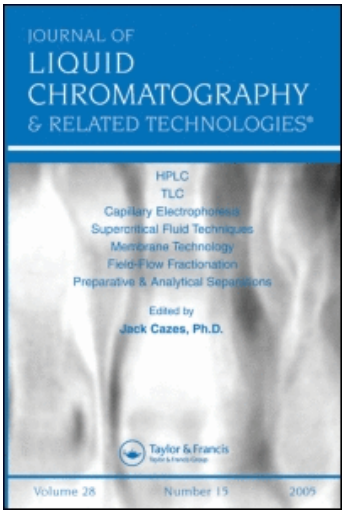


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Development and Validation of a Rapid HPLC Method for the Determination of Methadone and its Main Metabolite EDDP in Biological Fluids, Following SPE

V. F. Samanidou[a]; K. Anastasiadou[a]; I. N. Papadoyannis[a]
[a] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

To cite this Article Samanidou, V. F. , Anastasiadou, K. and Papadoyannis, I. N.(2006) 'Development and Validation of a Rapid HPLC Method for the Determination of Methadone and its Main Metabolite EDDP in Biological Fluids, Following SPE', Journal of Liquid Chromatography & Related Technologies, 29: 6, 889 — 902
To link to this Article: DOI: 10.1080/10826070500531433
URL: http://dx.doi.org/10.1080/10826070500531433

Journal of Liquid Chromatography & Related Technologies®, 29: 889–902, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500531433

Development and Validation of a Rapid HPLC Method for the Determination of Methadone and its Main Metabolite EDDP in Biological Fluids, Following SPE

V. F. Samanidou, K. Anastasiadou, and I. N. Papadoyannis
Laboratory of Analytical Chemistry, Department of Chemistry,
Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract: A reversed-phase high performance liquid chromatographic (RP-HPLC) method is developed and validated for the determination of methadone, a substitute of heroin used for drug addicts under heroin detoxication treatment in therapeutic communities, as well as its main metabolite EDDP in biological fluids. The analytical column, a Kromasil C_{18}, 5 μm, 250 × 4 mm, was operated at ambient temperature using isocratic elution with a mixture of CH_3CN – 0.025 M CH_3COONH_4 (90–10 v/v) at a flow rate 1.2 mL/min. Anthracene (1 ng/μL) was used as the internal standard. Inlet pressure was 220 kg/cm^2. A DAD detector was monitoring the column eluant at 220 nm.

The limit of detection was 10 ng for EDDP and 20 ng for methadone, per 20 μL injection volume. Linearity held up to 20 ng/μL for methadone and 15 ng/μL for its metabolite. The statistical evaluation of the method was performed in terms of within-day (n = 6) and between-day (n = 8) precision and accuracy, and was found to be satisfactory, with high accuracy and precision results.

The method was successfully applied to biological fluids. Blood serum samples after deproteinization with acetonitrile and solid phase extraction, yielded high recovery rates: 101.5% for EDDP and 103.3% for methadone. Direct analysis of urine provided recovery rates at 97.9% for EDDP and 94.9% for methadone. The developed method can be readily applied to monitoring the levels of methadone and EDDP in biological samples from patients undergoing methadone maintenance therapy, in order to individualize treatment.

Keywords: Methadone, EDDP, Biological fluids, Serum, Urine, HPLC, SPE

Address correspondence to I. N. Papadoyannis, Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, GR-54124 Thessaloniki, Greece. E-mail: papadoya@chem.auth.gr

INTRODUCTION

Methadone [6-(dimethylamino)-4,4-diphenyl-3-heptanone], formerly known as dolophine, is a synthetic opioid analgesic used to relieve chronic pain in cancer patients, and as a maintenance drug to control withdrawal symptoms in people undergoing treatment for opiate addiction. In the latter case, methadone blocks the opioid receptors of the brain that bind opiates such as heroin.[1,2]

Methadone maintenance programs are applied in many countries as the most common and effective means for treating heroin addiction. By administration of methadone to the narcotics abusers, negative and sometimes severe withdrawal symptoms are eliminated.[3]

The available methadone hydrochloride in the market is a racemic mixture of two stereoisomers. L-methadone is the pharmacologically active isomer and D-methadone has an antitussive activity.

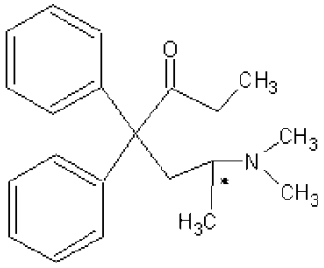
The pharmacokinetics of methadone varies greatly from person to person leading to differences in the optimum dosages for maintenance treatment. Methadone is mostly metabolised in the liver. Metabolism consists of N-demethylation by CYP3A4 to EDDP [2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine], an inactive metabolite. Since the activity of CYP3A4 varies considerably among individuals, large differences in methadone bioavailability are noticed. The major factor responsible for the variation of methadone bioavailability is the inter-individual difference in the expression of CYP3A4.[4–6] The chemical structures of methadone and EDDP are shown in Figure 1.

Methadone taken orally is detectable in the plasma about 30 min after administration. Body clearance of methadone varies widely among individuals, contributing to the large differences in methadone kinetics among drug addicts. The elimination of methadone and its metabolites occurs mainly through the kidneys: 15–60% during the first 24 h (20% as unmodified drug, and 13% as EDDP).[4]

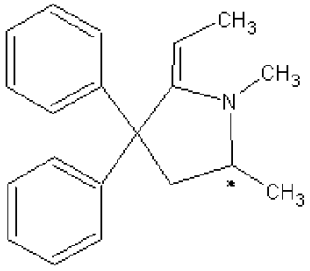
Despite its positive behaviour, adverse effects during methadone administration may also be noticed. The most important short term side effects of methadone are restlessness, vomiting, nausea, slowed breathing, itchy skin, pupil contraction, while long-term adverse effects are lung and respiration problems. Pregnancy complications may also be noticed in cases where dosages are reduced during pregnancy.[3]

Patients receiving methadone should be monitored for adverse reactions to this drug, and/or possible accidental overdose. For these reasons, the need for a sensitive, selective, accurate, and precise method is clearly justified.

Analytical methods found in literature for the determination of methadone and EDDP in biological fluids include gas chromatography[7–9] and capillary electrophoresis,[10–13] however, the vast majority of methods include liquid chromatographic techniques.[14–18] Chiral methods are also



Methadone



EDDP

Figure 1. Chemical structure of methadone and EDDP.

provided since methadone and EDDP can be found in two stereoisomeric forms (R− and S−).[19−30] Surface enhanced Raman scattering (SERS) spectroscopy has been employed to characterise different drugs and some of their degradation products contained in biomatrices after separation by HPLC.[31]

For sample preparation prior to the quantitation of methadone and its metabolite in biological fluids (e.g., urine, serum), either liquid−liquid extraction[11,19,20] or solid-phase extraction (SPE)[14,22,28] have been used, however, the latter leads to higher recovery rates within shorter preparation times. SPME has also been introduced recently for the screening of drugs in urine and confirmation analysis of suspected substances in serum.[9]

In the present paper, an accurate, precise, and rapid analytical method was developed and validated for the direct HPLC determination of methadone and EDDP in biological fluids: blood serum and urine. The developed method can be readily applied to the monitoring of methadone levels in biological fluids of patients undergoing methadone maintenance therapy.

EXPERIMENTAL

Instrumentation and Chromatography

A Shimadzu (Kyoto, Japan) quaternary low pressure gradient system was used for chromatographic determination of methadone and EDDP. The solvent lines were mixed in an FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the analytical column. Sample injection was performed by an SIL-9A autosampler, and detection was achieved by an SPD-M6A Photodiode Array Detector with Data acquisition software Class-M10A. Degassing of solvents was achieved by continuous helium sparking in the solvent flasks through a DGU-2A degassing unit.

A Kromasil C_{18} 5 μm, 250 × 4 mm, analytical column purchased from MZ-Analysentechnik (Mainz, Germany) was used.

A glass vacuum filtration apparatus obtained from Alltech Associates (Deerfield, IL, USA) was employed for the filtration of the ammonium acetate solution, using 0.2 μm membrane filters obtained from Schleicher & Schuell (Dassel, Germany).

Dissolution of compounds was enhanced by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). A Glass-col (Terre Haute, IN USA) small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pretreatment. All evaporations were performed with a 9 port Reacti-Vap™ evaporator, Pierce, Model 18780 (Rockford, IL, USA). The UV spectrum of methadone and EDDP for selecting the working wavelength of detection was taken using a Varian DMS 100S UV–Vis double beam spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

The mobile phase was CH_3COONH_4 0.025 M-CH_3CN 10 : 90 v/v. Inlet pressure, observed at a flow rate of 1.2 mL/min, was 220 kg/cm^2. The injection volume was 20 μL. Anthracene was used as an internal standard at a concentration of 1.0 ng/μL.

The SPE study was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, Varian (Harbor City, CA, USA). Nexus cartridges were supplied from Varian (Harbor City, CA, USA) and Discovery C_{18} from Supelco (Bellefonte, PA, USA).

Samples, Chemicals, and Reagents

Methadone standard solutions were prepared from tablets after fine powdering and dissolution in methanol. EDDP was purchased from Sigma (Sigma-Aldrich Chemie BV, The Netherlands). Methanol and acetonitrile of HPLC grade were purchased from Carlo Erba (Rodano, Italy). Ammonium acetate of analytical grade was supplied by Riedel-de-Haen (Seelze, Germany). Bis dionized water was used throughout the study.

Serum samples were kindly provided from the Blood Donation Unity of a State Hospital, while urine samples were provided by healthy volunteers.

Stock standard solutions (80.0 ng/μL for methadone and 100.0 ng/μL for EDDP) were prepared in methanol. Working methanolic standards were prepared by appropriate dilution at various concentrations covering the linear range from 0.5–20.0 ng/μL. These solutions, when kept refrigerated, were found to be stable for at least 3 months.

Anthracene (methanolic solution) at a concentration of 1.0 ng/μL was selected as the most suitable internal standard.

Validation of the Method

The sensitivity, specificity, linearity, within- and between-day precision and accuracy, and extraction recovery of the developed method were fully evaluated.

The linearity response was assessed in the range of ng/μL. Method validation regarding repeatability was achieved by replicate injections of standard solutions at three concentration levels (5, 10, and 15 ng/mL for EDDP and 5, 10, and 16 ng/mL for methadone); peak areas were measured versus peak area of the internal standard. Statistical evaluation revealed relative standard deviations at different values for six injections. Intermediate precision study was conducted during routine operation of the system over a period of eight consecutive days. In both validation processes, accuracy was determined by replicate analysis and it was expressed as relative error of measurement.

Samples of biological fluids spiked at three concentration levels (8, 12, and 15 ng/mL for methadone and 8, 10, and 14 ng/mL for EDDP) were used for estimating accuracy and precision in terms of recovery. Repeatability was checked by six replicates of spiked biological samples.

The sensitivity of the developed method was checked in terms of limits of detection (LOD) and quantitation (LOQ). The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal to-noise ratio.

Sample Preparation

Blood Serum and Urine

To an aliquot of 50 μL of pooled blood serum, proteins were precipitated using 500 μL of acetonitrile. After centrifugation for 15 min at 3500 rpm, the supernatant, after dilution with water, was transferred to the preconditioned SPE cartridges. Two types of sorbents were studied: Abselut Nexus from Varian and Discovery DSC-18 from Supelco, with various eluting

systems such as methanol, acetonitrile, and their mixtures with 1% acetic acid or 1% HCl. After evaporation to dryness at 45°C under a gentle nitrogen stream, the dry residue was reconstituted to volume with 100 μL of methanolic solution of internal standard, which in this assay was used as a chromatographic one.

Pooled urine samples were filtered and directly analyzed after addition of standards.

Calibration curves were constructed using spiked serum and urine samples at concentration levels in the range from 5.0 to 16.0 ng/μL.

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram obtained using the developed method conditions is illustrated in Figure 2. Retention times revealed were 3.3 min and 7.4 min for

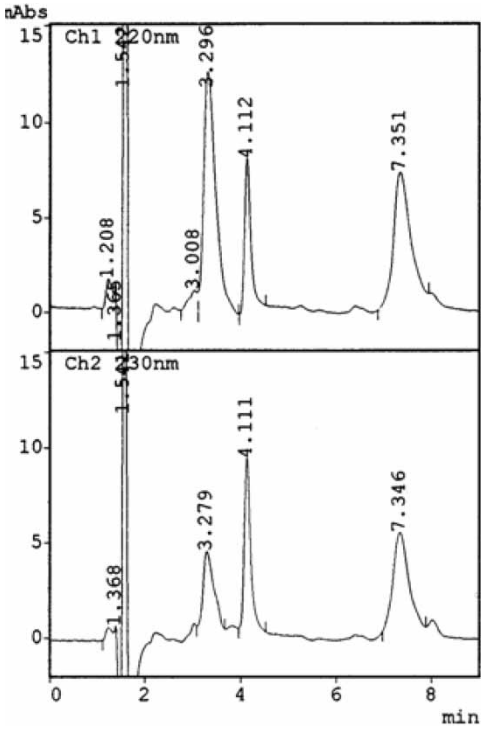


Figure 2. Chromatogram of standard solution of methadone (7.4 min) and EDDP (3.3 min). Chromatographic conditions are described in text. Anthracene (4.1 min) is used as internal standard.

Table 1. Linearity and sensitivity data for the determination of methadone and its major metabolite EDDP

Analyte	Regression equation	Correlation coefficient R	LOD (ng)	LOQ (ng)	Upper limit (ng)
Standard solutions					
EDDP	$y = (0.01305 \pm 0.00017)\,x + (4.05400 \pm 0.02865)$	0.9997	10	30	300
Methadone	$y = (0.01188 \pm 0.00040)\,x - (0.08473 \pm 0.07178)$	0.9983	20	60	400
Serum					
EDDP	$y = (0.01944 \pm 0.00167)\,x + (0.33368 \pm 0.26555)$	0.9963			
Methadone	$y = (0.01019 \pm 0.00025)\,x + (0.25701 \pm 0.04352)$	0.9997			
Urine					
EDDP	$y = (0.02516 \pm 0.00165)\,x - (0.76168 \pm 0.32314)$	0.9957			
Methadone	$y = (0.01170 \pm 0.00043)\,x - (0.14178 \pm 0.09209)$	0.9986			

$y =$ Peak area ratio of analyte versus internal standard.
$x =$ ng.

EDDP and methadone, respectively. The internal standard was eluted at 4.1 min. Resolution factor between EDDP and IS was 1.1, while the respective value for IS and methadone was 4.4, indicating an excellent separation.

Method Validation

Linearity and Sensitivity

Calibration curves were obtained by least squares linear regression analysis of the peak areas ratio of methadone and EDDP to the internal standard versus the analyte's absolute amount. The method exhibits linearity up to 15 and 20 ng/μL for EDDP and methadone, respectively.

The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three, and it was found to be 20 ng for methadone and 10 ng for its metabolite. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal-to-noise ratio and it was found to be 60 ng for methadone and 30 ng for the metabolite. Table 1 summarizes all calibration and sensitivity data.

Specificity

Five different individual lots of blank serum and urine were extracted and analyzed as blanks. The results showed that there were no measurable peak

Table 2. Accuracy and precision data of the determination of methadone and EDDP

Analyte	Added (ng)	Found ± SD (ng)	Bias (%)	RSD
Within-day repeatability (n = 6)				
EDDP	100	102.5 ± 2.2	+2.5	2.2
	200	195.1 ± 9.5	−2.5	4.8
	300	302.5 ± 3.4	+0.8	1.1
Methadone	100	94.2 ± 3.3	−5.8	3.5
	200	217.8 ± 7.7	+8.9	3.6
	320	347.2 ± 7.3	+8.5	2.1
Between-day precision (n = 8 days × 3 measurements)				
EDDP	100	104.1 ± 6.3	+4.1	6.1
	200	189.6 ± 11.7	−5.2	6.2
Methadone	300	288.2 ± 16.2	−3.9	5.6
	100	96.3 ± 9.0	−3.7	9.3
	200	200.5 ± 15.8	+0.2	7.9
	320	322.6 ± 14.7	+8.1	4.5

areas detected in the blanks for the analyte or internal standard that could affect quantitation.

Precision and Accuracy

The accuracy and precision of the method based on within-day repeatability was performed by replicate injections ($n = 6$) of three standard solutions, covering different concentration levels, low, medium, and high, where peak areas were measured in comparison to the peak area of the internal standard. The reproducibility (between-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days. Statistical evaluation revealed relative standard deviations, at different values. Table 2 summarizes the results of the method validation regarding accuracy, within-day, and between-day precision assays. The measured concentrations had RSD values $<5\%$ for within-day precision and $<10\%$ for between-day precision, with relative error (inaccuracy) in the range of -5.8 to $+8.9\%$.

Accuracy was expressed as relative error calculated by the equation:

$$\text{Relative error}\,(\%) = \frac{[\text{mean determined value} - \text{theoretical (added amount)}]}{\text{theoretical}} \times 100$$

Table 3. Methadone and metabolite recovery results using different SPE protocols

		Recovery (%)	
Cartridge	Elution solvent	EDDP	Methadone
Varian abselut Nexus	MeOH	36.2	71.9
	ACN	40.7	55.3
	MeOH-1% acetic acid	64.5	73.2
	ACN- 1% acetic acid	51.6	67.8
	MeOH –1% HCl	48.3	92.4
	ACN–1% HCl	60.4	84.7
	MeOH –1% HCl	72.4	89.9
Supelco discovery DSC-18	MeOH	4.3	56.6
	ACN	1.6	45.2
	MeOH –1% HCl	97.9	102.1
	ACN–1% HCl	35.7	46.1

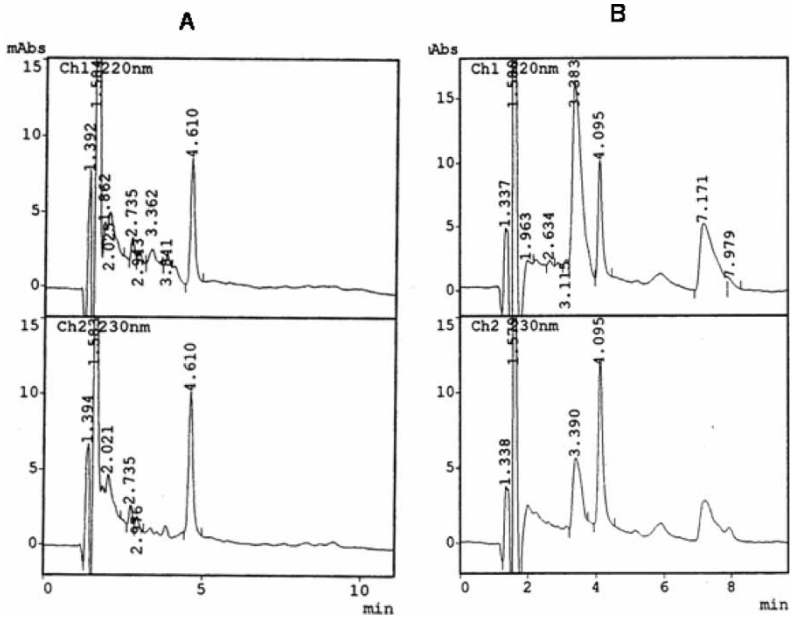


Figure 3. A. Blank chromatogram of serum sample. B. Chromatogram of methadone (7.2 min) and EDDP (3.4 min) determination in spiked serum sample in the presence of anthracene (4.1 min).

Application to Biological Fluids

The developed method was subsequently applied to biological fluids. SPE protocol development was executed studying their retention and recovery behavior using two sorbent types and seven elution systems as shown in Table 3. From the results tabulated it is obvious that Discovery C_{18} cartridges with CH_3OH-1% HCl as eluent yielded the maximum recovery rates.

Optimum protocol was subsequently applied to biological fluids. Serum samples were deproteinised by acetonitrile prior to SPE, while urine samples were simply filtered.

Typical chromatograms of blank and spiked samples of blood serum and urine are presented in Figure 3A, 3B, and Figure 4A and 4B, respectively. No endogenous interference is noticed in biological matrices.

The method was also validated using spiked samples of biological fluids. The results are summarized in Table 4. High recovery rates are obtained, ranging from 95.5 to 107.9% for blood serum samples and from 87.7 to 107.8% for urine samples. The assay procedures are simple, with satisfactory precision and accuracy (RSD $< 9\%$).

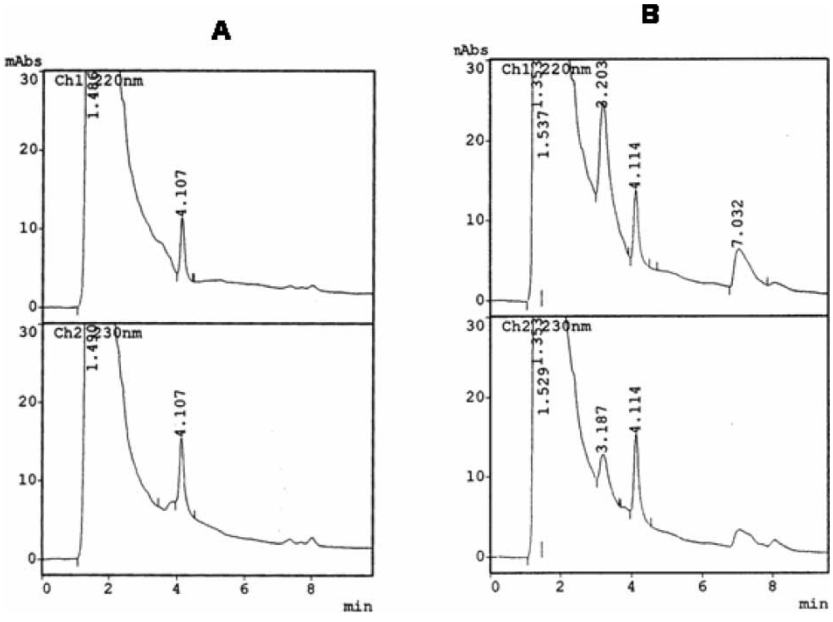


Figure 4. A. Blank chromatogram of urine sample. B. Chromatogram of methadone (7.0 min) and EDDP (3.2 min) determination in spiked urine sample in the presence of anthracene (4.1 min).

Table 4. Within-day precision data of methadone and EDDP isolation from biological fluids

Analyte	Added (ng)	Found ± SD (ng)	RSD	Recovery (%)
Serum				
EDDP	160	161.4 ± 13.3	8.2	100.9
	200	208.8 ± 7.9	3.8	104.4
Methadone	280	278.1 ± 5.7	2.0	99.3
	160	172.6 ± 11.8	6.8	107.9
	240	255.4 ± 11.4	4.5	106.4
	300	286.6 ± 1.4	0.5	95.5
Urine				
EDDP	160	141.7 ± 11.1	7.8	88.5
	200	195.1 ± 5.7	2.9	97.5
Methadone	280	302.0 ± 8.3	2.7	107.8
	160	140.3 ± 10.9	7.8	87.7
	240	257.7 ± 8.3	3.2	107.4
	300	268.8 ± 9.9	3.7	89.6

CONCLUSIONS

The method described herein is a simple, validated assay that can be readily used for the determination of methadone and its metabolite EDDP in biological fluids, such as human serum and urine. The assay procedures are simple with satisfactory precision and accuracy. Overall RSD values obtained were lower than 10%.

High percentage recoveries from biological fluids were noticed without endogenous interference. The method can be applied to monitor pharmacokinetics of methadone among different individuals, which is necessary in order to individualize doses to achieve optimum treatment.

REFERENCES

1. http://www.healthatoz.com/healthatoz/Atoz/ency/methadone.jsp. Access, May 2005.
2. http://en.wikipedia.org/wiki/Methadone. Access, February 2005.
3. http://www.cesar.umd.edu/cesar/drugs/methadone.asp. Access, June 2005.
4. Ferrari, A.; Coccia, C.P.R.; Bertolini, A.; Sternieri, E. Methadone—metabolism, pharmacokinetics and interactions. Pharmacol. Res. **2004**, *50* (6), 551–559.
5. Wolff, K.; Sanderson, M.; Hay, A.W.M.; Raistrick, D. Methadone concentrations in plasma and their relationship to drug-dosage. Clin. Chem. **1991**, *37* (2), 205–209.
6. Chikhi-Chorfi, N.; Pham-Huy, C.; Sandouk, P.; Sadeg, N.; Pecquart, C.; Massicot, F.; Galons, H.; Scherrmann, J.M.; Warnet, J.M.; Claude, J.R. Pharmacokinetic profiles of methadone enantiomers and its major metabolite in serum, saliva and urine of treated patients. Toxicol. Lett. **1998**, *95* (1), 103.
7. Chikhi-Chorfi, N.; Pham-Huy, C.; Galons, H.; Manuel, N.; Lowenstein, W.; Warnet, J.M.; Claude, J.R. Rapid determination of methadone and its major metabolite in biological fluids by gas–liquid chromatography with thermionic detection for maintenance treatment of opiate addicts. J. Chromatogr. B. **1998**, *718* (2), 278–284.
8. Moore, C.; Guzaldo, F.; Hussain, M.J.; Lewis, D. Determination of methadone in urine using ion trap GC/MS in positive ion chemical ionization mode. Forensic Sci. Intl. **2001**, *119* (2), 155–160.
9. Staerk, U.; Külpmann, W.R. High-temperature solid-phase microextraction procedure for the detection of drugs by gas chromatography–mass spectrometry. J. Chromatogr. B. **2000**, *745* (2), 399–411.
10. Molteni, S.; Caslavska, J.; Allemann, D.; Thormann, W. Determination of methadone and its primary metabolite in human urine by capillary electrophoretic techniques. J. Chromatogr. B. **1994**, *658* (2), 355–367.
11. Frost, M.; Koehler, H.; Blaschke, G. Enantioselective determination of methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in serum, urine and hair by capillary electrophoresis. Electrophoresis **1997**, *18* (6), 1026–1034.
12. Caslavska, J.; Allemann, D.; Thormann, W. Analysis of urinary drugs of abuse by a multianalyte capillary electrophoretic immunoassay. J. Chromatogr. A **1999**, *838* (1–2), 197–211.

13. Esteban, J.; de la Cruz Pellín, M.; Gimeno, C.; Barril, J.; Mora, E.; Giménez, J.; Vilanova, E. Detection of clinical interactions between methadone and anti-retroviral compounds using an enantioselective capillary electrophoresis for methadone analysis. Toxicol. Lett. **2004**, *151* (1), 243–249.
14. Cheng, Y.F.; Neue, U.D.; Woods, L.L. Novel high-performance liquid chromatographic and solid-phase extraction methods for quantitating methadone and its metabolite in spiked human urine. J. Chromatogr. B **1999**, *729* (1–2), 19–31.
15. Achilli, G.; Cellerino, G.P.; Melzi d'Eril, G.M.; Tagliaro, F. Determination of illicit drugs and related substances by high-performance liquid chromatography with an electrochemical coulometric-array detector. J. Chromatogr. A **1996**, *729* (1–2), 273–277.
16. Roy, I.M.; Jefferies, T.M. Performance evaluation of an aqueous organic phase separator for post-column reactions in high-performance liquid chromatography, and its application to the enhanced detection of some basic drugs of abuse. J. Pharmaceut. Biomed. Anal. **1990**, *8* (8–12), 831–835.
17. Choo, R.E.; Murphy, C.M.; Jones, H.E.; Huestis, M.A. 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. J. Chromatogr. B **2005**, *814* (2), 369–373.
18. Souverain, S.; Rudaz, S.; Ortelli, D.; Varesio, E.; Veuthey, J.-L. Use of large particles support for fast analysis of methadone and its primary metabolite in human plasma by liquid chromatography–mass spectrometry. J. Chromatogr. B **2003**, *784* (1), 117–123.
19. Schmidt, N.; Brune, K.; Geisslinger, G. Stereoselective determination of the enantiomers of methadone in plasma using high-performance liquid chromatography. J. Chromatogr. B. **1992**, *583*, 195–200.
20. Norris, R.L.G.; Ravenscroft, P.J.; Pond, S.M. Sensitive high-performance liquid chromatographic assay with ultraviolet detection of methadone enantiomers in plasma. J. Chromatogr. B **1994**, *661* (2), 346–350.
21. Riis, K.K.H.; Blemmer, A.T. Enantioselective high-performance liquid chromatographic method for the determination of methadone in serum using an AGP and a CN column as chiral and analytical column, respectively. J. Chromatogr. A **1994**, *666* (1–2), 283–287.
22. Rudaz, S.; Veuthey, J.L. Stereoselective determination of methadone in serum by HPLC following solid-phase extraction on disk. J. Pharmaceut. Biomed. Anal. **1996**, *14* (8–10), 1271–1279.
23. Pham-Huy, Ch.; Chikhy-Chorfi, N.; Galons, H.; Sadez, N.; Laqueille, X.; Aymard, N.; Massicot, Fr.; Warnet, J.-M.; Claude, J.-R. Enantioselective high-performance liquid chromatography determination of methadone enantiomers and its major metabolite in human biological fluids using a new derivatized cyclodextrin-bonded phase. J. Chromatogr. B **1997**, *700* (1–2), 155–163.
24. Angelo, H.R.; Beck, N.; Kristensen, K. Enantioselective high-performance liquid chromatographic method for the determination of methadone and its main metabolite in urine using an AGP and a C_8 column coupled serially. J. Chromatogr. B **1999**, *724* (1), 35–40.
25. Foster, D.J.R.; Somogyi, A.A.; Bochner, F. Stereoselective quantification of methadone and its major oxidative metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, in human urine using high-performance liquid chromatography. J. Chromatogr. B. **2000**, *744* (1), 165–176.

26. Rodriguez Rosas, M.E.; Preston, K.L.; Epstein, D.H.; Moolchan, E.T.; Wainer, I.W. Quantitative determination of the enantiomers of methadone and its metabolite (EDDP) in human saliva by enantioselective liquid chromatography with mass spectrometric detection. J. Chromatogr. B **2003**, *796* (2), 355–370.
27. Whittington, D.; Sheffels, P.; Kharasch, E.D. Stereoselective determination of methadone and the primary metabolite EDDP in human plasma by automated on-line extraction and liquid chromatography mass spectrometry. J. Chromatogr. B **2004**, *809* (2), 313–321.
28. He, H.; Sun, C.; Wang, X.R.; Pham-Huy, C.; Chikhi-Chorfi, N.; Galons, H.; Thevenin, M.; Claude, J.R.; Warnet, J.M. Solid-phase extraction of methadone enantiomers and benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis. J. Chromatogr. B **2005**, *814* (2), 385–391.
29. Rodriguez-Rosas, M.E.; Medrano, J.G.; Epstein, D.H.; Moolchan, E.T.; Preston, K.L.; Wainer, I.W. Determination of total and free concentrations of the enantiomers of methadone and its metabolite (2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine) in human plasma by enantioselective liquid chromatography with mass spectrometric detection. J. Chromatogr. A **2005**, *1073* (1–2), 237–248.
30. Kelly, T.; Doble, P.; Dawson, M. Chiral analysis of methadone and its major metabolites (EDDP and EMDP) by liquid chromatography–mass spectrometry. J. Chromatogr. B **2005**, *814* (2), 315–323.
31. Trachta, G.; Schwarze, B.; Sägmüller, B.; Brehm, G.; Schneider, S. Combination of high-performance liquid chromatography and SERS detection applied to the analysis of drugs in human blood and urine. J. Molec. Structure **2004**, *693* (1–3), 175–185.

Received November 29, 2005
Accepted December 27, 2005
Manuscript 6765