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Journal of Chromatography B, 814 (2005) 369–373
JOURNAL OF CHROMATOGRAPHY B
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Determination of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, 2-ethyl-5-methyl-3,3-diphenylpyraline and methadol in meconium by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry

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Received 12 August 2004; accepted 29 October 2004
Available online 8 December 2004

Abstract

This paper details a validated liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI–MS/MS) method for the quantification of methadone, and its metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) and methadol in human meconium. Limits of detection (LOD) were determined to be 1.0 ng/g for methadone, EDDP and EMDP and 2.5 ng/g for methadol. The limits of quantitation (LOQ) for methadone, EDDP, EMDP were 5 and 25 ng/g for methadol. Linearity ranged from 5.0 to 500 ng/g. Following solid-phase extraction, no matrix effect was observed. This method proved to be suitable for the quantification of methadone, EDDP and EMDP and the semi-quantitation of methadol in meconium. Literature review revealed no other published LC–APCI–MS/MS method for the detection of methadone and its three main metabolites in meconium specimens.
Published by Elsevier B.V.

Keywords: Methadone; Meconium; LC–APCI–MS/MS

1. Introduction

Sensitive and specific methods are needed to accurately determine the concentration of drugs and metabolites in infant meconium, which in turn, may be correlated to infant outcomes. Recent reports describe the value of various maternal and fetal matrices for the monitoring of prenatal drug exposure [1–5].

Liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods exist for the measurements of methadone and its primary metabolites in oral fluid, urine, plasma and sweat [1,6–8]. Some methods also have achieved high pg/ml sensitivity when quantifying the R and S enantiomers of methadone and EDDP [7–11]. However, meconium, a much more complex matrix due to the presence of bile acids and other excretory products, poses a difficult analytical challenge.

Meconium begins to form between the 12th and 16th weeks of gestation and acts as a reservoir for exogenous and endogenous compounds until birth [12]. Previous researchers have investigated the usefulness of meconium as a matrix for monitoring drug exposure. Meconium offers advantages in detecting drug exposure during the prenatal period due to the ease and non-invasiveness of collection and its long window of drug detection [13].

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1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2004.10.068

High concentrations of morphine, cocaine, cannabinoids and methadone and their metabolites have been reported in meconium [2,3,13–20]. Methadone and its primary metabolite, EDDP, have been quantified in meconium by liquid chromatography–photo diode array (LC–PDA) [21] and gas chromatography–mass spectrometry (GC–MS) [2,3,21]. Stolk et al. [21] reported a linear range of 460–3680 ng/g and 1000–6000 ng/g for methadone and EDDP, respectively, with LC–PDA. The LODs of this method were 99 ng/g for methadone and 113 ng/g for EDDP, respectively. The GC–MS method demonstrated LOQs of 25 ng/g for methadone, EDDP and EMDP with a linear range from 25 to 2000 ng/g [3].

In this report, we describe the first application of LC–APCI–MS/MS for the simultaneous quantification of methadone, EDDP, EMDP and methadol in meconium after methanolic extraction followed by solid-phase extraction (SPE).

2. Experimental

2.1. Chemicals

(±)Methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP), 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP), (−)-alpha-methadol HCl, (±)methadone-D9 and EDDP-D3 perchlorate were purchased from Cerilliant™ (Austin, TX, USA). All standards were >99.9% pure, as described by the manufacture and verified within our laboratory. Reagent grade ammonium formate and formic acid were obtained from Sigma Chemical Co. (Milwaukee, WI, USA). All other solvents were of HPLC grade or better. Certified methadone and illicit drug free meconium was purchased from ElSohly Laboratories (Oxford, MS, USA) and verified as negative within our laboratory.

2.2. Specimen collection

Meconium specimens, containing methadone and metabolites, were obtained from infants born to methadone-maintained mothers participating in a study at the Center for Addiction and Pregnancy (CAP) in Baltimore Maryland. Meconium specimens were obtained within the first 3 days of birth and stored at −20°C until the time of analysis. The Johns Hopkins Bayview Medical Center (JHBMC) and NIDA Institutional Review Board (IRB) reviewed the study and written informed consent was obtained from all participants. Mothers were compensated for their participation; however, no compensation was given for infant specimens.

2.3. Instrumentation

Sample preparation utilized an ultrasonic disrupter (Tekmar, Cincinnati, OH, USA) and Eppendorf® Refrigerated Multipurpose Centrifuge, Model 5804R, (Brinkmann, Westbury, NY, USA). Samples were dried under nitrogen using a TurboVap® LV (Zymark, Hopkinton, MA, USA).

An LCQ Deca XP Ion Trap Mass Spectrometer, equipped with an orthogonal APCI source, was interfaced to a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA). Data acquisition was carried out using Xcalibur™ Software, version 1.2. The analytical column was a Phenomenex (Torrance, CA, USA) Synergi Hydro-RP 80A (50 mm × 2.0 mm, 4 μm), fitted with a C_{18} ODS Octadecyle (4.0 mm × 2.0 mm) guard column, also obtained from Phenomenex. The column oven was maintained at 30 °C and the autosampler tray at 15°C. Optimal separation of the analytes of interest was accomplished by gradient elution, with mobile phase consisting of (A) 10 mM ammonium formate in water with 0.001% formic acid (pH 4.5) and (B) acetonitrile, at a flow rate of 300 μL/min. The initial gradient conditions were 40% B for 2 min, increasing to 90% over 7 min, and maintenance at this concentration for an additional 2 min. The column was then re-equilibrated for 6 min for a total run time of 17 min. HPLC flow was directed to the mass spectrometer from 1 to 12 min; during the remaining time, flow was diverted to waste.

Mass spectrometry data were collected in positive ion mode, with the following APCI–MS parameters: corona discharge needle voltage, 4.5 kV; vaporizer temperature, 450 °C; sheath gas setting (high purity nitrogen), 70; no auxiliary gas was utilized; and transfer capillary temperature, 220 °C.

Identification and quantification of the analytes were based on selected reaction monitoring (SRM). Precursor and product ions were established by direct infusion of individual analytes at a concentration of 5 μg/mL.

2.4. Calibration standards, internal standards and quality control samples

Stock standard solutions (0.1 mg/mL) of all native analytes were prepared in methanol. Working solutions were prepared by diluting stock solutions in water yielding a final working concentration range of 20–10,000 ng/mL for the calibrators. Internal standards (IS), methadone-D_9 and EDDP-D_3 were prepared from the stock in water for final working concentrations of 200 ng/mL. Methadone-D_9 was the IS for methadone, EMDP and methadol and EDDP-D_3 was the IS for EDDP. Quality control samples were prepared at 10, 40 and 400 ng/g using certified methadone negative meconium. Quality control samples were prepared from vials obtained from the same vendor and lot number as the working solutions but prepared on different days from different vials.

Calibration curves were constructed by spiking 25 μL of working calibrator solution, containing each analyte of interest into 0.5 g meconium. There were nine calibration points over a concentration range of 1.0–500 ng/g (1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 125.0, 250.0 and 500.0 ng/g).

Xcalibur™ LCQuan software version 1.2. was utilized to calculate the linear regression. Parent and transition ions were monitored for methadone (310.9; 265.3), EDDP (278.0; 249.2), EMDP (264.3; 235.2) and methadol (312.3; 223.0, 171.2, 105.2). Peak-area ratios of target analytes were calculated.

2.5. Sample preparation

Approximately 0.5 g of meconium was transferred to a polypropylene centrifuge tube, 25 μL of internal standard was added and tubes were vortexed for approximately 30 s. Two milliliters of methanol was added followed by ultrasonic disruption at 60 amps, 60% duty cycle for 1 min, while on ice. Samples were placed in an ultrasonic bath at 24 °C for 30 min to fully homogenize the specimen. Specimens were centrifuged at 6831 × *g* for 10 min. The supernatant was decanted into a clean glass tube. Solvent volume was reduced under N_2 at 37 °C to approximately 0.5 mL using a TurboVap® LV. In preparation for SPE, samples were reconstituted in 4 mL of 2 N sodium acetate buffer (pH 4.0).

Solid-phase extraction was performed by a modification of the ElSohly method [22]. Briefly, reconstituted extracts were applied to preconditioned mixed mode solid-phase extraction columns with 200 mg of stationary phase (Clean Screen ZSDAU020, United Chemical Technologies Inc., Bristol, PA, USA). Columns were washed successively with 2 mL deionized water, 1.5 mL of 0.1 N HCl and 2 mL of methanol and dried under vacuum for 3 min. Analytes of interest were eluted with 4 mL of methylene chloride/isopropanol/ammonium hydroxide (78:20:2). The eluate was evaporated to dryness under nitrogen at 37 °C using a TurboVap® LV and reconstituted in 100 μL mobile phase "A". Forty microliters was injected onto the LC–MS/MS.

When the concentration of analytes in the clinical specimens exceeded the linear range, samples were diluted 1:100. Sample size was reduced from 0.5 to 0.05 g of meconium for a 1:10 dilution and further diluted 1:10 after liquid extraction by using only 200 μL of elute rather than 2 mL and diluting to 4 mL with 2 N sodium acetate buffer (pH 4.0). The dilution was carried out prior to the SPE. Dilution integrity was accessed at 500 ng/g for each analyte.

2.6. Method validation

The following criteria were used to evaluate the method: sensitivity, LOD, LOQ, linearity, specificity, imprecision, accuracy, recovery, carryover effect, stability and matrix effects. Method validation for the assay consisted of four runs on different days.

The LOD for each analyte was the lowest concentration yielding a signal-to-noise ratio of at least 3:1, with adequate peak shape, presence of all ions and a retention time within ±10%. The limit of quantification (LOQ) was defined as the lowest concentration with a signal-to-noise ratio of 10:1, in addition to the same criteria described above. The linearity of the method was investigated by calculation of the regression line by the method of least squares and expressed by the correlation coefficient (R^2). A 1/*x*-weighting factor was applied to compensate for heteroscedasticity. Precision and accuracy were determined over the linear dynamic range using three concentration levels (10, 40 and 400 ng/g). Imprecision (intra-day $n = 5$ and inter-day $n = 20$) was expressed as the relative standard deviation (R.S.D.). Accuracy of the method was calculated as the percent difference (%diff) from the target value. Carryover was assessed by injecting a blank specimen following a 500 ng/g calibrator.

Extraction efficiency was assessed at three (10, 40 and 400 ng/g) concentrations, with five replicates at each level. Standard/internal standard ratios were compared between samples in which internal standards were added before and after solid-phase extraction. The percent expected concentration (actual amount divided by the expected amount) of each analyte of interest was calculated.

Matrix effect was evaluated by injecting a blank pretreated meconium specimen into the LC with simultaneous post column infusion of the analyte of interest, methadone (50 ng/mL) [23]. The average of three replicates was determined. Stability, at a concentration of 125 ng/g of each analyte of interest, was evaluated over 24 h under different conditions (24, 4 and −20 °C). The conditions were applied to both fortified specimens and SPE extracts. Additionally, fortified meconium specimens were subjected to three freeze thaw cycles.

3. Results and discussion

Separation of the four analytes of interest and internal standards was achieved within 11 min (Table 1). Precursor and product ions and collision energy (*V*) for each analyte are described in Table 1. Due to the fragmentation of methadol, it was necessary to monitor three transition ions to increase sensitivity.

The LODs, LOQs and representative linearity results are detailed in Table 2. The LODs were found to be 1.0 for all analytes except methadol, which had an LOD of 2.5 ng/g. The linear dynamic ranges were 5.0–500 ng/g for Methadone, EDDP and EMDP and 25.0–500 ng/g for methadol with correlation coefficients of >0.99 (R^2, weighting factor, 1/*x*). The

Table 1
LC–APCI–MS/MS parameters for the quantification of methadone and metabolites in meconium

Analyte	*V*	Precursor ion	Product ion	Retention time
Methadone	40	310.9	265.3	7.2
Methadone-D9	40	319.9	268.3	7.1
EDDP	30	278.0	249.2	6.0
EDDP-D3	30	281.0	249.2	5.9
EMDP	30	264.3	235.2	10.5
Methadol	35	312.3	223; 171.2; 105.2	6.9

Table 2
Limits of detection (LODs), limits of quantitation (LOQs), and calibration results for methadone and metabolites in meconium by LC–APCI–MS/MS

Compound	Internal standard	LOD (ng/g)	LOQ (ng/g)	Equation	R^2
Methadone	MT-D9	1.0	5.0	$Y = -1.82517 + 0.14217X$	.999
EDDP	EDDP-D3	1.0	5.0	$Y = -0.04640 + 0.11012X$	.994
EMDP	MT-D9	1.0	5.0	$Y = 0.19755 + 0.01613X$	.992
Methadol	MT-D9	2.5	25.0	$Y = -0.00080 + 0.01054X$	.997

MT = methadone; RT = retention time.

Table 3
Imprecision and accuracy of methadone and metabolites in meconium as determined by LC–APCI–MS/MS

Analyte	Intra-day ($n = 5$)			Inter-day ($n = 20$)		
	Concentration (ng/g)	Mean (ng/g)	Imprecision (R.S.D.)	Mean (ng/g)	Imprecision (R.S.D.)	Accuracy (%expected)
Methadone	10	9.8	5.1	9.5	6.9	95.0
	40	40.8	4.4	43.8	12.6	109.5
	400	404.1	12.4	381.6	16.2	95.4
EDDP	10	10.2	4.7	9.4	13.8	94.0
	40	40.9	5.0	38.4	12.4	96.0
	400	349.7	2.8	377.1	9.1*	94.3
EMDP	10	10.9	13.7	10.8	15.2**	108.0
	40	38.1	6.7	46.0	14.7	115.0
	400	339.4	15.8	348.9	18.7	87.2
Methadol	10	ND	ND	ND	ND	ND
	40	36.3	2.8	37.3	14.0	93.3
	400	379.2	2.9	386.4	22.9	96.6

ND = not determined.
* $n = 15$.
** $n = 17$.

linear dynamic range covered two-orders of magnitude, while LOQs for methadone, EDDP and EMDP achieved a five-fold increase in sensitivity over previous results reported by GC–MS [3]. Additionally, LC–MS/MS permitted the semi-quantification of methadol, a metabolite previously not included in LC–PDA or GC–MS analysis.

Imprecision was evaluated over the linear dynamic range at three concentrations (10, 40 and 400 ng/g) for methadone, EDDP and EMDP. Due to the LOQ of 25 ng/g, imprecision for methadol was accessed at 40 and 400 ng/g. Intra-day imprecision was <20% for all analytes. Inter-day imprecision was <20% for methadone, EDDP, and EMDP, while for methadol imprecision was slightly higher at 23%. The increase in imprecision is likely due to the less efficient ionization of methadol. Accuracy of the method was based on percent difference from target value and was between 87 and 115% for all analytes at all concentrations (Table 3).

Extraction efficiency was >82% for methadone, >70% for EDDP, >94% for EMDP and >95% for methadol at all tested concentrations. All analytes were stable for 24 h at tested temperatures and were reduced by less than 32% by three freeze-thaw cycles except SPE extracted EMDP at −20 °C, 24 h which had a 48% loss (Table 4).

Post column infusion of methadone was used to determine the effect of sample matrix on ionization of the compound. It was determined that there was no significant suppression or enhancement of methadone with LC–APCI–MS/MS analysis due to the biological matrix.

This method is used in ongoing clinical studies for the analysis of methadone and metabolites in meconium. A representative meconium specimen from an infant whose mother was maintained on methadone for 19 weeks of gestation was evaluated with the new method and results are shown in Fig. 1b. The meconium contained 2492 ng/g of methadone, 13,188 ng/g of EDDP and 27.0 ng/g of EMDP. Fig. 1a rep-

Table 4
Stability of methadone and metabolites at a concentration of 125 ng/g (%found)

	Fortified meconium			SPE extracted		
	24 °C, 24 h	4 °C, 24 h	3 × Freeze/thaw	24 °C, 24 h	4 °C, 24 h	−20 °C, 24 h
Methadone	93.6	100.7	109.5	104.1	105.6	92.7
EDDP	111.7	89.5	78.6	118.2	82.2	113.0
EMDP	106.9	68.1	ND	100.3	82.4	52.1
Methadol	101.9	111.1	111.3	116.1	116.1	93.4

ND = not determined.

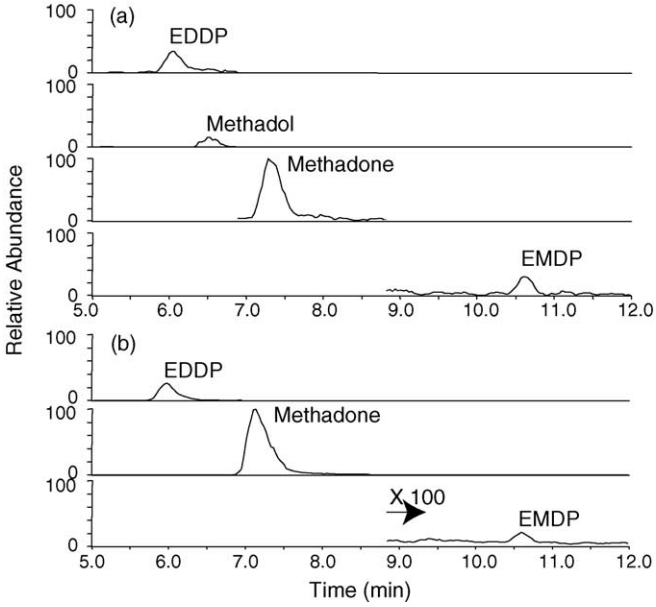

Fig. 1. (a) Chromatogram representative of LOQs at 10 ng/g for methadone, EDDP and EMDP and 40 ng/g for methadol. (b) Chromatogram of a representative meconium specimen from an infant whose mother was maintained on methadone for 19 weeks; calculated concentration of methadone (2492 ng/g), EDDP (13,188 ng/g) and EMDP (27.0 ng/g).

resents a blank chromatogram with the addition of internal standards.

4. Conclusion

Methanolic extraction followed by SPE in combination with LC–APCI–MS/MS detection offers sufficient sensitivity, selectivity and simultaneous quantification of methadone and its three primary metabolites, EDDP, EMDP and methadol in a complex meconium matrix. This LC–APCI–MS/MS method represents an advancement over previous methods in sensitivity and specificity for methadone and its metabolites, EDDP, EMDP and methadol in a complex meconium matrix. Methanolic extraction followed by solid-phase extraction offered sufficient clean up of the matrix with good recoveries.

This method has been shown to be useful for the quantification of methadone and metabolites in meconium from infants whose mothers were maintained on methadone during pregnancy. It is hoped that accurate and sensitive measurements of methadone and metabolites in meconium may help to elucidate the relationship between methadone concentrations and infant outcomes.

Acknowledgements

This study was supported by the National Institute on Drug Abuse Extramural Research Grant DA12403-02 and intramural research funds. We would like to acknowledge the staff at the Center for Addiction and Pregnancy, and Labor and Delivery and Post-partum ward at Johns Hopkins Bayview Medical Center for all of their assistance during the course of this study.

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