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Original Article

VALIDATION FOR QUANTITATIVE OF METHADONE ENANTIOMERS AND ITS MAJOR METABOLITE USING VANCOMYCIN COLUMN COUPLED WITH MASS SPECTROMETRIC DETECTION AND ITS APPLICATION TO CLINICAL SAMPLES

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ABSTRACT

Objective: To develop method to measure both methadone enantiomers and its major metabolite 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) in clinical samples

Methods: Five hundredmicroliters plasma/serum was extracted using solid phase extraction (mixed mode SPE-C8/SCX). The eluent was evaporated, reconstituted in mobile phase (95:5, 0.003% formic acid in methanol: 20 mM* ammonium formate) and injected.

Result: The recoveries of methadone enantiomers and EDDP were 97% and 89% respectively. Under this condition, methadone enantiomers were successfully separated at baseline but not EDPP. Precision of spiked plasma for intra-day and inter-day was less than five for both methadone enantiomers and less than 12 for EDDP at medium and high quality control samples. Linear relationship between peak area ratio and internal standard were obtained for methadone in the range 5-1000ng/ml, and for EDDP from 5-500ng/ml with correlation coefficients greater than 0.99. The limit of quantification was 5ng/ml.

Conclusion: The assay was used to analyse serum samples obtained from patients enrolled in a methadone maintenance treatment program.

Keywords: Methadone, Vancomycin, LC/MS/MS, Enantiomers.

INTRODUCTION

Methadone was discovered in the 1930's for use as an analgesic and has been used since 1960's for the stabilization and maintenance of patients with addictive disorders [1]. With the advent of HIV/AIDS in the 1980's, methadone became a widely used to substitute for illicit injectable opiates that propelled HIV spread. Over the past 10 years, interest in its use for pain treatment has also increased. Methadone however has complex pharmacokinetics and pharmacodynamics. This contribute to the poor relationship between dose, plasma levels and effects and the use of therapeutic concentrations. Earlier studies demonstrated that methadone doses from 60 to 100mg/day were effective; however it is now increasingly acknowledged that doses larger than 100mg/day may be required [2]. Thus, although 100mg/day is considered a maximum by many physicians, doses of more than 100mg/day are used in an increasing number of centres. On average, researchers have affirmed the benefit of a 150 to 600 ng/mL trough level to suppress opioid craving and a trough level at or above 400 ng/mL to provide opioid blockade during methadone maintenance[3, 4].

Methadone has an asymmetrical carbon atom in its structure. It exists as two enantiomers, having the same chemical composition but different spatial arrangements. It is marketed as a racemic mixture (50:50 mixture) of (R) or *levo* or *l*-methadone and (S) or *dextro* or *d*-methadone. R-methadone has 10-fold higher affinity than S-methadone for μ and δ opioid receptors [5]. It possesses up to 50 times the analgesic activity of S-methadone in human and in animal models[6]. R-methadone prevents opioid withdrawal but notS-methadone[7]. HoweverS-methadone blocks the potassium channel 3.5-fold more potently than R-methadone to cause prolonged QTC and sudden death [8]. It is proposed that the concentration of methadone enantiomers in serum of patients on MMT is higher than a certain maximum to cause prolonged QTC. It is also proposed that a therapeutic range exists for R-methadone for optimal effectiveness. Availability of a method to simultaneously measure methadone enantiomers would therefore be useful to test these hypotheses.

The objective of this study was to develop method to measure both methadone enantiomers and its major metabolite 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) in clinical samples.Methadone is mainly cleared via hepatic metabolism by cytochrome P450 (CYP) to the inactive metabolite EDDP through *N*-demethylation pathway. EDDP has no pharmacological activity and been reported to be of lower concentrations in plasma during therapeutic usage. However, measurement of EDDP was imperative in order to determine whether preferential metabolism of methadone had occurred.

MATERIAL AND METHODS

Chemicals and reagents

S-Methadone, R-methadone, EDDPand deuterium-labeled (R,S)-[2H3]-Met, were purchased from Cerilliant (Austin, TX, USA). Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur and formic acid (98-100%) was purchased from Merck Millipore (Merck KGaA, Darmstadt, Germany),ammonium formate 99% was obtained fromAcros Organic (Geel, Belgium), and ultra-pure water wasobtained, using a Milli-Q water-purification system (Millipore, Milford, MA, USA).

Pooled drug-free human plasmawas donated from Blood Bank, Hospital Universiti Sains Malaysia, Malaysia.

For the SPE procedures, mixed mode Si-SCX/C8 columns (SampliQ) (100 mg sorbent mass, 1 ml column volume) were purchased from Agilent Technologies (USA). Phosphate buffer 0.01 M was prepared by dissolving disodium hydrogen phosphate (1.42 g) in water in a one litre volumetric flask. pH was adjusted to 6.0 (±0.1)either with sodium hydroxide 1.0 M or phosphoric acid. The acetic acidic solution 1 M, was prepared by an appropriate dilution of

concentrated aceticacid with water. The eluent was acetonitrile-ammonia (95:5, v/v) and was prepareddaily.

Instrumentation

Equipment comprised a Sorvall™ ST 16 Centrifuge (Thermo Fisher Scientific™ Inc, Germany);a SIGMA 1-14 microcentrifuge (Sartorius Stedim Biotech GmbH, Germany); a Eutech, CyberScan ph1100 pH Meter (Fisher Scientific™ Inc, Germany); a Thermolyne vortex shaker (Thermo Fisher Scientific™ Inc, Germany) and N-EVAP 24 nitrogen evaporator manifold (Organomation Associates, MA, USA). Chromatography was performed on an Agilent 1260 infinity with G1312B binary pump, G1379B 1260µ degasser, G1316A thermostated column compartment, G1367E auto sampler and 6460 MS QQQ mass spectrometer (Agilent Technologies GmbH, Germany). An Astec Chirobiotic V2 (5 µm, 4.6 mm*×250 mm, Sigma-Aldrich Co. LLC) was used to separate the compounds.

Working solution and spiked plasma

Standard stock solutions of 1 mg/mL R-methadone and S-methadone were prepared in methanol by appropriately weighing the drugs. It were stored at -80ºC until use. Working solutions of R-methadone, S-methadone, EDDP and methadone-D3 at 10ug/ml in methanol were prepared by appropriately diluting the stock in methanol. For the preparation of calibration and quality control (QC) samples, blankplasma was seeded with appropriate amounts of (R,S)-methad one working solutions to obtain a concentration range of 5–1000 ng/mL for each methadone enantiomers and 5–500ng/mL for EDDP. Control samples were included in every run.

Sample Collection

This study was approved by Universiti Sains Malaysia Ethical Committee. Patients in Methadone Maintenance Therapy program at Psychiatric Clinic, Hospital Universiti Sains Malaysia and willing to sign an informed consent form were enrolled in this study after two weeks stabilized on any dose of methadone. Five millilitres blood were taken before subsequent dosing. After each collection, blood samples were spun at 3,500rpm and kept the serum at -20C until analysis

Sample preparation

Prior to solid phase extraction (SPE), all plasma or serum was centrifuged at 10,000rpm for 10 minutes. Five hundred microlitres of plasma or serum was diluted with 2500 µl* dipotassium phosphate pH 6 before it was loaded into the SPE column (mixture of C8 and SCX) that was pre-conditioned with 1 ml* methanol and 1 ml* of dipotassium phosphate pH 6. The column was then washed three times. The first wash comprised 1 ml* dipotassium phosphate pH 6, the second1 ml* of 1M acetic acid and finally 1 ml* methanol. We used a full vacuum pressure for 30 sec* at the final washing step to dry out the column. One ml of 5% ammonium hydroxide in acetonitrile was used to elute the drugs, applying a pressure of 2.5 mm*Hg. The eluent was dried under a nitrogen stream at room temperature. The residue was reconstituted with 25 µl* 50% methanol in water. The mixture was then transferred into an HPLC micro vials and placed in the auto-injector receptacles for injections into the HPLC.

LC and MS conditions

The final optimized LC separation was performed on a Chirobiotic V2 column (5 µm, 4.6 mm× 250 mm, Astec, Whippany, NJ) using amobilephase of 5:95 20 mM* ammonium formate: 0.003% formic acid in methanol at a flow rate of 0.4 ml*/min and column temperature was of6ºC. Injection volume was 1 µL*. The mass spectrometric measurements were performed with a 6460QQQ-MS (Agilent Technologies), equipped with an electrospray ionization (ESI) source and the Agilent Jet Stream technology. It was operated in the positive mode with multiple-reaction monitoring(MRM) under optimized conditions for the detection of methadone, EDDP and methadone-D3. The monitored mass transitions for methadone and methadone-D3 were set at m/z 310.2 → 265.1and 313.2 → 268.1 (dwell time 20 ms, fragmentor voltage 78 V and collision energy 9 V). The monitored mass transitions for EDDP were set at m/z 278.2 → 234.1 (dwell time 20 ms, fragmentor voltage 84 V and collision energy 29 V).Nitrogen was used as nebulizer, turbo (heater) gas, curtain, and collision-activated dissociation gas. A Jet Stream ESI source was operated with capillary voltage of 3,500 V, nozzle voltage of 500 V, drying gas temperature of 300 °C, gas flow of 5 L/min, nebulizer gas pressure of 45 psi, sheath gas temperature of 250 °C and sheath gas flow rate of 11 L/min. Data collection and integration were performed using the Mass Hunter workstation software (version B.05.00)

Method Validation

Linearity

To establish linearity, a series of calibration curves were constructed by analyzing drug free plasma seeded with EDDP to give theoretical concentrations of 5, 25, 50, 100, 200, 300, 500 ng/ml, and R- and S-methadone concentrations of 5, 50, 200, 300, 400, 500, 1000 ng/ml of each enantiomer. Quality control (QC) samples in plasma(10, 75, 150, 400 ng/ml EDDP; 25, 150, 250, 750 ng/ml each *R*- and *S*-methadone) were prepared using separate dilutions of stocks. Calibration and QC samples were aliquotted and stored at −20ºC until extracted. Calibration and QC samples were analysed daily together with the analytical samples. Standard curves were constructed using linear regression. Acceptance criteria for the calibration curves was a regression coefficient ($r2$) >0.95and back-calculated values of calibrations standards that deviated≤15% from nominal and less than 20% at the limit of quantification. The limit of detection (LOD) was defined as a signal to noise ratio of 2:1. The limit of quantification (LOQ) was the lowest concentration on the standard curve with an acceptable level of variation (<20%) and a signal to noise ratio >10:1.

Matrix Effect and Recovery

The matrix effect (ME) was studied by comparing the concentrations of quality control standards injected directly in mobile phase (set A) with the concentration found of the same analytes spiked after extraction (set B)at three levels (LQC, MQC, and HQC). The formula used was: ME= set B/set A×100. The recovery (RE) was studied by analyzing quality control standards at three levels and comparing the concentration of these analytes before extraction (set C) with another set of the same analytes after extraction (set B). The formula used was: RE = Set C/Set B×100.

Stability

Effect of freeze and thaw cycles on the stability of plasma samples containing R-methadone, S-methadone and EDDP were determined by subjecting six aliquots of low, mid and high unprocessed QC samples stored at−70±5 ◦C to three freeze thaw cycles. After the completion of third cycle, the samples were analyzed. The accuracy of this sample set was determined by comparison of untreated QC samples extracted and run in the same session. The sample was stable if the % change in concentration of the stability samples was within ±15% of the theoretical value.

Accuracy and precision

Intra-day accuracy and precision were evaluated by replicate analysis of R-methadone, S-methadone and EDDP at different concentrations. The run consisted of a calibration curve plus six replicates each of lower limit, low, medium (mid) and high QC samples. The inter-day accuracy and precision were assessed in a similar manner on five separate occasions. The evaluation of precision was based on the criteria that the, coefficient of variation for each concentration level should not be more than 15.0% except for the LLOQ, for which it should not be more than 20.0%. Similarly, for accuracy, mean values should not deviate by ±15.0% of theoretical concentration except for the LLOQ where it should not deviate by more than ±20.0% of the actual concentration.

RESULTS AND DISCUSSIONS

Methods have been described for chiral determination of methadone alone or in combination with EDDP and EDMP in various neat sample types, including sera, plasma and urine [9-12]. Most methods used alpha$_1$-acid glycoprotein (AGP) column as chiral selector but these columns tend to gradually lose separation efficiency with prolonged use. In this study we choose macrocyclic glycopeptide as

chiral selector because it is capable of separating a broad variety of enantiomeric compounds with good efficiency, good column loadability, high reproducibility, and long-term stability.

As recommended by the manufacturer, Chirobiotic V2 column has been used as CSP (chiral stationary phase) [13]. With modifications of the mobile phase, flow rates and temperature (Table 1), we managed to resolve R- and S-methadone characterised by a resolution factor of 2.2.

The final mobile phase composition for the method was set at (5:95) 20 mM* ammoniumformate: 0.003% formic acid in methanol. Under this condition, analysis was completed in 28 min. Retention times forR- and S-methadone were 20.27 and 22.03, respectively and the observed enantioselectivity (α) was 1.09(Fig. 1(B)). However, under this condition, R- and S-EDPP were only partially resolved.

The retention time for EDPP was 24.02 min (Fig. 1(C)). Only total EDDP concentration could be quantified using this method. There is no evidence that EDDP contributes to the adverse effects of methadone. In vitro evidence suggests CYP3A4 as the main enzyme in the formation of EDDP and this reaction is not enantiospecific [14].

Table 1: The effect of the formic acid concentration in the mobile phase (5% ammonium formate:95% 0.003 formic acid in methanol), flow rate and column temperature; retention time (rt); α: enantioselectivity; *Rs*: resolution.

Flow rate (ml/min)	Concentration formic acid in methanol (%)	Column temperature (°C)	*R*-met (*rt*)	*S*-met (*rt*)	Met (α)	Met (*Rs*)
0.5	0.005	10	11.79	12.63	1.07	1.6
0.5	0.003	10	15.72	16.86	1.07	1.7
0.4	0.003	6	20.27	22.03	1.09	2.2

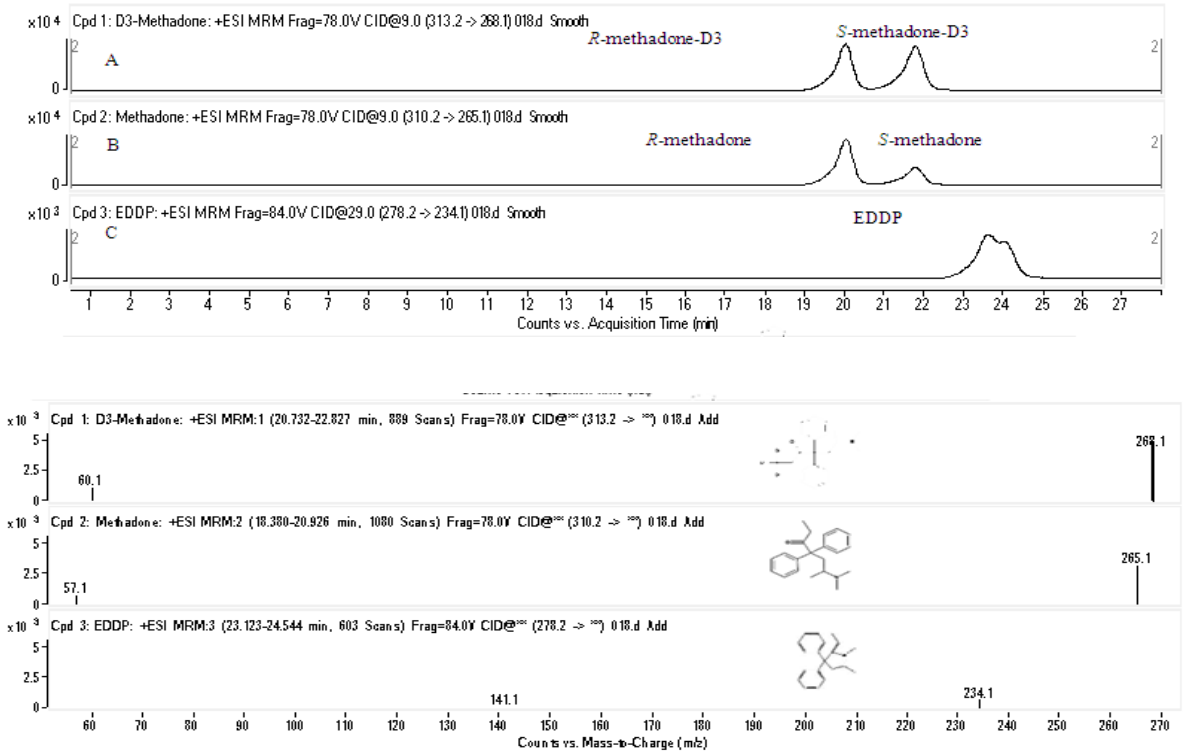

Fig. 1: Representative chromatogram of the serum sample collected before subsequent dosing from a patient who had received methadone 95mg/day. The measured concentrations were R-methadone 105ng/ml, S-methadone 39.8ng/ml, EDDP 11.6ng/ml. The chromatographic trace obtained using MRM at *m/z*: (A) 313.2, (B) 310.2, and (C) 278.2

For the simultaneous quantification of R- and S-methadone, as well as EDDP, calibration standards were prepared by seeding plasma with known amounts of racemic methadone and EDPP. The linearity for each enantiomer was determined by performing linear regression analysis on the plot of the peak area ratios of each enantiomer to internal standard versus concentration. The calibration curves of seeded plasma exhibited good linearity for the concentration of interest (5-1000 ng/ml for each methadone enantiomers, 5-500 ng/ml for EDDP) with correlation coefficients greater than 0.99 (Table 2).

Recovery (RE) of the analytes using mixed-mode (C8/strong cation exchange (SCX)) solid phase extraction from plasma was 97% ±0.5 for R-methadone, 99% ±6.6 for S-methadone and 89% ±2.3; Table 3. The matrix effects of R-, S- methadone and EDDP form different pool of plasma were investigated using quality control samples at three levels (LQC, MQC and HQC). Results are shown in Table 3. The average matrix effects (ME) for *R*-, *S*- methadone was 90% and for EDDP was 95%.

Precision and accuracy for intra-day and inter-day quality control samples are summarized in Table 2, Table 3 and Table 4. The coefficients of variation (CV) for both inter-day and intra-day determination were less than five for both methadone enantiomers and less than 12 for EDDP at medium and high quality control samples. For low quality control samples the CV was higher but it was still in acceptance limit(Table 4, 5 and 6). Accuracy was≥ 92% for methadone and≥88% for EDDP. Stability was assessed by comparing newly extracted quality control samples and quality control samples that underwent three freeze/thaw cycles with those extracted and reconstituted in mobile phase. There were no significant differences in the mean values between the sample sets.

Table 2: Linearity of inter-day calibration samples

n=5	Slope	Intercept	R-squared
R-methadone			
Mean	0.008581	-0.002405	0.9989
SD	0.000270	0.004110	0.0005
CV (%)	3.15	-170.86	0.05
S-methadone			
Mean	0.008617	-0.004937	0.9991
SD	0.000243	0.007503	0.0005
CV (%)	2.82	-151.97	0.05
EDDP			
Mean	0.005160	-0.003409	0.9948
SD	0.000443	0.003323	0.0037
CV (%)	8.59	-97.49	0.37

Linear weighted 1/*x*. The three curves were prepared and run on five different days.

Table 3: Results of the extraction recovery, matrix effects and stability after three cycles freeze and thawed on the extraction of methadone and EDDP.

	Extraction Recovery (%)			Matrix effects (%)			Freeze and thaw (3 cycles) (%)		
	Methadone		EDDP	Methadone		EDDP	Methadone		EDDP
	R	S		R	S		R	S	
LQC	97	96	91	95	93	99	97	103	91
MQC	98	94	90	90	93	96	94	88	86
HQC	97	106	87	85	84	91	95	97	86
Mean	97	99	89	90	90	95	95	96	88
SD	0.5	6.6	2.3	4.9	5.5	4.1	1.7	7.6	3.0
CV	0.5	6.7	2.6	5.5	6.1	4.3	1.8	7.9	3.4

Table 4: Accuracy and precision of quality control samples for R-methadone

Intraday (n=6)		LLQC	LQC	MQC	HQC
(ng/ml)		25	150	250	750
Day 1	Mean (+ SD)	26.6 + 1.7	153.1 + 3.9	261.0 + 9.1	716.0 + 15.5
	CV	6.4	2.5	3.5	2.2
	Accuracy (%)	106.3	102.1	104.4	95.5
	Bias %	6.3	2.1	4.4	-4.5
Day 2	Mean (+ SD)	24.6 + 1.2	147.8 + 7.1	245.7 + 12.7	688.7 + 21.5
	CV	5.1	4.8	5.2	3.1
	Accuracy (%)	98.4	98.5	98.3	91.8
	Bias %	-1.6	-1.5	-1.7	-8.2
Day3	Mean (+ SD)	24.6 + 1.2	150.6 + 5.1	249.9 + 5.3	706.3 + 19.7
	CV	4.8	3.4	2.1	2.8
	Accuracy (%)	98.4	100.4	100.0	94.2
	Bias %	-1.6	0.4	0.0	-5.8
Day4	Mean (+ SD)	25.4 + 0.9	151.89 + 3.6	249.6 + 3.0	724.3 + 12.0
	CV	3.4	2.4	1.2	1.7
	Accuracy (%)	101.6	101.3	99.8	96.6
	Bias %	1.6	1.3	-0.2	-3.4
Day5	Mean (+ SD)	25.2 + 0.7	143.4 + 3.7	242.7 + 9.8	712.1 + 24.7
	CV	2.7	2.6	4.0	3.5
	Accuracy (%)	100.8	95.6	97.1	94.9
	Bias %	0.8	-4.4	-2.9	-5.1
Inter- day (n=5)	Mean (+ SD)	25.3+ 0.8	149.4+ 3.9	249.8+ 6.9	709.5+ 13.3
	CV	3.2	2.6	2.8	1.9
	Accuracy (%)	101.1	99.6	99.9	94.6
	Bias %	1.1	-0.4	-0.1	-5.4

Table 5: Accuracy and precision of quality control samples for S-methadone

Intraday (n=6)		LLQC	LQC	MQC	HQC
(ng/ml)		25	150	250	750
Day 1	Mean (+ SD)	26.4 + 1.0	157.8 + 8.1	260.4 + 10.3	712.2 + 4.9
	CV	3.7	5.1	3.9	0.7
	Recovery %)	105.4	105.2	104.2	95.0
	Bias %	5.4	5.2	4.2	-5.0
Day 2	Mean (+ SD)	25.0 + 0.8	150.8 + 5.1	251.8 + 11.3	706.0 + 24.6
	CV	3.1	3.4	4.5	3.5
	Recovery %)	99.9	100.5	100.7	94.1

	Bias %	-0.1	0.5	0.7	-5.9
Day 3	Mean (+ SD)	27.7 + 1.3	148.7 + 4.9	245.8 + 6.4	700.2 + 18.5
	CV	4.7	3.3	2.6	2.6
	Recovery %)	110.8	99.1	98.3	93.4
	Bias %	10.8	-0.9	-1.7	-6.6
Day 4	Mean (+ SD)	24.8 + 1.1	149.9 + 5.6	249.5 + 11.3	706.7 + 15.1
	CV	4.5	3.8	4.5	2.1
	Recovery %)	99.0	99.9	99.8	94.2
	Bias %	-1.0	-0.1	-0.2	-5.8
Day 5	Mean (+ SD)	24.6 + 1.0	150.9 + 9.6	251.8 + 10.7	698.2 + 28.84
	CV	4.1	6.4	4.2	4.1
	Recovery %)	98.4	100.6	100.7	93.1
	Bias %	-1.6	0.6	0.7	-6.9
Inter-day (n=5)					
	Mean (+ SD)	25.7+ 1.3	151.6+ 3.6	251.9+ 5.4	704.7+ 5.6
	CV	5.2	2.4	2.1	0.8
	Recovery (%)	102.7	101.1	100.7	94.0
	Bias %	2.7	1.1	0.7	-6.0

Table 6: Accuracy and precision of quality control samples for EDDP

Intraday (n=6)		LLQC	LQC	MQC	HQC
(ng/ml)		10	75	150	400
Day 1	Mean (+ SD)	10.0 + 0.7	77.5 + 3.5	147.4 + 9.5	406.1 + 47.8
	CV	7.4	4.5	6.5	11.8
	Accuracy (%)	100.1	103.3	98.3	101.5
	Bias %	0.1	3.3	-1.7	1.5
Day 2	Mean (+ SD)	10.3 + 0.8	77.2 + 6.6	153.7 + 14.0	369.2 + 28.4
	CV	7.6	8.6	9.1	7.7
	Accuracy (%)	102.7	102.9	102.4	92.3
	Bias %	2.7	2.9	2.4	-7.7
Day 3	Mean (+ SD)	9.8 + 1.6	71.1 + 4.8	140.5 + 14.1	363.1 + 41.9
	CV	16.1	6.8	10.0	11.5
	Accuracy (%)	98.5	94.8	93.7	90.8
	Bias %	-1.5	-5.2	-6.3	-9.2
Day 4	Mean (+ SD)	11.2 + 1.1	75.8 + 2.4	149.0 + 16.6	357.7 + 32.9
	CV	9.3	3.1	11.1	9.2
	Accuracy (%)	112.4	101.1	99.3	89.4
	Bias %	12.4	1.1	-0.7	-10.6
Day 5	Mean (+ SD)	9.5 + 1.2	71.4 + 4.3	132.6 + 6.4	353.8 + 34.0
	CV	13.0	6.1	4.8	9.6
	Accuracy (%)	94.8	95.2	88.4	88.5
	Bias %	-5.2	-4.8	-11.6	-11.5
Inter- day (n=5)					
	Mean (+ SD)	10.2 + 0.7	74.6 + 3.1	144.6 + 8.2	370.0 + 21.0
	CV	6.5	4.2	5.7	5.7
	Accuracy (%)	102.0	99.5	96.4	92.5
	Bias %	1.7	-0.5	-3.6	-7.5

Table 7: The concentration of methadone (met) enantiomers and EDDP in serum samples from patients in a methadone maintenance program with different dose.

Subjects	Age (year)	Dose (mg)	R-met (ng/ml)	S-met (ng/ml)	R/Smet ratio	EDDP (ng/ml)	Metabolic ratio
1	40	70	127.5	93.7	1.4	15.2	8.4
2	38	80	196.6	177.0	1.1	27.0	7.3
3	39	65	76.2	54.5	1.4	5.6	13.6
4	23	20	132.0	233.6	0.6	8.2	16.0
5	31	120	127.2	72.5	1.8	17.6	7.2
6	42	70	164.6	124.7	1.3	17.2	9.5
7	37	65	85.5	60.2	1.4	12.3	6.9
8	42	80	222.2	155.1	1.4	39.7	5.6
9	40	95	105.0	39.8	2.6	11.6	9.1
10	37	25	60.1	65.3	0.9	4.5	13.4
Mean	37	69	129.7	107.6	1.4	15.9	9.7
SD	5.8	29.6	52.2	63.4	0.5	10.7	3.5
CV (%)	15.8	42.9	40.3	58.9	39.2	67.0	35.7

Applications to clinical samples

The validated method was applied to serum samples of patients on the national methadone maintenance treatment (MMT) program. In this study, serum samples were obtained at steady state before the next dose. The average *R/S*-met ratio was 1.4±0.5 with a range 0.6-2.6 (Table 7). Our results are consistent with previous observations

[9, 10]. It will be applied to measure concentrations of methadone enantiomers and its main metabolite (EDDP).

CONCLUSIONS

To the best ofour knowledge, this is the first report of the use of V2 column in the LC/MS/MS quantitation of methadone enantiomers in human plasma and serum without matrix effects even though the baseline separation of the enantiomers were achievable after 20 minutes. This validated assay offers same sensitivity, linear range and ruggedness over previously published method using different chiral selector.

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REFERENCES

1. Kreek MJ, Vocci FJ, History and current status of opioid maintenance treatments:blending conference session. J Subst Abuse Treat 2002;23(2):93-105.
2. Donny EC, Brasser SM, Bigelow GE, Stitzer ML, Walsh SL, Methadone doses of 100 mg or greater are more effective than lower doses at suppressing heroin self-administration in opioid-dependent volunteers. J Addiction 2005;100(10):1496-509.
3. Eap CB, Buclin T, Baumann P, Interindividual variability of the clinical pharmacokinetics of methadone:implications for the treatment of opioid dependence. J Clin Pharmacokinet 2002;41(14):1153-93.
4. Leavitt SB, Shinderman M, Maxwell S, Eap CB, Paris P, When "enough" is not enough:new perspectives on optimal methadone maintenance dose. Mt Sinai J Med 2000;67(5-6):404-11.
5. Kristensen K, Christensen CB, Christrup LL, The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. J Life Sci 1995;56(2):PL45-50.
6. Scott CC, Robbins EB, Chen KK, Pharmacologic comparison of the optical isomers of methadon. J Pharmacol Exp Ther 1948;93(3):282-6.
7. Isbell H, Eisenman AJ. The addiction liability of some drugs of the methadon series. J Pharmacol Exp Ther 1948;93(3):305-13.
8. Eap CB, Crettol S, Rougier JS, Schlapfer J, Sintra Grilo L, Deglon JJ, *et al*. Stereoselective block of hERG channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. J Clin Pharmacol Ther 2007;81(5):719-28.
9. Foster DJ, Somogyi AA, Dyer KR, White JM, Bochner F, Steady-state pharmacokinetics of (R)-and (S)-methadone in methadone maintenance patients. Br J Clin Pharmacol 2000;50(5):427-40.
10. Rodriguez-Rosas ME, Medrano JG, Epstein DH, Moolchan ET, Preston KL, Wainer IW, 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):237-48.
11. Etter ML, George S, Graybiel K, Eichhorst J, Lehotay DC. Determination of free and protein-bound methadone and its major metabolite EDDP:enantiomeric separation and quantitation by LC/MS/MS. J Clin Biochem 2005;38(12):1095-102.
12. Foster DJ, Morton EB, Heinkele G, Murdter TE, Somogyi AA. Stereoselective quantification of methadone and a d(6)-labeled isotopomer using high performance liquid chromatography-atmospheric pressure chemical ionization mass-spectrometry: application to a pharmacokinetic study in a methadone maintained subject. J Ther Drug Monit 2006;28(4):559-67.
13. Lee JT, Brian HE, Beesley TE. Enhanced Chiral Selectivity by Chemical Modification of Chiral Stationary Phases For Pharmaceutically Important Drugs and Drug Metabolites. Int J Symposium on Chiral Discrimination 2004.
14. Foster DJ, Somogyi AA, Bochner F. Methadone N-demethylation in human liver microsomes:lack of stereoselectivity and involvement of CYP3A4. Br J Clin Pharmacol 1999;47(4):403-12.