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BIOANALYSIS OF METHADONE IN HUMAN PLASMA AND URINE BY LC/MS/MS

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A rapid LC/MS/MS method for bioanalysis of methadone in human plasma and urine was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a 45:55 (v/v) mixture of acetonitrile and 0.2% (v/v) formic acid in water, at 45 ºC with a flow rate of 1 mL/min. The detection of metadone was in the MRM mode (m/z 310→ m/z 265). The human plasma samples were deproteinisated with methanol and the urine samples were diluted with bidistilled water. Aliquots of 3 μL from supernatants obtained by centrifugation were injected in the chromatographic system. The method showed a good linearity, precision and accuracy over the range of 10-1000 ng/mL in plasma and 20-2000 ng/mL in urine, respectively. The validated method is very simple and rapid and was successfully applied to the quantification of methadone from real plasma and urine samples.

INTRODUCTION

Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone; CAS-76-99-3) is a sintetic μ-opioid-receptor agonist widely used in the treatment of severe pain and in maintenance treatment of opioid addicts[1]. The pharmacokinetics and the pharmacodynamics of methadone have large inter-individual variability concerning the metabolism, the protein binding and the receptor affinity. [2,3] For this reason it is necessary that the doses are individualized to achieve optimum treatment.[5] Moreover, several reports warned about the danger of uncontrolled methadone therapy in addicts, particularly due to the tendency to mixed ingestion of methadone and other drugs of abuse. By consequence, the monitoring of methadone levels in blood and urine samples during MMT is important.[6] In MMT patients, 20–60% of the methadone dose is excreted in urine in 24 h, with up to 33% as unchanged drug.[5] Methadone excretion in urine is pH-dependent, being increased in acid urine (pH 5-6).[4,7]

Several HPLC methods are available for methadone quantification in different biological samples, which involve the isolation of methadone by liquid–liquid extraction (LLE)[8-10] or solid-phase extraction (SPE) [6,10-15] prior to quantification. Unlike classical HPLC techniques, LC/MS/MS techniques showed major advantages by their high specificity and sensitivity. Concerning sample pretreatment, in LC/MS/MS assay the sample preparation is more simple and rapid, and may include only protein precipitation or sample dilution and centrifugation. [7,16,17]

The aim of the present study was to develop and validate a simple, rapid and efficient new LC/MS/MS method for the quantification of methadone in human plasma and urine that can be optimum for application in methadone level monitoring in MMT addicts.

RESULTS

Several mobile phases were tested for the methadone chromatographic separation (Table 1).

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The best sensitivity (signal/noise ratio, S/N) was obtained with a mixture of ACN and 0.2% formic acid (45:55, v/v), this mobile phase being selected. No significant effect of ion suppression was observed for both matrices. No significant interference at the retention time of methadone (1.05 min) was observed (Fig. 1 and 2) due to the specificity of selected signal (Fig. 3).

The method was validated (Tables 2, 3 and 4) and applied for methadone determination in real plasma and urine samples obtained from healthy volunteers (Table 5).

Table 1

Mobil phases tested for the chromatographic methadone separation:

Solvent A	Solvent B	Ratio (v/v)	Detector signal
ACN[1]	Water	45:55	1.7×10^5
ACN	0.1% FA[2]	45:55	8.2×10^5
ACN	0.2% FA	45:55	8.6×10^5
ACN	0.3% FA	45:55	6.8×10^5
ACN	0.01% TFA[3]	45:55	5.2×10^5
ACN	1 mM NH_4Ac[4]	45:55	7.9×10^5
ACN	3 mM NH_4Ac	45:55	6.5×10^5

[1]ACN – acetonitril, [2]FA – formic acid, [3]TFA – trifluoroacetic acid, [4]NH_4Ac – ammonium acetate

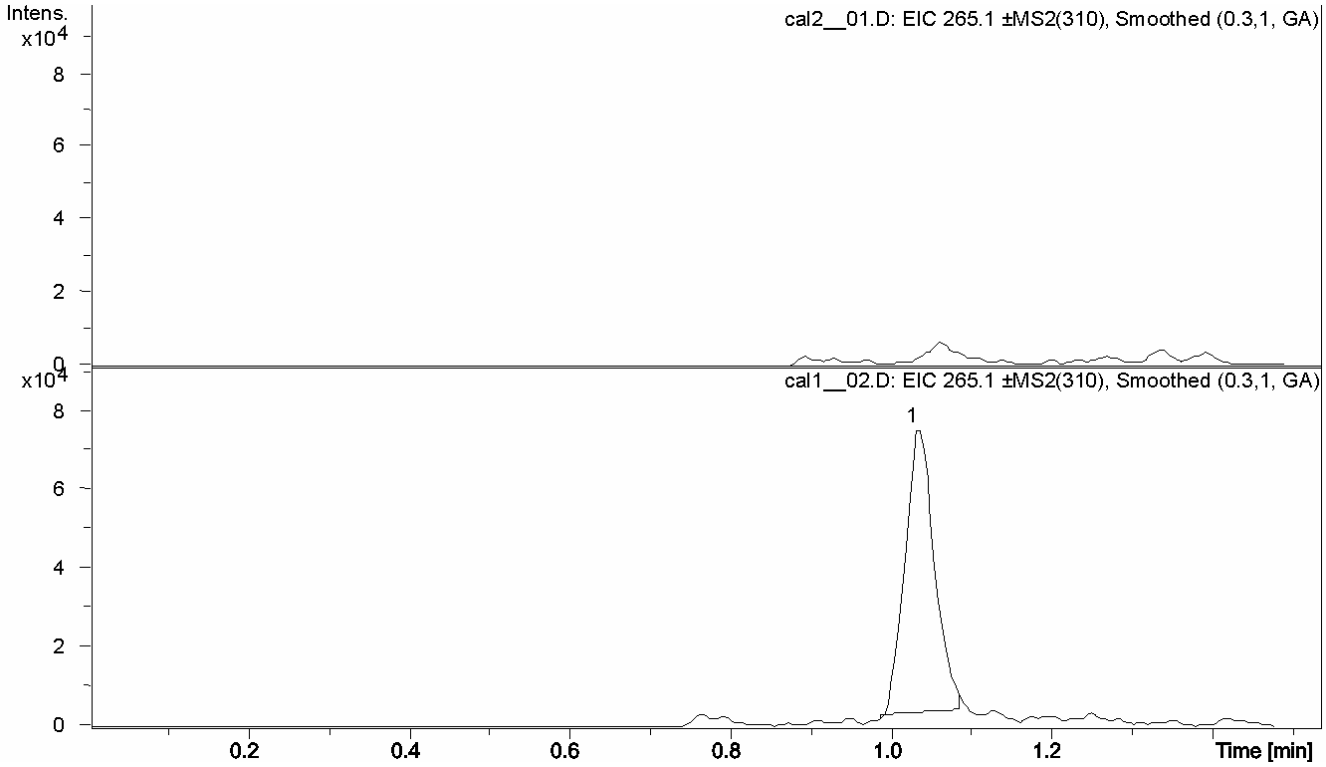

Fig. 1 – Chromatograms of plasma blank and of spiked plasma at LLOQ level (10 ng/mL methadone).

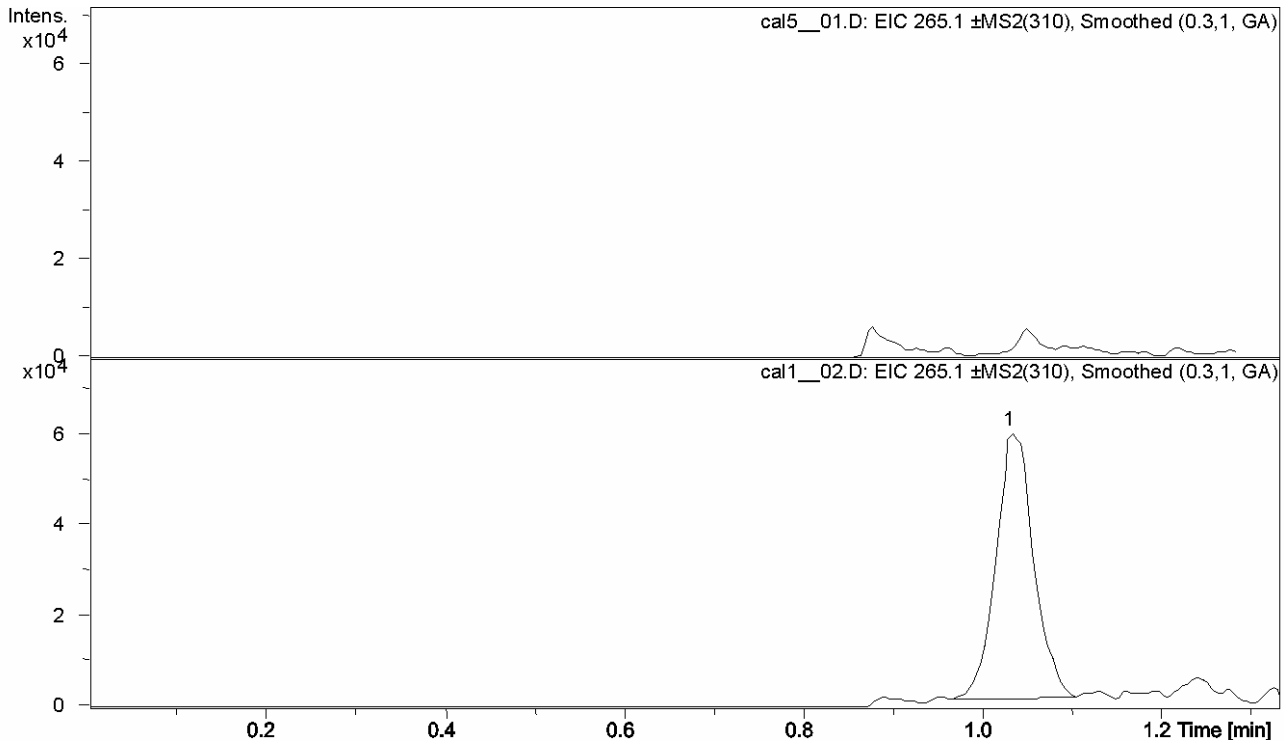

Fig. 2 – Chromatograms of urine blank and of spiked urine at LLOQ level (20 ng/mL methadone).

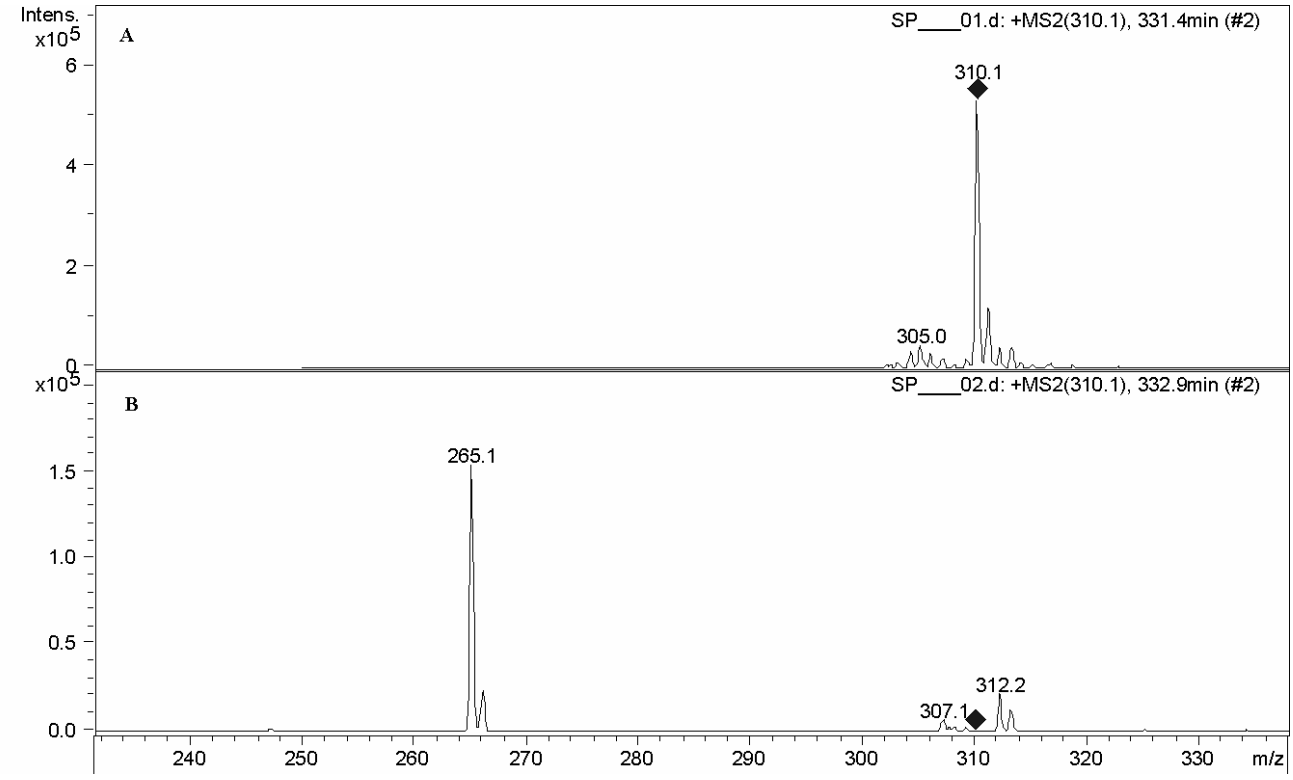

Fig. 3 – (A) (+) ESI / MS spectra methadone (B) CID fragmentation of the molecular ion of methadone.

Table 2

Precision, accuracy and recovery for methadone in plasma (n=5)

C nominal (ng/mL)	Mean C found (ng/mL) (±S.D.)		CV %		Bias %		Recovery % (±S.D.)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
10.00	9.54 (0.85)	9.98 (0.57)	8.9	5.7	-4.6	-0.2	91.7 (7.0)	94.5 (7.7)
30.00	31.12 (1.25)	30.62 (1.82)	4.0	5.9	3.7	2.1	92.5 (3.5)	93.4 (3.6)
200.00	200.55 (15.40)	186.63 (6.69)	7.7	3.6	0.3	-6.7	105.4 (8.0)	98.8 (4.9)
600.00	631.45 (7.38)	647.16 (6.30)	1.2	1.0	5.2	7.9	101.0 (1.2)	103.4 (2.5)

Table 3

Precision, accuracy and recovery for methadone in urine (n=5)

C nominal (ng/mL)	Mean C found (ng/mL) (±S.D.)		CV %		Bias %		Recovery % (±S.D.)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
20.00	21.85 (0.90)	22.25 (1.02)	4.1	4.6	9.3	11.2	94.2 (3.1)	104.1 (7.6)
80.00	77.85 (5.82)	75.99 (6.39)	7.5	8.4	-2.7	-5.0	106.2 (5.4)	94.8 (8.1)
200.00	205.13 (23.84)	200.60 (15.85)	11.6	7.9	2.6	0.3	102.5 (11.3)	93.3 (7.3)
600.00	587.02 (59.99)	649.49 (54.75)	10.2	8.4	-2.2	8.2	93.5 (8.8)	105.6 (7.5)

Table 4

Precision, accuracy and recovery for methadone dilution in plasma and urine samples (n=5)

Sample	C nominal (ng/mL)	Mean C found (ng/mL) (±S.D.)		CV %		Bias %	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Plasma	200.00	211.6 (15.1) 499.2	212.4 (17.6)	7.2	8.3	5.8	6.2
Urine	500.00	(52.4)	440.7 (19.9)	10.5	4.5	-0.2	-11.9

Table 5

Methadone concentrations found in plasma and urine samples from healthy volunteers after oral administration of methadone (single dose)

Subject	Age	Gender	Body Weight (kg)	Methadone intake (mg)	Concentration found (ng/mL)						
					in plasma after			in urine after			
					2 h	6 h	10 h	4.5 h	8 h	10 h	11 h
A	30	M	102	20	134.4	94.7	74.5	744.1	1974.6		2468.9
B	39	F	46	10	89.5	65.1	55.7			1390.0	

DISCUSSION

The method showed a good linearity over the studied concentration range for both biological matrices (10-1000 ng/mL plasma, 20-2000 ng/mL urine; r>0.992). Intra- and inter-day accuracy and precision were lower that ± 15% for all samples, not diluted (Tables 2 and 3) or diluted (Table 4), in agreement to international regulations regarding bioanalytical methods' validation.[18,19] The lower limit of quantification (LLOQ) was established at 10.0 ng/mL methadone for plasma and at 20.0 ng/mL methadone for urine, respectively, with accuracy and precision less than 20% (Tables 2 and 3). The mean recoveries means at the LLOQ level and for the three concentration levels tested for each biological matrix varied between 94-105% in plasma (Table 2) and between 93-105% in urine, respectively (Table 3). Methadone showed good stability in biological matrix for 4 h at room-temperature before processing and post-preparation for at least 24 hours at 25ºC in autosampler before the chromatographic analysis (CV between 3.5% at high tested level and 13.1% at LLOQ in plasma, and between -2.4% at high tested level and -7.9% at LLOQ in urine, respectively).

The methadone levels found in the real plasma and urine samples from healthy volunteers (Table 5) were in the linearity range of the validated method and in agreement with those reported in the literature. [4,6,20] Fig. 4 shows typical chromatograms of the plasma and urine samples from a volunteer after oral administration of a single dose of 20 mg methadone.

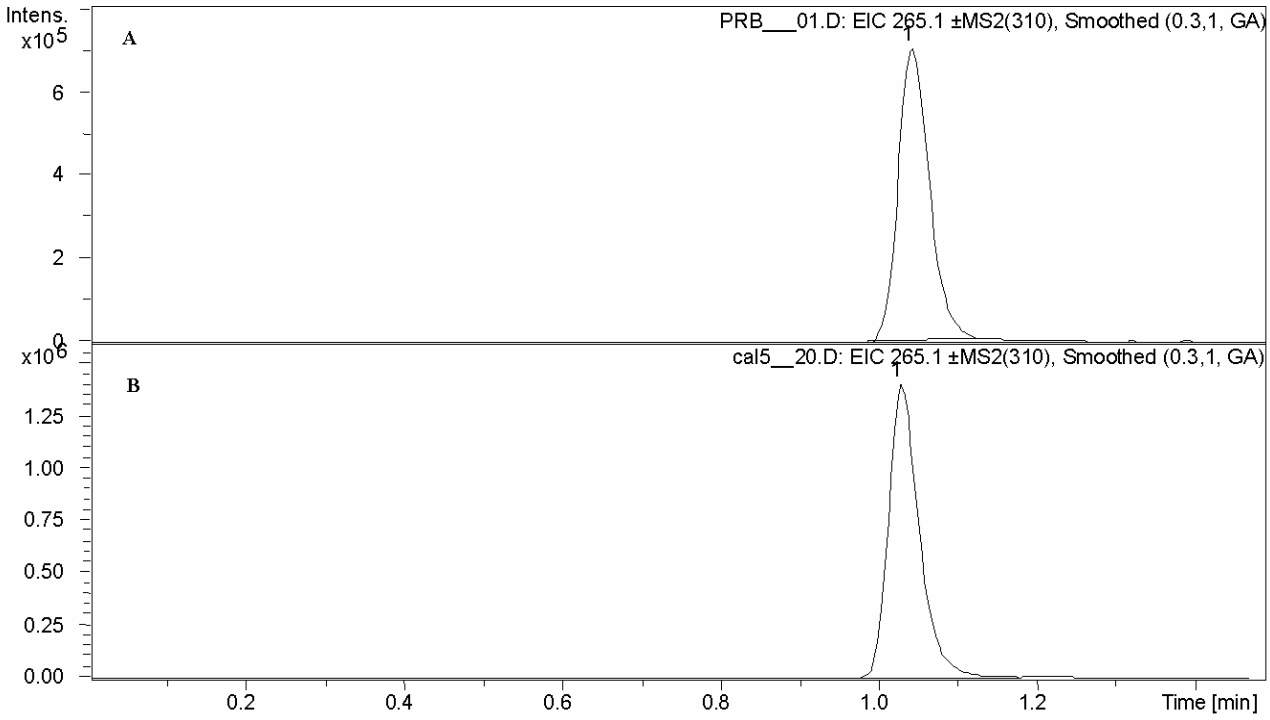

Fig. 4 – Chromatograms of plasma and urine sample from a healthy volunteer, at 2 h and 4.5 h after administration of a single oral dose of 20 mg methadone.

In comparison with previously published LC/MS methods for methadone quantification in plasma (Table 6), the method's sensitivity is not better (LLOQ = 10 ng/mL), but it is inferior to plasma therapeutic levels (0.05-1.0 mg/L),[4] so the method can be successfully applied in methadone treatment monitoring. However, the method offers quantitative recoveries (> 93.4%) and is fast (run time < 2 min, sample pretreatment time < 8 min). Gao *et al.*[19] have studied the influence of the volatile ion-pair reagents on the LC/MS analysis of methadone in plasma. In order to reduce ion suppression, the ion-pair reagent was added to the supernatant obtained after protein precipitation and centrifugation. But the run time and the assay cost increased and the frequent use of ion-pair reagents can impair the column in time. Moreover, the linearity of Gao's method was studied for a narrow concentration range (0.1–50 ng/mL), being recommended for the detection of methadone in

plasma, but not for quantitative analysis in clinical studies as well as in monitoring methadone treatment in addicts. With respect to the method elaborated for methadone quantification in urine, its sensitivity (LLOQ = 20 ng/ml) is better compared to another published HPLC/MS methods, in which solid-phase extraction [14] or protein precipitation and centrifugation [7] were applied. The simple urine dilution followed by centrifugation offers good recoveries (> 93.3%) with a minimum cost and a shorter analysis time.

Table 6

Analytical characteristics of reported HPLC methods with MS detection for the determination of methadone in plasma and/or urine:

References	Biological samples	Column	Mobile phase constituents and flow (mL/min)	Pretreatment/ extraction[a]	LOQ (ng/mL)	Time run (min)	Recovery (%)
Bogusz[6]	Serum, blood, urine	C18	ACN / 50 m*M* ammonium formate, pH 3.0 (45:55, v/v) isocratic mode, 0.4 mL/min	SPE	NA[c]	10.0	87
Bernard et al.[7]	Urine	C18	ACN / 5 mM ammonium acetate buffer pH 5 (35:65), isocratic mode	PP	40	10.0	NA
Cheng et al.[14]	Urine	C18	ACN / 0.01 M ammonium formate, pH 3 (38:62, v/v), isocratic mode, 0.2 mL/min	SPE	40-60	10.0	87.2
Shakleya et al.[16]	Plasma	C18	10mM ammonium acetate in water, 0.001% formic acid, pH 4.5/ ACN, gradient mode, 0.2 mL/min	PP	1.0	16.0	87.5
Gao et al.[17]	Plasma	C18	0.1% formic acid in deionized water / 0.1% formic acid in ACN, gradient mode, 0.3 mL/min	PP[b]	0.1	4.5	82.1-93.7
Ganssmann et al.[21]	Plasma, urine	C8	4mM ammonium acetate pH 4.6 /1:1, *v/v,* methanol-ACN (35:65), isocratic mode, 0.25 mL/min	LLE	*	5.0	*
Quintela et al.[22]	Plasma	C18	ACN / 0.1% formic acid, gradient mode, 0.6 mL/min	SPE	2	2.5	83.6-83.9
Rook et al.[23]	Plasma	C18	5mM Ammonium formate in water (pH 4.0) / ACN, gradient mode, 1 mL/min	SPE	5	15.0	77-86

[a]LLE, liquid–liquid extraction; SPE, solid-phase extraction; PP, protein precipitation. [b]Ion-pairing reagent was added to the supernatant. [c]NA, not available. [*]Methadone was used as internal standard.

EXPERIMENTAL

Reagents

Methadone was reference standard from Lipomed AG (Arlesheim, Switzerland). All chemicals were of analytical-reagent grade. HPLC-grade acetonitrile (ACN), HPLC-grade methanol, 98% formic acid (FA), trifluoroacetic acid (TFA) and ammonium acetate (NH_4Ac) were purchased from Merck (Darmstadt, Germany). Bidistilled, deionised water pro injections was purchased from Infusion Solution Laboratory of University of Medecine and Pharmacy Cluj-Napoca (Roumania). The human blank plasma was supplied by the Bleeding Centre Cluj-Napoca (Roumania), and the blank urine from the healthy volunteers.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 (Scientific Industries, New York, USA); ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent model (Darmstadt, Germany) consisting of a G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 VL (Darmstadt, Germany).

Chromatographic and spectrometric conditions

Chromatographic separation was performed at 45ºC on a Zorbax SB-C18 (100 mm x 3.0 mm, 3.5 µm I.D.) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 45:55 (v/v) mixture of acetonitrile and 0.2% (v/v) formic acid in water and a flow rate of 1 mL/min. The detection of methadone was in the multiple-reaction monitoring (MRM) mode (monitored ion transition: m/z 310→ m/z 265) using an ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionisation ion source (capillary 4000 V, nebulizer 70 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350ºC).

Standard solutions and sample preparation

A stock solution of methadone (0.5 mg/mL in methanol) and two working solutions for each biological matrix (0.02 mg/mL and 0.001 mg/mL in plasma, and 0.05 mg/mL and 0.002 mg/mL in urine, respectively) were used. These solutions were used to prepare plasma standards with the concentrations ranging between 10.00 – 1000.00 ng/mL (n=7), and urine standards with the concentrations ranging between 20.00 – 2000.00 ng/mL (n=7), respectively. Before the chromatographic analysis, standard and sample plasma (0.2 mL) were deproteinizated with methanol (0.6 mL), and standard and sample urine (0.2 mL) were diluted with bidistilled water (0.6 mL). After vortex-mixture (10 s) and centrifugation (6 min at 5000 rpm), the supernatant (0.15 mL) was transferred in autosampler vials and 3 µL were injected into the HPLC system.

Method validation

The method was validated in agreement to international regulations.[18,19] Specificity was verified using different plasma blanks (n=6) and different urine blanks (n=6) obtained from healthy human volunteers who have not previously taken any medication. The concentration of methadone was determined automatically by the instrument data system using peak areas and the external standard method; the calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted ($1/y$) linear regression, where y – peak area and x – analyte concentration. The accuracy and precision of the method were verified using plasma standards with concentrations of 30.00 ng/mL (low), 200.00 ng/mL (medium) and 600.00 ng/mL (high) methadone, and urine standards with concentrations of 80.00 ng/mL (low), 200.00ng/mL (medium) and 600.00 ng/mL (high) methadone, respectively. The intra- and inter-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias%) were determined by analysis on the same day of five different samples at each of the lower, medium and higher levels of the considered concentration range and one different sample of each on five different occasions, respectively. The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%. The relative recoveries were measured by comparing the response of the treated plasma and urine standards with the response of standards in solvent with the same concentration of methadone as the plasma and urine samples. The methadone stability in plasma (at 30.00 ng/mL and 600.00 ng/mL, n=5) and urine (at 80.00 ng/mL and 600.00 ng/mL, n=5) was investigated. For the room-temperature stability (RTS) study, the samples were prepared and kept at room temperature 4 h, then they were processed and analyzed by HPLC. For the post-preparative stability (PPS) study, the samples were prepared, processed and kept 24 h in the autosampler of the HPLC system at 25ºC before the chromatographic analysis. Samples stability is assumed when the difference between mean concentrations of the tested samples in various conditions and nominal concentrations was placed within ±15% interval against the theoretical concentration value. The dilution sample validation (in a 1:10 ratio) was determined for the standards of 2000 ng/mL methadone in plasma and 5000 ng/mL methadone in urine, respectively (n = 5). The requirement was that the accuracy and the precision for the same series of determinations or for different series was in the ±15% range.

The validated method was applied for the determination of methadone concentrations in plasma and urine samples obtained from healthy volunteers after oral administration of a single dose of methadone tablets (Sintalgon®, Sicomed, Roumania).

CONCLUSIONS

A new LC/MS/MS method for the bioanalysis of methadone in human plasma and urine was developed and validated. The method is very simple and rapid, the method specificity and efficiency being ensurred by a simple sample preparation procedure (protein precipitation or urine dilution and centrifugation) and by the selected signal used for monitoring. The method has clinical applicability and can be used in routine bioanalysis, especially for methadone level monitoring in MMT addicts, as well as in other pharmaco-toxicological studies.

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REFERENCES

1. D.A.Fiellin, P.G.O'Connor, M.Chawarski, J.P.Pakes, M.V.Pantalon and R.S.Schottenfeld, *Journal of the American Medical Association,* **2001**, *286*, 1724-1731.
2. D.C.Lehotay, S.George, M.L.Etter, K.Graybiel, J.C.Eichhorst, B.Fern, W.Wildenboer, P.Selby and B.Kapur, *Clinical Biochemistry*, **2005**, *38*, 1088-1094.
3. J.Hanna, D.J.R.Foster, A.Salter, A.A.Somogyi, J.M.White and F.Bochner, *British Journal of Clinical Pharmacology*, **2005**, *60*, 404-413.
4. A.C.Moffat, M.D.Osselton and B.Widdop, "Clarke's Analysis of Drugs and Poisons in pharmaceuticals, body fluids and postmortem material", Pharmaceutical Press, London, **2004**, vol. 2, 3rd edition, p.1231-1233.
5. T.Kelly, P.Doble and M.Dawson, *Journal of Chromatography B*, **2005**, *814*, 315-323.
6. M.J.Bogusz, *Journal of Chromatography B,* **2000**, *748*, 3-19.
7. J.P.Bernard, M.S.Opdal, R.Karinen, J.Mørland and H.Z.Khiabani, *European Journal of Clinical Pharmacology*, **2007**, *63*, 777-782.
8. C.Joyce, W.F.Smyth, V.N.Ramachandran, E.O'Kane and D.J.Coulter, *Journal of Pharmaceutical and Biomedical Analysis*, **2004**, *36*, 465-476.
9. H.R.Liang, R.L.Foltz, M.Meng and P.Bennett, *Journal of Chromatography B*, **2004**, *806*, 191-198.
10. H.He, C.Sun, X.R.Wang, C.Pham-Huy, N.Chikhi-Chorfi, H.Galons, M.Thevenin, J.R.Claude and J.M.Warnet, *Journal of Chromatography B*, **2005**, *814*, 385-391.
11. Y.F.Cheng, U.D.Neue and L.L.Woods, *Journal of Chromatography B*, **1999**, *729*, 19-31.
12. P.Fernandez, L.Morales, C.Vazquez, A.M.Bermejo and M.J.Tabernero, *Forensic Science International*, **2006**, *161*, 31-35.

13. L.Mercolini, R.Mandrioli, M.Conti, C.Leonardi, G.Gerra and M.A.Raggi, *Journal of Chromatography B*, **2007**, *847*, 95-102.
14. W.C.Cheng, T.S.Yau, M.K.Wong, L.P.Chan and V.K.K.Mok, *Forensic Science International*, **2006**, *162*, 95-107.
15. F.M.Wylie, H.Torrance, R.A.Anderson and J.S.Oliver, *Forensic Science International*, **2005**, *150*, 191-198.
16. D.M.Shakleya, L.M.Jansson and M.A.Huestis, *Journal of Chromatography B*, **2007**, *856*, 267-272.
17. S.Gao, S.Bhoopathy, Z.P.Zhang, D.S.Wright, R.Jenkins and H.T.Karnes, *Journal of Pharmaceutical and Biomedical Analysis*, **2006**, *40*, 679-688.
18. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, published in the Federal Register 66/100, **2001**.
19. Note for Guidance on the Investigation of Bioavailability and Bioequivalence, The European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products, CPMP/EWP/QWP/1401/98, **2001**.
20. R.J.Flanagan, *Annals* of *Clinical Biochemistry*, **1998**, *35*, 261-267.
21. B.Ganssmann, A.Klingmann, J.Burhenne, Y.Tayrouz, R.Aderjan and G.Mikus, *Chromatographia*, **2001**, *53*, 656-660.
22. O.Quintela, P.Lopez, A.M.Bermejo and M.Lopez-Rivadulla, *Journal of Chromatography B*, **2006**, *834*, 188-194.
23. E.J.Rook, M.J.X.Hillebrand, H.Rosing, J.M.van Reeb and J.H.Beijnen, *Journal of Chromatography B*, **2005**, *824*, 213-221.