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Validated method for the simultaneous determination of methadone and its main metabolites (EDDP and EMDP) in plasma of umbilical cord blood by gas chromatography–mass spectrometry

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ABSTRACT

A sensitive and specific GC/MS method for the determination of methadone (MDN) and its two main metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP), in plasma samples obtained from venous and arterial umbilical cord blood and maternal blood has been developed, optimized and validated. Specimen preparation includes protein precipitation with acetonitrile and simultaneous solid-phase extraction of the three analytes. Methadone-d9 was used as internal standard for the determination of MDN and EMDP, while EDDP-d3 for EDDP. Limits of detection were 0.6 μg/L for MDN and 0.3 μg/L for EDDP and EMDP, while limits of quantification were 2.0 μg/L for MDN and 1.0 μg/L for EDDP and EMDP. The calibration curves were linear up to 2000 μg/L for MDN and up to 1000 μg/L for EDDP and EMDP. Absolute recovery ranged from 94.8 to 99.7% for all three analytes. Intra- and interday accuracy was less than 5.3 and 5.5%, respectively, while intra- and interday precision was less than 3.5 and 5.0%, correspondingly, for all analytes. The method proved suitable for the determination of MDN and its two main metabolites in plasma samples obtained from umbilical cord and maternal blood of a woman participating in a MDN maintenance program, during the prenatal and postpartum period.

1. Introduction

Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) (MDN), a synthetic opiate, is widely used in maintenance programs for opioid-dependent patients due to the mild withdrawal signs and symptoms that occur after abrupt discontinuation [1–3]. Furthermore, MDN administration is the only treatment approved for opiate substitution during pregnancy and in the postpartum period [4], in Greece and in other parts of the world.

MDN is a lipophilic, weakly basic (pK_a 8.25) compound, that is highly protein bound [5]. The main metabolic pathway of MDN is dealkylation to form its major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and a minor metabolite 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) [5]. In plasma, therapeutic concentrations usually range between 50.0 and 1000 μg/L, with peak plasma levels at about 4 h after oral administration [6]. In patients during a MDN maintenance treatment, plasma concentrations considerably fluctuate day by day and the interindividual MDN half life varies significantly (15–25 h) [7,8].

In cases of patients who need special medical care, such as women in pregnancy or in postpartum period, there could be interindividual differences in MDN pharmacokinetics [9,10]. Determination of MDN and its metabolites in plasma is important, in order to monitor and maintain plasma concentrations of MDN within an effective range, to obtain maximum treatment efficacy, to prevent toxicity, to investigate the mechanisms involved in MDN metabolism [1,8,10,11] and to define the relationship between the given dose and the resulting drug concentration in plasma [12].

The equilibrium between maternal and fetal blood actively distributes drugs, through the placenta, in the fetus. The transfer of lipids and lipoproteins from maternal blood to the developing fetus favors the passive diffusion of bounded drugs across the placental membrane. Maternal MDN is transferred across the placenta and can induce significant withdrawal symptoms in the newborn. In such a case, treatment of the newborn is required [13,14]. However, there is no consistent relationship between the maternal MDN

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dose and the severity of neonatal symptoms [13]. Additionally, the umbilical cord and neonatal blood levels of MDN, as well as the neonatal plasma MDN levels, are consistently lower than those of the mother's [13,15].

Several analytical methods based on gas chromatography [1,9,10,12,16–28] or liquid chromatography [2,5,8,29–31], coupled with MS [1,5,8,9,12,16–31] or UV [2,29] detection, have been published for the determination of MDN and its metabolites in a variety of biological matrices, such as urine [10,19–21], plasma or blood [1,8,9,22,23,30,31], hair [12,24–26], saliva [10,27], oral fluid [28], meconium [18] and breast milk [2,5,16,17,29]. Liquid–liquid extraction (LLE) [8,22,23,30] or solid-phase extraction (SPE) [1,9] techniques, without an initial preparative step of protein precipitation, have been used for the isolation of MDN and/or its major metabolites from plasma or blood samples before any chromatographic determination. A review of the most recent literature has revealed an increasing trend in using SPE instead of LLE, due to the advantages of the former [9]. Only a few methods that include a protein precipitation step for the determination of MDN and its metabolites in breast milk or plasma have been published [5,31].

In the current literature there are no validated analytical GC/MS methods for the quantification of MDN, EDDP and EMDP in umbilical cord blood. The aim of the present study was to develop, optimize and validate a sensitive and accurate GC/MS method for the determination of MDN and its main metabolites EDDP and EMDP in plasma samples obtained from umbilical cord blood or maternal blood. This method could be used to investigate the uptake of MDN and its metabolites by infants born to MDN maintained women.

2. Experimental

2.1. Materials

MDN, EDDP, EMDP, methadone-d9 and EDDP-d3 were purchased from LGC Promochem (Molsheim, France). All standards were >99.9% pure, as described by the manufacturer. HPLC-grade methanol, acetonitrile and dichloromethane were obtained from Merck (Darmstadt, Germany). Analytical-grade ammonium hydroxide, glacial acetic acid and extra pure sodium-dihydrogen phosphate dihydrate were obtained from Merck (Darmstadt, Germany). Phosphate buffer (pH 6.00; 0.1 M) was prepared by dissolving the appropriate amount of sodium-dihydrogen phosphate dihydrate in water and accurately adjusting the pH to 6.00 with sodium hydroxide (0.1N). Pooled, drug free, human plasma was obtained from local healthy volunteers ($n = 6$), verified as negative for drugs by GC/MS and was used for the development and validation of the method.

2.2. Instrumentation

Chromatographic analysis was performed on a Shimadzu QP 2010S GC/MS system, equipped with a DB-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), and helium was used as carrier gas at a flow rate of 1.30 mL/min. Injections of 1 μL were carried out in the splitless mode using a Shimadzu AOC-20i autosampler system. The initial column temperature of 80 °C was increased, at a rate of 40 °C/min, to the intermediate step temperature of 270 °C with 2 min hold, followed by an increase at a rate of 40 °C/min to the final column temperature of 300 °C and held for 4 min. The injector, ion source and interface temperatures were maintained at 260 °C, 200 °C and 280 °C, respectively. The above GC conditions were chosen after optimization of the developed GC/MS method. The mass spectrometer was operated in electron impact ionization/selective ion monitoring (EI/SIM) mode for this assay. The mass spectra of MDN, EDDP, EMDP, methadone-d9 and EDDP-d3 show a base peak of m/z 72, 277, 208, 78 and 280, respectively, and consequently these ions were used for quantitation.

Confirm HCX (Isolute) and Nexus (Varian) SPE columns were used. A vortex (Chiltern, Model MT 19) was used for the mixing of samples and standards. The pH-meter used was a 691 digital model (METROHM, Switzerland) with a glass combination electrode. An evaporating device using nitrogen (Reacti-Vap PIERCE, Model 18780, Rochford, Illinois) and a cooled centrifuge (Sigma 4K10, Germany) were used.

2.3. Calibrators and controls

Stock standard solutions (100 mg/L) of analytes were prepared in methanol for MDN and EDDP and in acetonitrile for EMDP. Stock standard solutions (100 mg/L) of methadone-d9 and EDDP-d3 were prepared in methanol.

Nine aqueous combined working standard solutions containing MDN at 0.04–40 mg/L as well as EDDP and EMDP at 0.02–20 mg/L, were prepared by mixing the appropriate volumes of the corresponding stock solutions of each compound and dilution with water. Spiked plasma standards for calibration curves (calibrators) were constructed by spiking 1.00 mL aliquots of blank human plasma with 50 μL of the combined working standard solutions. The nine calibrators contained MDN at 2.00, 4.00, 10.0, 40.0, 100, 200, 400, 1000 and 2000 μg/L, while EDDP and EMDP at 1.00, 2.00, 5.00, 20.0, 50.0, 100, 200, 500 and 1000 μg/L.

Additional combined aqueous working standard solutions (in three different concentrations) containing MDN, EDDP and EMDP were prepared from different stock solutions from what was used for calibrators, in order to prepare plasma quality control (QC) samples. The three plasma QC samples contained MDN at 6.00, 900 and 1600 μg/L, as well as EDDP and EMDP at 3.00, 450 and 800 μg/L, and were prepared on a similar way with that of calibrators. Fresh working solutions were prepared on a daily basis.

Methadone-d9 was used as internal standard for the determination of MDN and EMDP, while EDDP-d3 for EDDP. A combined aqueous working internal standard solution containing methadone-d9 at 4.00 mg/L and EDDP-d3 at 2.00 mg/L was prepared by mixing the appropriate volumes of the corresponding methanolic stock standard solutions and dilution with water.

Calibration curves, based on the peak area ratio of analyte to corresponding internal standard against analyte concentration, were constructed for each day of analysis and were used for the calculation of the analyte concentration in the QC and patient plasma samples.

2.4. Sample preparation

To 1.00 mL of plasma (calibrator, QC or patient sample) 50 μL of the combined working internal standard solution were added and vortex mixed for 15 s. Therefore, all calibrators, QC and patient samples contained methadone-d9 and EDDP-d3 at 200 and 100 μg/L, respectively. Afterwards, the samples were diluted with 1.00 mL of deionized water. Consequently, 3.00 mL of acetonitrile were added dropwise while vortex mixing, for the protein precipitation, and the specimens were centrifuged at 2000 rpm for 5 min. The supernatant organic phase was decanted into a clean glass tube and solvent was evaporated under a gentle stream of N_2 at 40 °C to approximately 1.5 mL. The pH of the samples was adjusted to 6.00 with the addition of 3.00 mL of phosphate buffer (pH 6.00) 0.1 M.

The SPE columns were conditioned with 3 mL of methanol, 3 mL of deionized water and 1 mL of phosphate buffer 0.1 M (pH 6.00) prior to sample loading. The samples were applied to the columns at a flow rate of approximately 1.0 mL/min. Then, the columns were washed successively with 3 mL of deionized water, 1 mL of acetic acid, 1.0 M and 3 mL of methanol and after that they were dried under maximum vacuum for 5 min. The analytes were eluted twice with 1.5 mL of freshly prepared mixture of dichloromethane:methanol:ammonium hydroxide (90:10:2, v/v/v). The eluates were collected in silanized tubes and evaporated to dryness under a gentle stream of N_2 at 40 °C. The residue was reconstituted with 60 μL of acetonitrile and an aliquot of 1 μL of the resulting solution was injected onto the GC/MS system.

2.5. Method validation

The following criteria were used to evaluate the GC/MS method: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, recovery, specificity, robustness and stability. Selectivity, linearity, precision and accuracy of the method were validated through six analytical runs on six different days.

Selectivity was accomplished by analyzing six different blank samples and the matrix effect was evaluated.

The *LOD and LOQ* for each analyte was determined as the lowest concentration yielding a signal-to-noise ratio of at least 3:1 and 10:1, respectively.

Linearity was investigated by calculation of the regression line by the method of least-squares with a weighting factor of $1/x^2$ and expressed by the correlation coefficient (R^2). Each calibrator was back calculated against the total curve.

Precision and accuracy of the method was evaluated by analyzing three QC samples, across each analyte's linear range (6.00, 900 and 1600 μg/L for MDN, while 3.00, 450 and 800 μg/L for EDDP and EMDP). The concentration of the QC samples was calculated with the equation of the total calibration curve of the corresponding day of analysis. Precision (intraday $n = 6$ and interday $n = 36$) was expressed as the relative standard deviation (% RSD). Accuracy of the method was calculated as the percent difference from the expected concentration (% E_r).

Extraction efficiency for QC samples was assessed with five replicates at the low and high concentrations and with eight replicates at the intermediate concentration. Extraction recovery of the method was calculated as the percentage of each analyte response at the sample compared to that of a solution containing the analyte at the corresponding concentration.

Specificity was accomplished by analyzing a standard mixture of commonly used illicit and licit drugs or their metabolites (morphine, codeine, 6-acetyl-morphine, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, cocaine, ecgonine methylester, benzoyloecgonine, diazepam, nordiazepam, bromazepam, alprazolam, 7-amino-flunitrazepam, phenobarbital, amitriptyline, clomipramine, amphetamine, methamphetamine, MDMA, ephedrine and ketamine) at a concentration of 100 mg/L. These drugs could be used by participants in MDN maintenance programs and consequently could interfere with our analysis. Spiked human plasma samples with these substances ($n = 6$) at a concentration of 1 mg/L were also analyzed.

Robustness of the entire method was studied for several variations in the procedure (pH of samples was adjusted to 6.50 instead of 6.00, different lot number of SPE columns and different ratio of the solvents in the mixture of elution) and in the chromatographic parameters (flow rate of carrier gas: 1.26 ml/min, injector temperature: 257 °C, intermediate step column temperature: 273 °C and 3% lower detector voltage).

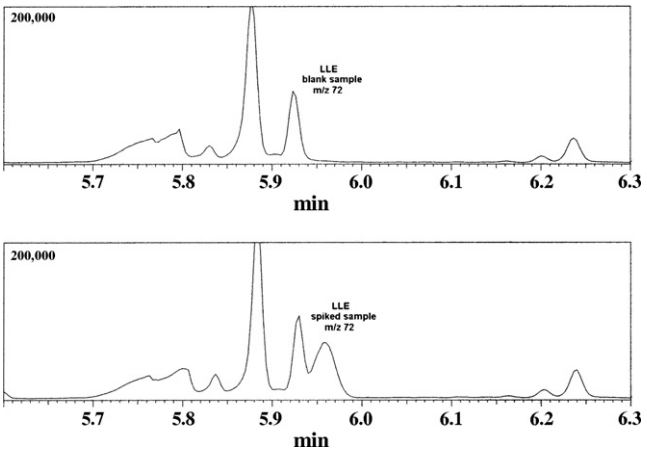

Fig. 1. Representative SIM chromatograms (m/z 72) of a liquid–liquid extracted blank and spiked (MDN: 25 μg/L) human plasma sample.

Stability study was assessed by analyzing fortified human plasma with MDN and its metabolites at low and high concentrations (9.00 μg/L and 1400 μg/L for MDN, while 4.50 μg/L and 700 μg/L for EDDP and EMDP) and keeping the samples at different temperature conditions (at room temperature and at −20 °C). Furthermore, fortified human plasma specimens were subjected to three freeze–thaw cycles.

2.6. Method application

The method was applied to plasma samples of a MDN maintained woman who was receiving MDN for the last three months of pregnancy. Maternal whole blood samples were collected during prenatal and postpartum period. Venous and arterial umbilical cord blood, as well as the corresponding maternal blood was also collected during the delivery. The umbilical cord blood samples were taken from a small double-clammed segment of the umbilical cord. The arterial blood was collected from the umbilical vein, whereas the venous blood from the two umbilical arteries. Plasma samples were obtained after centrifugation of blood at 3500 rpm for 10 min.

3. Results and discussion

3.1. Method development and optimization

A gas chromatography–mass spectrometric method has been developed and optimized, in order to determine the concentrations

Table 1
% Absolute extraction recovery of MDN, EDDP and EMDP, from spiked human plasma quality controls samples

	% Mean absolute recovery ± S.D.
MDN	
6.00 μg/L ($n = 5$)	94.8 ± 3.6
900 μg/L ($n = 8$)	95.6 ± 2.4
1600 μg/L ($n = 5$)	97.5 ± 4.4
EDDP	
3.00 μg/L ($n = 5$)	96.4 ± 2.8
450 μg/L ($n = 8$)	99.7 ± 1.8
800 μg/L ($n = 5$)	96.1 ± 5.5
EMDP	
3.00 μg/L ($n = 5$)	97.9 ± 4.1
450 μg/L ($n = 8$)	99.1 ± 3.8
800 μg/L ($n = 5$)	99.3 ± 4.7

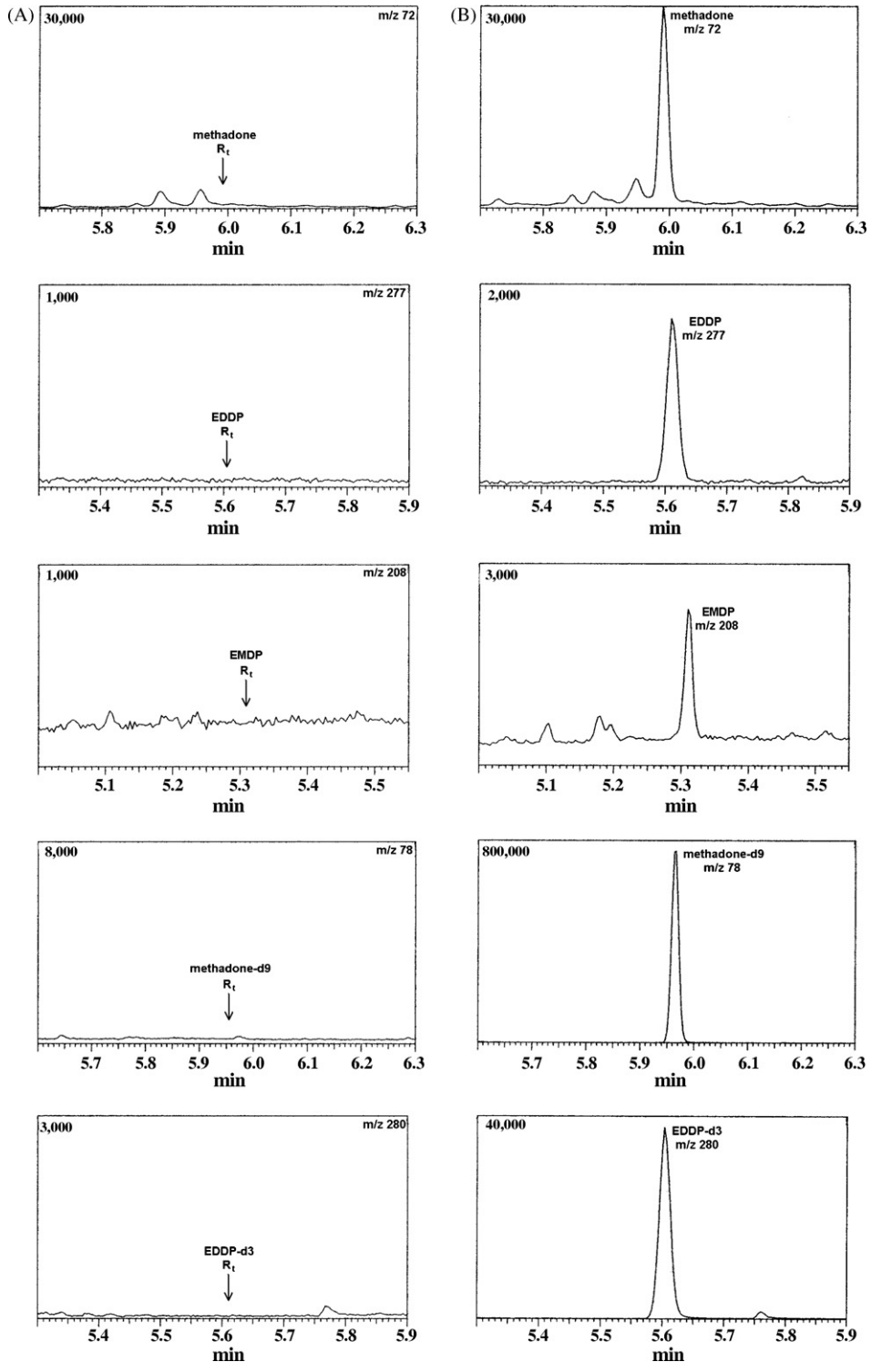

Fig. 2. Representative SIM chromatograms of a solid-phase extracted blank (A) and a spiked (B) human plasma sample at the lowest QC concentration (MDN: 6.00 μg/L, EDDP and EMDP: 3.00 μg/L).

Table 2
Mean regression equations, interday precision of slopes (%RSD) and linearity for MDN, EDDP and EMDP, in human plasma

Compound	Concentration range (μg/L)	Mean regression equations ($n = 6$)	R^2	% RSD of slopes ($n = 6$)
MDN	2.00–2000	$y = 0.00584\ (\pm 0.00019) + 0.00403\ (\pm 0.00065)$	>0.996	3.2
EDDP	1.00–1000	$y = 0.01059\ (\pm 0.00036) + 0.00906\ (\pm 0.00093)$	>0.996	3.4
EMDP	1.00–1000	$y = 0.000576\ (\pm 0.000027) + 0.000574\ (\pm 0.000068)$	>0.991	4.7

y: peak area ratio of analyte/corresponding internal standard.

Table 3
Intra- and interday accuracy and precision of MDN, EDDP and EMDP from plasma quality controls samples

Analyte	Concentration added (μg/L)	Intraday (n = 6)			Interday (n = 36)		
		Concentration found, mean ± S.D. (μg/L)	Accuracy (% E_r)	Precision (% RSD)	Concentration found, mean ± SD (μg/L)	Accuracy (% E_r)	Precision (% RSD)
MDN	6.00	6.02 (±0.21)	0.3	3.5	6.16 (±0.23)	2.7	3.7
	900	898.0 (±7.1)	−0.2	0.8	901 (±14)	0.1	1.5
	1600	1515 (±33)	−5.3	2.2	1587 (±80)	−0.8	5.0
EDDP	3.00	3.05 (±0.09)	1.7	2.9	3.04 (±0.09)	1.3	3.0
	450	459.8 (±3.6)	2.2	0.8	461.6 (±6.2)	2.6	1.3
	800	793 (±17)	−0.9	2.1	822 (±36)	2.8	4.4
EMDP	3.00	2.93 (±0.04)	−2.3	1.4	2.97 (±0.08)	−1.0	2.7
	450	464.7 (±6.5)	3.3	1.4	466 (±21)	3.6	4.5
	800	832 (±21)	4.0	2.5	844 (±27)	5.5	3.2

of MDN, EDDP and EMDP in plasma samples obtained from umbilical cord and maternal blood. The isolation of these three analytes from the specimens was achieved after protein precipitation with acetonitrile and SPE.

The developed GC/MS method was optimized for: column temperature program, flow rate of carrier gas and temperatures of injector, ion source and interface. The final optimized GC separation of analytes of interest and their respective internal standards was achieved within 6 min and the total chromatographic run time was less than 12 min. The retention times of MDN, EDDP and EMDP were 5.99, 5.61 and 5.31 min, correspondingly.

In a biofluid, such as plasma or blood, sample pretreatment is essential in order to remove endogenous materials, like proteins. Protein precipitation using organic solvents, such as methanol, acetone and acetonitrile, was tested in plasma samples of MDN maintained individuals and acetonitrile showed the best extraction efficiency.

During the optimization of the extraction procedure, LLE of plasma samples was tested using many systems of organic solvents, as well as SPE with two different types of columns (Nexus and HCX), which are suitable for analysis of basic drugs. In all LLE procedures significant matrix effect was observed nearby the retention time of MDN in m/z 72, which resulted to insufficient resolution of the peaks (Fig. 1). For the above reasons, LLE proved to be an ineffective procedure. When plasma samples were applied to Nexus SPE columns (mixed-mode SPE columns, polar and non-polar) the flow rate was irregular and there were also problems of matrix effect in m/z 72. When other ions of MDN (e.g. 294) were used for quantification [17,18,25], the sensitivity was considerably decreased, in both LLE and SPE procedures. Matrix effect was minimal when HCX SPE columns [mixed mode (non-polar and strong cation exchange) columns] were used. Furthermore, the use of acetate buffer pH 4.00 in the washing step of HCX columns [7,19,32,33], instead of acetic acid 1.0 M [1,9], resulted in extremely low recovery for EMDP due to the elution of EMDP with methanol during the washing step. The extraction efficiency during SPE was also improved by using different mixture of elution solvents and dichloromethane:methanol:ammonium hydroxide (90:10:2) showed the best results for the elution of all analytes.

In order to investigate, in this study, if analytes need some time to equilibrate with plasma after spiking, freshly-spiked plasma samples as well as spiked plasma samples equilibrated for 24 h were analyzed. In both cases, we received the same results.

3.2. Method validation

The combination of protein precipitation and SPE of plasma samples, succeeded in better cleaning up of the specimens. All blank plasma samples were free of co-eluting peaks at the retention times of the analytes of interest. The selectivity of the method was adequate with minimal matrix effect at all blank samples. Furthermore, the combination of protein precipitation and SPE improved the extraction efficiency of the method and the absolute recovery of all analytes, estimated at three concentrations levels, was higher than 94.8% (Table 1). Representative selected ion monitoring chromatograms of a blank sample and a spiked sample at low QC concentration (6.00 μg/L for MDN, while 3.00 μg/L for EDDP and EMDP) are shown in Fig. 2.

Calibration curves of spiked plasma samples (calibrators) showed excellent linearity, for all analytes. The linear dynamic ranged from 2.00 to 2000 μg/L for MDN, while from 1.00 to 1000 μg/L for EDDP and EMDP, with correlation coefficients (R^2) exceeding 0.991. LODs and LOQs were 0.6 and 2.0 μg/L, respectively, for MDN, while 0.3 and 1.0 μg/L, correspondingly, for EDDP and EMDP. Representative linearity results are shown in Table 2.

Intra- and interday accuracy and precision of the method were evaluated at three concentrations over the linear dynamic range (6.00, 900 and 1600 μg/L for MDN, while 3.00, 450 and 800 μg/L for EDDP and EMDP). Intra- and interday accuracy was less than 5.3 and 5.5%, respectively, while intra- and interday precision was less than 3.5 and 5.0%, correspondingly, for all analytes (Table 3).

Specificity of the method was evaluated by analyzing spiked plasma sample with licit and illicit drugs which are commonly used by individuals participating in MDN maintenance programs. Our interference study documented that plasma concentrations of 1 mg/L of these drugs did not interfere with the accurate determination of MDN, EDDP and EMDP in human plasma.

Robustness of the method was studied and the differences of the average area of each analyte, for each variation used in the procedure or in the chromatographic parameters, as well as their standard deviation were calculated. Neither a single variation nor a combination of the ones tried, showed a significant influence on the method, which proved to be sufficiently robust against the chosen variations.

Table 4
Assessment of MDN and its metabolites stability in human plasma based on % loss for each analyte, at two concentration levels and at different conditions

	Analyte					
	MDN		EDDP		EMDP	
	9.00[a]	1400[a]	4.50[a]	700[a]	4.50[a]	700[a]
25 °C, 4 h	−0.1	−0.7	−0.2	−0.7	−0.7	−0.3
−20 °C, 30 days	−2.1	−1.1	−1.4	−1.7	−2.5	−1.1
Three freeze–thaw cycles	−2.6	−0.6	−0.9	−0.7	−4.4	−3.2

[a] Concentration (μg/L).

Table 5
Concentrations (μg/L) of MDN, EDDP and EMDP in plasma samples of a MDN maintained woman before and 4 h after MDN administration, during two days of prenatal period (70 mg MDN/day) and the first two days of postpartum period (40 mg MDN/day)

Prenatal period (70 mg MDN/day)	Day 1 (32nd week)		Day 2 (33rd week)	
	Before	4 h after	Before	4 h after
MDN (μg/L)	212.1	360.1	156.9	229.7
EDDP (μg/L)	32.42	62.70	30.50	41.3
EMDP (μg/L)	[b]	[b]	1.20	[b]

Postpartum period (40 mg MDN/day)	1st day		2nd day	
	Before	4 h after	Before	4 h after
MDN (μg/L)	85.31	188.5	91.55	173.4
EDDP (μg/L)	15.00	28.46	13.83	21.34
EMDP (μg/L)	[b]	[b]	[a]	[b]

[a] Concentration below LOD.
[b] Concentration above LOD and below LOQ.

MDN and its metabolites in plasma were found to be stable at room temperature for at least 4 h, while at −20 °C for at least one month and after three freeze–thaw cycles. The loss for each analyte, at two concentrations (low and high) was calculated less than 0.7, 2.5 and 4.4% at room temperature, at −20 °C and at three freeze–thaw cycles, respectively (Table 4).

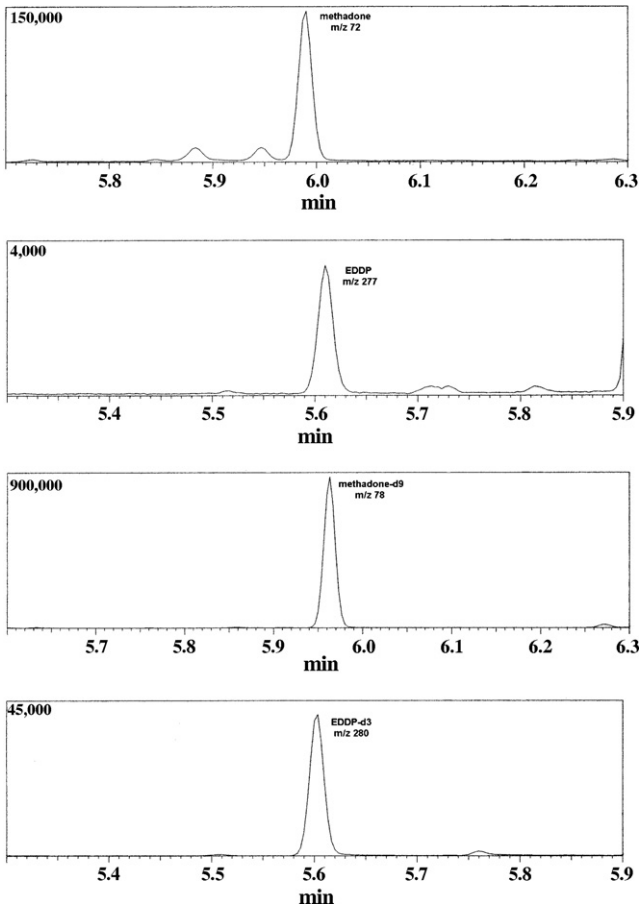

Fig. 3. SIM chromatograms of an extracted plasma sample obtained from arterial umbilical cord blood of a MDN (40 mg/day) maintained woman (MDN: 35.70 μg/L and EDDP: 9.01 μg/L).

Table 6
Concentrations (μg/L) of MDN, EDDP and EMDP in plasma samples obtained during delivery from umbilical cord blood and maternal blood of a MDN maintained woman (40 mg MDN/day)

Administration of 40 mg MDN/day	Umbilical cord blood		Maternal blood
	Arterial blood	Venous blood	
MDN (μg/L)	35.70	28.67	85.31
EDDP (μg/L)	9.01	8.04	15.00
EMDP (μg/L)	[a]	[a]	[b]

[a] Concentration below LOD.
[b] Concentration above LOD and below LOQ.

3.3. Method application

The method has been applied for the determination of plasma levels of MDN, EDDP and EMDP of a heroin addicted woman who had started MDN maintenance with a single daily dose of 70 mg on the 29th week of gestation. The dose was gradually reduced to 40 mg MDN during the last two weeks before delivery. Plasma samples were collected before and 4 h after oral MDN administration for two days at prenatal period (32nd and 33rd week) and for the first two days of postpartum period and were analyzed by the method developed. MDN and EDDP plasma concentrations determined before and 4 h after MDN administration were higher at the 32nd week compared with the corresponding concentrations of the 33rd week (Table 5). During the prenatal and postpartum period, MDN and EDDP plasma levels that were determined 4 h after MDN administration were higher than the corresponding levels before MDN administration. This was expected since peak plasma levels are usually observed 4 h after oral MDN administration [6]. The concentrations of EMDP in plasma were in most samples of our case below LOQ.

Plasma samples, obtained from venous and arterial umbilical cord blood and the corresponding maternal blood during delivery, were analyzed and the concentrations found are shown in Table 6. Plasma concentrations of MDN and EDDP in umbilical cord were found to be lower than those in the corresponding maternal plasma, while EMDP was not detected in umbilical cord blood. Furthermore, MDN and EDDP levels in arterial umbilical cord blood were slightly higher than those in venous umbilical cord blood. It has to be noticed that the MDN and EDDP umbilical cord plasma levels, in our case, are lower than the mother's. Representative selected ion monitoring chromatogram of plasma sample obtained from arterial umbilical cord blood is presented in Fig. 3.

4. Conclusions

The method developed in this study, when compared with previously described GC/MS or LC/MS methods for the determination of MDN and its metabolites in blood or plasma shows improved sensitivity, wider linearity range, enhanced accuracy and precision data, higher extraction recoveries and is robust in a combination of minor variations in the procedure or in the chromatographic conditions. To our knowledge, it is the only published GC/MS method that determines simultaneously MDN and its two metabolites in umbilical cord blood providing full validation data. The combination of protein precipitation with ACN and SPE, used in the procedure of the method, proved to be useful for the determination of total MDN plasma concentrations, since no interference from endogenous and exogenous compounds was observed. Previously reported validated methods for the determination of MDN and its metabolites, that included protein precipitation in the extraction procedure, achieved comparable sensitivity, but were based on LC/MS/MS analysis. However, it has to be mentioned that although

LC/MS/MS, due to its benefits, is mostly used for research purposes, it remains a more expensive analytical technique, when compared with GC/MS, and is not so widely available for routine analysis in the toxicological laboratories, clinical or forensic, worldwide.

The method developed allows the determination of MDN, EDDP and EMDP in plasma samples obtained from venous and arterial umbilical cord blood taken from newborns of women addicts in MDN maintenance programs and can be used in order to investigate the overall MDN exposure of these infants. Estimation of MDN uptake during intrauterus exposure of the infant could be utilized for the assessment of possible toxicity or neonatal abstinence syndrome (NAS) symptoms and the adjustment of the relative treatment. Furthermore, this method can be also used for therapeutic drug level monitoring in patients that follow MDN maintenance programs. The above method has been successfully applied in one real case of an infant born to a MDN maintained woman.

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