.3	−11.2	−5.2	−6.2
6AM	75	−1.3	5.7	−5.1	−0.9	6.8
	375	−6.4	1.6	2.4	−11.2	14.0
	7.5	6.3	5.4	−7.5	0.2	1.9
BE	75	−1.9	2.0	3.0	13.1	5.2
	375	−6.1	5.9	−9.3	3.0	−2.7
	7.5	−1.2	−1.1	10.7	−6.8	−4.9
Cocaine	75	−3.3	8.3	−1.0	−1.8	−5.4
	375	0.1	6.2	−6.9	−3.1	−9.7
	7.5	5.9	−1.2	4.3	−4.8	0.7
EDDP	75	−3.5	−4.7	0.6	−12.6	−8.1
	375	−4.0	−10.3	4.4	−15.4	−0.7
	30	3.8	−2.2	8.2	−5.4	−6.8
Methadone	750	−5.1	−8.8	1.8	5.3	1.9
	1500	−2.4	−1.6	−4.2	0.1	12.7

run averages to obtain a pure inter-assay component of imprecision. Using the proposed equations, it is possible to obtain inter-day imprecision values equal to zero when the mean square inter-assay imprecision is lower than the mean square intra-assay imprecision [34]. Table 2 summarizes results for the imprecision and analytical recoveries at low, medium and high QC concentrations. Intra- and inter-assay and total imprecision ranged from 4.0% to 11.4%, 0.0% to 9.1% and 4.2% to 12.7%, respectively. Intra- and inter-assay analytical recoveries ranged from 85.9% to 112.7% of target and 92.4% to 109.4% of target, respectively.

Extraction efficiency was >75% for most analytes, except for 6AM and EDDP, with values >59.2%, at the three different QC concentrations. Matrix effect experiments showed ion suppression of signal ranging from 4.2% to 28.9% for most analytes, except for EDDP, for which a 39.5% signal enhancement was observed at the low QC concentration. These values were similar to those obtained for their respective deuterated IStd, with differences <20% in all cases. Employing deuterated IStd compensated for the variability in extraction efficiency and matrix effect for all analytes at the different concentrations tested (Table 3). Process efficiency ranged from 48.6% to 97.5%, similar to extraction efficiency, due to the low matrix effect found for most analytes.

No drug carryover above the LOD was found in an IStd-fortified blank specimen injected after the analysis of an umbilical cord sample fortified at double the upper LOQ.

Minimal hydrolysis of cocaine and 6AM was observed during specimen treatment when analyzing specimens containing only these two analytes at the high QC concentration. Cocaine and 6AM were quantified with imprecision <5.2% and analytical recovery >85.8%, and the percentage formation of BE and morphine were 0.19% and 1.87%, respectively.

Stability in the autosampler for 24 and 72 h was demonstrated by re-injected concentrations of 0.1–11.4% of original values. Stability of the analytes in umbilical cord tissue after 24 h at room temperature, 72 h at 4 °C and three freeze/thaw cycles also was verified with % analyte loss <15.4% in all cases (Table 4).

The validated method was employed to analyze an umbilical cord specimen from an opioid-dependent pregnant woman receiving daily 75 mg methadone at the time of delivery. Methadone and EDDP concentrations were 190.2 and 43.6 ng/g, respectively. This method also permitted simultaneous analysis of exposure to other common drugs of abuse. This specimen also contained BE (449.8 ng/g), morphine (40.1 ng/g) and codeine (3.0 ng/g). Extracted ion chromatograms for analytes are displayed in Fig. 1c. These preliminary results corroborate the utility of this alternative matrix for identifying fetal drug exposure.

4. Conclusion

The first method for the simultaneous determination of methadone, EDDP, cocaine, BE, 6AM, morphine and codeine in umbilical cord was developed and fully validated. Homogenization of the specimen in acidic conditions and subsequent extraction with mixed mode cation exchange cartridges provided adequate sensitivity and selectivity for quantification of analytes of interest. Simultaneous analysis of methadone, cocaine and opiate analytes provided considerable time and cost savings over three separate analyses for each drug class. In general, umbilical cord tissue is not limited, thus, specimen volume is not an issue, and simultaneous analysis does not provide an advantage in this respect.

This method will be applied to the analysis of umbilical cord specimens from opioid-dependent women under pharmacological treatment with methadone, or women suspected of cocaine or opiate abuse. Quantification of methadone and EDDP in umbilical cord specimens from women receiving known methadone doses permits determination of potential correlations between drug concentrations in umbilical cord and administered doses, and whether umbilical cord drug concentrations predict maternal and neonatal outcomes. Determination of other common drugs of abuse will help us to understand the usefulness of umbilical cord analysis for identifying *in utero* drug exposure.

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