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Journal of Chromatography B, 856 (2007) 267–272
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Validation of a LC–APCI-MS/MS method for quantification of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) in infant plasma following protein precipitation

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Received 20 February 2007; accepted 12 June 2007
Available online 16 June 2007

Abstract

A validated, quantitative LC–APCI-MS/MS method for methadone, EDDP and EMDP in 200-μL plasma is presented. Specimen preparation was limited to protein precipitation and centrifugation. Chromatographic separation was achieved on a Synergi Hydro-RP 80A (50 mm × 2.0 mm, 4 μm) column with gradient elution. The assay was linear from 1 to 500 ng/mL, with intra- and inter-assay accuracy ≥87.5% and intra- and inter-assay precision <13.4% R.S.D. and recovery ≥87.5% for all analytes at 40 ng/mL. This analytical method is suitable for the accurate and precise determination of methadone and metabolites in human plasma specimens.
Published by Elsevier B.V.

Keywords: Methadone; Plasma; LC–APCI-MS/MS; EDDP; EMDP

1. Introduction

Methadone maintenance therapy is currently the standard of care for opiate dependency during pregnancy in the US and is associated with better compliance with obstetrical care, reduced risk of HIV infection and improved infant outcomes and parenting than in an untreated cohort [1–5]. The practice of lactation in this group frequently provokes controversy, principally due to a lack of clear guidelines for this population and limited information regarding the degree of exposure of infants to methadone and metabolites through human breast milk. Thus, there exists an important need to monitor the concentrations of these analytes in small plasma volumes of methadone-exposed infants.

Methadone is metabolized in the liver mainly by cytochrome P450 3A4, 2B6 and 2C19 [6–8] through *N*-demethylation, ring cyclization and conjugation to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) [8,9]. Quantification of methadone and metabolites in biological fluids requires a sensitive and specific analytical method. Various methods have been developed for quantification of methadone and metabolites by liquid chromatography-photo diode array (LC-PDA) [10], gas chromatography–mass spectrometry (GC–MS) [10–12] and LC/MS/MS [13–16]. Gas chromatography can achieve quantification of methadone and the metabolites; however, an extensive derivatization process was necessary for the specimen preparation [17]. Furthermore, the high temperature in gas chromatography injectors may produce decomposition of methadone to EDDP, creating an artifact [18].

The coupling of liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS/MS) provides a specific, selective and sensitive means for analyzing compounds with a wide polarity range in a broad spectrum of different biological fluids and tissues [14,19]. Although numerous LC/MS methods have been published, almost none were able to combine selectivity, sensitivity, quantification of methadone and its two metabolites (EDDP and EMDP), less specimen volume and shorter specimen processing

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1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.06.004

time [20–27]. Quintela et al. reported an LC/MS assay that measured concentrations of methadone and EDDP in plasma [20]. The assay was sensitive for methadone and EDDP, having limit of quantification of 2 ng/mL for both the analytes. However, the metabolite EMDP was not monitored in the assay. This method by Quintela et al. required a large specimen volume (0.4–0.6 mL) and labor-intensive solid phase extraction [20]. The effectiveness of using liquid/liquid [24] or solid phase extraction [23] for plasma specimen preparation, without protein precipitation prior to injection, has been demonstrated for methadone and EDDP; however, these methods did not address the other metabolite (EMDP), and lacked sufficient sensitivity. A protein precipitation step before on-line injection improved the limit of detection [28]. Based on several methadone extraction procedures, we selected the protein precipitation method published by Souverain et al. [28] with a slight modification on the volume of acetonitrile.

We present a rapid, specific and sensitive validated LC–APCI-MS/MS method for simultaneous quantification of methadone, EDDP and EMDP in small volume infant plasma specimens. The method should prove useful due to improved sensitivity and reduced specimen handling prior to LC/MS/MS as compared to existing analytical techniques.

2. Experimental

2.1. Materials

(±) Methadone, EDDP, EMDP, (±) methadone-D_9 and EDDP-D_3 were purchased from Cerilliant™ (Austin, TX, USA). Reagent grade ammonium acetate and formic acid were obtained from Sigma Chemical Co. (Milwaukee, WI, USA). All other solvents were of HPLC grade. Different pools of human plasma were obtained from the Department of Transfusion Medicine, National Institute of Health (Bethesda, MD, USA).

2.2. Instrumentation

LC/MS experiments were carried out on an LCQ DECA XP Ion Trap Mass Spectrometer, equipped with an orthogonal APCI source, and interfaced to a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA). Data were acquired with a Surveyor Autosampler and MS Pump and analyzed with Xcalibur™ Software, version 2.0. Centrifugation of plasma specimens was performed with an Eppendorf Centrifuge 5415C (Hamburg, Germany). Evaporation of solvents under nitrogen was carried out on a TurpoVap® LV evaporator from Zymark (Hopkinton, MA, USA).

Mass spectrometry data were collected in positive ion mode, with the following APCI-MS parameters: corona discharge needle voltage, 4.5 kV; vaporizer temperature, 475 °C; sheath gas setting (high purity nitrogen), 20; no auxiliary gas was utilized; and the transfer capillary temperature was 250 °C.

Chromatographic separation was performed on a Synergi Hydro-RP 80A (50 mm × 2.0 mm, 4 μm) column with an identically packed guard column (4 mm × 2.0 mm) (Phenomenex, Torrance, CA, USA). Gradient elution with (A) 10 mM ammonium acetate in water, 0.001% formic acid (pH 4.5) and (B) acetonitrile at a flow rate of 200 μL/min was used with a gradient program of 40% B for 2 min, increasing to 90% over 7 min and hold for 2 min. The HPLC column was re-equilibrated for 6 min, giving a total run of 17 min. The valve was set to direct LC flow to the mass spectrometer from 2 to 12 min, with the remaining LC eluent diverted to waste. Identification and quantification of analytes were based on selected reaction monitoring (SRM). Precursor and product ions were established by direct infusion of individual analytes at a concentration of 1000 ng/mL in methanol.

2.3. Preparation of calibrators, internal standards and quality control samples

For calibrators, a stock solution of 100,000 ng/mL of each analyte was prepared in methanol and stored at −20 °C until use. Working solutions, ranging from 10 to 5000 ng/mL, were prepared by independent dilution with methanol from the main stock solution. Blank human plasma specimens (200 μL) were fortified with 20 μL aliquots of working solutions to yield an eight-point calibration curve (1, 2.5, 5, 10, 50, 100, 250 and 500 ng/mL) for methadone, EDDP and EMDP.

Quality control samples were prepared in a similar way from independently prepared stock solutions. Quality control stock solutions were prepared from different vial lots than that used for the calibrators. Blank human plasma specimens were fortified with 20 μL aliquots of quality control working solutions to yield 4, 40 and 400 ng/mL (low, medium and high, respectively) quality control samples.

Individual stock deuterated internal standard (methadone-D_9 and EDDP-D_3) 1000 ng/mL solutions were prepared in methanol and stored at −20 °C until use. A 100 ng/mL working solution containing both internal standards was prepared in methanol. Twenty microliters working internal standard was added to 200 μL of plasma, yielding a final internal standard concentration of 10 ng/mL. Quantification was accomplished by comparing peak area ratios of target analytes to internal standards over a concentration range from 1.0 to 500 ng/mL. Methadone-D_9 was the internal standard for methadone and EMDP, and EDDP-D_3 for EDDP. Data were fit to a linear least-squares regression curve with a weighting factor of $1/x$.

2.4. Specimen preparation

A 200 μL aliquot of each plasma specimen, quality control sample or calibrator was combined with 20 μL internal standard working solution and vortexed for 10 s. Protein precipitation was performed by addition of 600 μL of acetonitrile, vortexing for 10 s, and centrifugation at 6831 × g for 10 min. The supernatant was evaporated to dryness under nitrogen at 37 °C, reconstituted in 200 μL water and 20 μL injected into the LC/MS system with APCI.

2.5. Method validation

The following criteria were used to evaluate the method: sensitivity, specificity, limits of detection (LOD) and quantification (LOQ), linearity, precision, accuracy, recovery, carryover effect, matrix effect and stability. Method validation was accomplished in 4 days with four unique assays.

Specificity of the method was tested by analyzing blank human plasma specimens from six different individuals to evaluate endogenous interference. In addition, potential interference from common licit and illicit drugs was evaluated by adding drugs to 4 ng/mL quality control samples. Final interferent concentrations were 1000 ng/mL of cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC, 11-nor-9-carboxy-THC, morphine, normorphine, morphine-3-beta-D-glucuronide, morphine-6-beta-D-glucuronide, codeine, norcodeine, 6-acetylmorphine, 6-acetylcodeine, hydrocodone, hydromorphone, oxycodone, noroxycodone, oxymorphone, noroxymorphone, diazepam, lorazepam, oxazepam, alprazolam, imipramine, clomipramine, fluoxetine, norfluoxetine oxalate, paroxetine maleate, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, clonidine, ibuprofen, pentazocine, caffeine, diphenhydramine, clorpheniramine, brompheniramine, aspirin, acetaminophen, phencyclidine and nicotine. No interference was noted if analyte concentration quantified within ±20% of the 4 ng/mL target concentration.

Sensitivity was evaluated by determining LOD and LOQ. Limit of detection was defined as the lowest concentration with acceptable chromatography, the presence of precursor and product ions with a signal-to-noise ratio (determined by peak height) of at least 3, and relative retention time within ±2% of average retention time of the calibrators. Limit of quantification was the lowest concentration that met LOD criteria with a signal-to-noise ratio of at least 10 and acceptable precision and accuracy [relative standard deviation (R.S.D.) and percent difference, within ±20%]. Both parameters were determined empirically by triplicate analysis of a series of decreasing concentrations of drug-fortified human plasma.

Linearity was investigated by calculation of the regression line by the method of least squares ($1/x$ weighting factor) and expressed as coefficient of determination (R^2). Linearity (R^2) was required to be >0.98 and was determined with eight calibrators. Concentrations of each calibrator were required to be within ±15% of target except at the LOQ, where ±20% was acceptable, when calculated against the full eight-point curve.

Precision (% relative standard deviation, R.S.D.) and accuracy were evaluated over the linear dynamic range at three concentrations, low (4 ng/mL), medium (40 ng/mL) and high (400 ng/mL). Intra- and inter-batch precision was assessed with five determinations per concentration over 4 days ($n_{total} = 20$), respectively. An estimate of precision was obtained using a one-way analysis of variance (ANOVA), using Excel, with day as the grouping variable. Precision was required to be within ±15% for the medium and high quality control samples and ±20% for the low quality control.

Accuracy was determined by comparing the mean ($n = 20$) measured concentration of all analytes to the target value. Accuracy was expressed as the percent of target concentration. The acceptance criterion was ±20% of target. Recovery for each analyte was determined at 40 ng/mL ($n = 5$) by comparing the peak areas of analytes spiked into blank plasma samples followed by protein precipitation, to neat samples. Carryover was assessed by injecting negative plasma samples after the 500 ng/mL calibrator and calculating analyte concentrations if present. Carryover was assumed to occur if the concentration of any analyte was greater than the LOD.

The absence of ion-suppression/enhancement was demonstrated with the following procedure: six different sources of drug free plasma were extracted as described previously. The extracts were then fortified with all drugs at a concentration of 10 ng/mL. A reference solution containing (A) 10 mM ammonium acetate in water, 0.001% formic acid (pH 4.5) and (B) acetonitrile (50:50, v/v) was also fortified with all drugs to the same nominal concentration. The reconstituted extracts and reference solutions were injected into the LC/MS system. Peak areas obtained from the extracts were compared with the corresponding peak areas produced by the reference solutions.

Stability was evaluated using human plasma fortified with analytes of interest at 40 ng/mL ($n = 3$). One day stability was tested for samples stored at room temperature, 4, 40 and −20 °C and after three freeze–thaw cycles.

2.6. Method application

The method will be used to analyze infant specimens collected in an IRB-approved protocol of nursing methadone-maintained mothers. Mothers provided written informed consent to participate in this study evaluating transfer of methadone through breast milk to their infants.

3. Results and discussion

In forensic and toxicological analysis, considerable effort is devoted to the development of fast, selective and sensitive analytical methods for the determination of drugs and metabolites in complex matrices. Therefore, LC/MS is often considered the method of choice.

Mass spectrometric optimization of methadone, EDDP and EMDP was performed by direct infusion of single analyte solutions of interest to APCI-MS. For maximum sensitivity, fragmentor voltages were chosen separately for each ion product. Identification and quantification were based on the following transitions: m/z 310.9–265.3 for methadone, m/z 278.0–249.2 for EDDP and m/z 264.3–235.2 for EMDP. The chromatographic run was divided into six scan events, each containing a set of optimized MS parameters for compounds of interest eluting within a given time period to ensure the highest sensitivity. Table 1 shows precursor and product ions, and retention times for all analytes. Deuterated internal standards (methadone-D_9 and EDDP-D_3) were employed to minimize the effects of analyte loss during specimen preparation. Methadone, EDDP and EMDP were adequately resolved within 11 min (Fig. 1). Total chromatographic

Table 1
LC–APCI-MS/MS chromatographic and mass spectrometric parameters

Compound	Precursor ion *m/z*	Product ion *m/z*	Retention time (min) mean ± S.D. (*n* = 35)
Methadone-D9	319.9	268.3	7.28 ± 0.037
Methadone	310.9	265.3	7.33 ± 0.032
EDDP-D3	281.0	249.2	6.58 ± 0.051
EDDP	278.0	249.2	6.59 ± 0.038
EMDP	264.3	235.2	9.85 ± 0.027

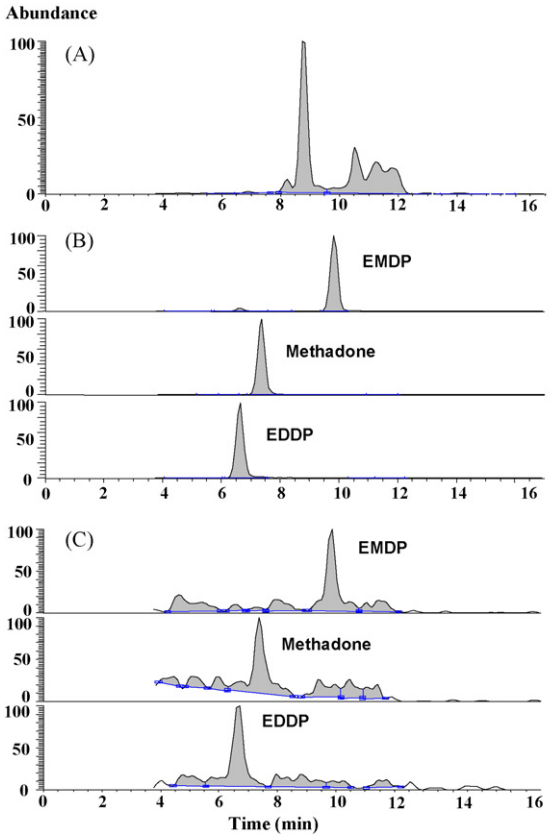

Fig. 1. (A) Total ion chromatogram (TIC) of extracted blank plasma, (B) selected reaction monitoring (SRM) of blank plasma spiked with 10 ng/mL of each analyte and (C) SRM of infant's plasma containing 2.4 ng/mL methadone, 1.9 ng/mL EDDP and 1.0 ng/mL EMDP.

run time was 17 min. Stability of the LC method was evaluated by calculating retention time variability. The percent relative variation for retention times were ≤0.78% for all compounds over 35 consecutive runs (Table 1).

LODs, LOQs and representative linearity results are detailed in Table 2. LODs and LOQs were 0.5 and 1.0 ng/mL for all analytes. Linear calibration curves were obtained with an average correlation coefficient (R^2; $n = 4$) of >0.99 for all analytes (Table 2).

There was no endogenous signal for any analyte in six blank human plasma specimens, demonstrating acceptable specificity for the method. In addition, no commonly used over-the-counter or abused drugs at a concentration of 1000 ng/mL interfered with the quantification of 4 ng/mL methadone, EDDP or EMDP. Table 3 contains precision and accuracy data for the method at three concentrations (4, 40 and 400 ng/mL) over the linear dynamic range. Intra-assay (within-run) precision and accuracy were determined over 4 days ($n = 5$ each day). Intra-assay precision was less than 13.4% R.S.D. and accuracy ≥87.5% of the target analytes across the linear range of the assay for all analytes on four separate days. Inter-assay (between-run) precision and accuracy, assessed with 20 specimens at each quality control concentration on four separate days, ranged from 4.6 to 11.1% R.S.D. and 87.5 to 107% of the target analytes, respectively, for all analytes at all three concentrations. Results of the ANOVA study demonstrated significant effects of day of analysis on the low, medium or high quality control measurements with maximum $F_{3,16} = 3.63$, 3.02 and 3.75, for low, medium and high concentration quality control samples, respectively; $P < 0.05$, for all the analytes. However, the differences in daily mean analyte concentrations did not exceed 11.1% of target and were considered clinically insignificant.

Recovery for each analyte ($n = 5$) was estimated by comparing LC/MS peak area of five different blank plasma specimens fortified at 40 ng/mL to five neat 40 ng/mL samples for each analyte. Mean percent recoveries for methadone, EDDP and EMDP were all above 87.5% at 40 ng/mL with R.S.D. of 5.0, 3.2 and 5.9%, respectively. There was no carryover observed at the LOD of the analysis. The absence of ion-suppression and matrix effect was demonstrated according to the procedure described in the experimental section. The mean area ratios (reconstituted extract in plasma/reference solution, $n = 3$) are presented in Table 4. Thus, no excessive ion-suppression/enhancement was observed for each analyte. Analyte concentrations at 40 ng/mL were stable above 85% of expected values under all four storage conditions, indicating stability at room temperature, 4, 40 and −20 °C for 24 h, and after three freeze–thaw cycles (Table 5).

Fig. 1A represents the total ion chromatogram (TIC) of the extracted blank plasma and 1B depict the selected reaction monitoring of blank plasma fortified with 10 ng/mL concentration of methadone, EDDP and EMDP. Fig. 1C shows the SRM of methadone, EDDP and EMDP in one infant's plasma specimen after 14 days of exclusive breastfeeding from a mother

Table 2
Limits of detection, limits of quantification and calibration results for methadone, EDDP and EMDP in plasma by LC–APCI-MS/MS

Compound	Internal standard	LOD (ng/mL)	LOQ (ng/mL)	Equation (±S.D.) $n = 4$
Methadone	Methadone-D9	0.5	1.0	$Y = -3.1247\ (\pm 0.557) + 2.8587\ (\pm 0.381)X$
EDDP	EDDP-D3	0.5	1.0	$Y = -0.1385\ (\pm 0.079) + 0.3382\ (\pm 0.094)X$
EMDP	Methadone-D9	0.5	1.0	$Y = 0.03977\ (\pm 0.663) + 2.3647\ (\pm 0.454)X$

Table 3
Intra- and inter-assay precision and accuracy of methadone, EDDP and EMDP in human plasma

Analyte	Expected concentration (ng/mL)	Accuracy (% of target)		Precision (% R.S.D.)	
		Intra-assay $n=5$	Inter-assay $n=20$	Intra-assay $n=5$	Intra-assay $n=20$
Methadone	4	87.5 (80–95)	87.5 (80–95)	6.7	4.6
	40	89.0 (85–90)	87.5 (81–99)	2.5	5.3
	400	95.5 (87–112)	95.8 (81–112)	9.7	8.3
EDDP	4	87.5 (83–103)	90.0 (80–113)	9.1	9.4
	40	92.5 (89–100)	87.8 (81–100)	5.1	5.9
	400	99.0 (92–103)	100 (84–117)	5.9	9.2
EMDP	4	87.5 (80–95)	92.5 (80–118)	5.5	11.1
	40	104 (80–115)	105 (80–115)	13.4	10.6
	400	112 (107–17)	107 (86–119)	4.0	8.1

Table 4
Results of the ion-suppression/enhancement study

Compound	Mean area ratios ($n=3$)	% R.S.D.
Methadone	1.012	6.27
EDDP	0.993	9.33
EMDP	1.009	11.90
Methadone-D9	1.061	7.06
EDDP-D3	0.996	10.72

maintained on a daily dose of 70 mg of methadone during gestation and in the post-partum period. Concentrations of the three analytes were 2.4 ng/mL methadone, 1.9 ng/mL EDDP and 1.0 ng/mL EMDP.

The LC–APCI-MS/MS method fulfilled our analytical standard criteria. Shorter specimen processing time, sensitivity and linear dynamic range were clinically relevant to monitor drug use in plasma. Precision and accuracy of the method proved to be acceptable for quantifying methadone, EDDP and EMDP in a small plasma sample volume. Protein precipitation with acetonitrile resulted in effective sample preparation as evidenced by minimal ion-suppression/enhancement. Analytes were stable during sample preparation and storage under the stated conditions. The method was applicable to clinical plasma specimens and proved to be a valuable analytical tool in testing of methadone and the principle metabolites (EDDP and EMDP). The method is being applied to infant's plasma specimens collected in an IRB-approved protocol of nursing methadone-maintained mothers.

Table 5
Stability of methadone and metabolites in human plasma for 24 h at different temperatures

Stability condition, $n=3$	% Analyte found		
	Methadone	EDDP	EMDP
Room temperature	97	93	89
4 °C	97	92	87
40 °C	96	92	85
−20 °C	98	95	93
After three freeze–thaw cycles	98	96	90

4. Conclusion

Compared to many published methods for the analysis of methadone and metabolites, this validated LC–APCI-MS/MS assay achieves better sensitivity, reduced pre-analysis specimen extraction time, and its small specimen volume is advantageous for infant and adult clinical specimens. The assay provides simultaneous quantitative analysis of methadone and metabolites EDDP and EMDP in human plasma with an LOQ of 1 ng/mL with minimal specimen preparation. This method was shown to be useful for monitoring plasma methadone and metabolite concentrations in 200-μL plasma specimens from methadone-exposed infants.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health and NIDA K08DA00495.

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