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Simultaneous enantioselective quantification of methadone and of 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine in oral fluid using capillary electrophoresis

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Abstract

A capillary electrophoresis method was developed for the enantioselective quantification of methadone (MTD) and its main metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP). The enantiomers of MTD and EDDP were resolved by CE in 5 min using 0.2% highly sulphated gamma-cyclodextrins as chiral selectors and a 50 mM phosphate solution at pH 4.5 as background electrolyte. The optimized method was applied and validated for oral fluid testing. Linear relationships were obtained for MTD enantiomers in the range of 8.1–625 ng/mL and in the range of 7.6–500 ng/mL for EDDP enantiomers. The detection limits ranged from 2.3 to 2.4 ng/mL, whereas the limits of quantification ranged from 7.6 to 8.1 ng/mL. Intra- and inter-assay precision and accuracy were acceptable, respectively. The method was applied to the analyses of 60 oral fluid specimens obtained from patients enrolled in a MTD maintenance programme. Our data pointed out that higher concentrations of (*R*)-MTD and the enantioselective excess of (*S*)-EDDP in OF may reflect the free fraction of MTD and EDDP enantiomers in plasma.

Keywords: Oral fluid; Methadone; 2-Ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine; Capillary electrophoresis; Highly sulphated γ-cyclodextrin

1. Introduction

Methadone (MTD) is the most widely used synthetic analgesic for the treatment of opioid dependence and has also an important use in the management of pain. MTD possesses an asymmetric carbon, but it is generally administered orally as a racemate. In humans, the (*R*)-enantiomer presents higher affinity towards the μ and δ-opioid receptors and its analgesic effect is about 25–50 times greater than its antipode [1,2]. Furthermore, MTD isomers differ also in their protein binding, as (*S*)-MTD is binding more extensively to α_1-acid glycoprotein (AGP) than (*R*)-MTD. In addition, (*R*)-MTD has a longer elimination half-life and a larger total volume distribution than (*S*)-MTD [2,3]. The main metabolic pathway of MTD leads to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), and to 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP). Neither of these metabolites possesses pharmacological activity.

It has been largely demonstrated that there exists high inter-individual variability in the MTD metabolism [4–9]. Therefore, the enantioselective quantification of MTD is frequently required in order to determine the level of the active (*R*)-MTD necessary to obtain maximum treatment efficacy, to prevent toxicity and/or to exclude additional consumption of (*R*)-MTD available on the illicit market.

The use of oral fluid (OF) as alternative specimen for drugs of abuse testing has received increased attention because it offers many advantages: OF is the most accessible specimen obtained by non-invasive techniques and it contains many analytes of interest useful for purposes (monitoring the compliance on drug maintenance programme, roadside drug testing, work place drug testing, etc.). OF or mixed saliva is a clean matrix (98% water) resulting from ultra-filtration of interstitial fluid. Oral fluid contains predominately the parent drug rather than drug metabolites, and therefore is a good indicator of intoxication states [10–13].

Several analytical methods such as high performance liquid chromatography (HPLC) [1,3,4,7,14–23], gas chromatography [9] or capillary electrophoresis (CE) [24–26] have been applied

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to the enantioselective studies of MTD in biological matrices. However, only two HPLC-mass spectrometry (MS) methods have been reported for the enantioselective study of MTD in OF [3,4]. Only one of them performed the analysis of both enantiomers of EDDP [3]. These chiral HPLC assays employed AGP columns for the chiral separation step. However, these columns are rather expensive and particularly, they tend to gradually lose separation efficiency proportional to the number of injections, which leads necessary to poor sensitivity and reproducibility and needs their frequent replacement [17,19,23,27].

Enantioseparation using CE with chiral additives has been established as an attractive and a relatively inexpensive alternative to conventional chromatographic techniques. Cyclodextrins (CDs) additives have mostly been used in the enantioselective separation of MTD and/or EDDP. CDs including heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) [26,28], (2-hydroxylpropyl)-β-cyclodextrin (HP-β-CD) [24,29–31], carboxylmethyl-β-cyclodextrin [31–33], highly sulphated β-cyclodextrin (HS-β-CD) [34] and sulphobutyl ether-β-cyclodextrin [31] have been applied for the chiral separation of MTD and/or EDDP in urine, plasma or hair. Recently, Rudaz et al. [35] obtained optimal resolution of methanolic solutions of racemic MTD by CE/MS using highly sulphated gamma cyclodextrin (HS-γ-CD) as chiral selector. However, the enantioselective separation of EDDP by CE using HS-γ-CD has not been studied yet.

The purpose of this study was to develop a rapid and specific method for the determination of enantiomeric ratios of MTD and EDDP in OF by capillary electrophoresis using HS-γ-CD as a chiral additive. The method was applied to the enantioselective quantification of MTD and EDDP in OF of addicts under maintenance programme for narcotic dependence.

2. Materials and methods

2.1. Reagents

Racemic MTD and racemic EDDP were purchased from Cerilliant (Austin, TX, USA). (*R*)-MTD hydrochloride was a gift from the Unit of Medicinal and Drug Analysis of the National Laboratory of Health (Luxembourg). HS-γ-CD (20%, w/v) aqueous solution was obtained from Beckman (Fullerton, CA, USA). Phosphoric acid (H_3PO_4), mesityl oxide (>90%), ammonia (NH_3, 25%), sodium hydroxide (NaOH), cyclohexane and (*R*)-(+)-1-phenylethylamine ((*R*)-PEA, $R{:}S \geq 99.5{:}0.5$) were purchased from Sigma–Aldrich (Bornem, Belgium). Ultrapure water (H_2O and methanol HPLC grade (MeOH) were purchased from Lab Scan (Dublin, Ireland)).

2.2. Specimen preparation

2.2.1. Collection of oral fluid

Mixed Saliva was obtained from patients undergoing a MTD treatment in collaboration with the "Jugend-and Drogenhëllef Foundation" (Luxembourg). The study was approved by the Ethic Research Committee of Luxembourg and each volunteer provided informed consent. Racemic methadone was administered orally either as syrup solution or as a tablet and the doses ranged between 3 and 140 mg.

OF was collected just before the administration of the daily dose of MTD. Specimens were collected with Salivette devices (Sarstedt, Nümbrecht, Germany); after being soaked for 2 min by OF, the cotton swab was placed back into the Salivette. The OF was recovered from the Salivette by centrifugation at $5000 \times g$ for 10 min. Finally, the OF specimens were stored at −20 °C until analysis. Drug free OF was obtained from healthy volunteers at the authors' laboratory.

2.2.2. Extraction procedure

A 200 μL aliquot of saliva was transferred to a microcentrifuge tube and the internal standard (IS), 10 μL of (*R*)-PEA 10 ng/μL was also added. The mixture was alkalinized with 0.2 mL of an ammonia solution (25%), and extracted with 3 mL of cyclohexane for 2 min. After centrifugation ($5000 \times g$ for 10 min), the upper organic layer was evaporated to dryness under nitrogen at 37 °C. The residue then was diluted in MeOH:H_2O (50:50, v/v) and vortex-mixed for 10 s.

2.3. Quantitation procedure and method validation

For calibration, drug-free OF were spiked with methanolic standards solutions, covering the range from 8.1 to 625 ng/mL for MTD enantiomers and from 7.6 to 500 ng/mL for EDDP enantiomers, respectively. The IS was added at a fixed concentration of 500 ng/mL. The peak area ratios between each enantiomer and the corresponding IS versus the concentrations ratio were used for calculations.

The efficiency of the liquid–liquid extraction was evaluated in five replicates by calculating the recoveries of blank OF spiked with the target substances at the concentrations of 40, 125 and 250 ng/mL. The recoveries of the enantiomers were then calculated by comparing the peak areas of the extracted OF specimens with those obtained by adding the same amounts of reference substances after extraction.

Three replicates of blank OF spiked with 25 ng/mL of MTD and EDDP enantiomers were used for the estimation of the limits of detection (LOD) and the limits of quantification (LLOQ). LOD and LLOQ were determined as the 3- and 10-fold standard deviation of the base line noise, respectively [36].

Intra- and inter-assay precision (relative standard deviation expressed as percentage) and accuracy (expressed as percentage error of concentration found compared to target concentrations) were determined by using blank OF ($n = 10$) spiked for each enantiomer at a low concentration (50 ng/mL) and at a higher concentration (250 ng/mL). Inter-day precision and accuracy were determined at the same concentrations during 5 days.

2.4. CE equipment and conditions

All CE separations were carried out on a Beckman P/ACE System MDQ equipped with a photodiode array detector (Beckman Coulter, Fullerton, CA, USA). The 32 Karat software from Beckman was used for data acquisition. Uncoated fused silica capillary of 375 μm outer diameter (o.d.), 50 μm inside diame-

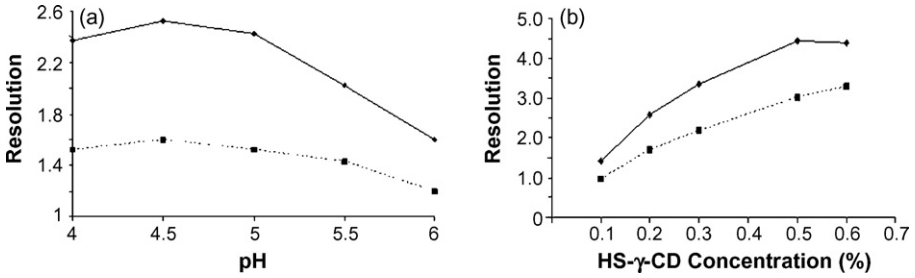

Fig. 1. Effect of the pH of BGE on the resolutions (a); influence of the HS-γ-CD concentration on the resolutions (b); curve identification, (♦) MTD, (■) EDDP; conditions: fused-silica capillary, 50 μm i.d., 40.2 cm total length (effective length 32.8 cm); 50 mM phosphate buffer, applied voltage, 20 kV; temperature, 20 °C; UV detection at 200 nm.

ter (i.d.) and 40.2 cm total length (effective length 32.8 cm) were used for separations. The capillary was conditioned before use by successively washing for 20 min with 0.1 M NaOH, 10 min H_2O followed by a 20 min flushing with the running buffer.

The applied voltage was +20 kV, the capillary temperature was maintained at 20 °C, and the detection wavelength was 200 nm. The data collection frequency was 4 Hz. The background electrolyte consisted of 50 mM H_3PO_4 buffer, pH 4.5. Separations were performed after rinsing the capillary for 0.5 min with the rinsing electrolyte (50 mM H_3PO_4, pH 4.5) containing 0.2% HS-γ-CD (w/v). Between consecutive analyses, the capillary was rinsed with 0.1 M NaOH (2 min) and deionized H_2O (1 min). Specimens' solutions were injected electrokinetically for 8 s with 10 kV.

2.5. Data treatment

The resolution (R_s) of each enantiomer pair was calculated according to the following equation:

$$R_s = \frac{2(t_2 - t_1)}{\omega_2 + \omega_1} \quad (1)$$

where t_2 and t_1 are the migration times of two adjacent peaks and ω_2 and ω_1 are the corresponding baseline peak width.

The electrophoretic mobility of the compounds was calculated with the following equation:

$$\mu_{ep} = \mu_{ap} - \mu_{eof} = \frac{lL_{tot}}{Vt} - \frac{lL_{tot}}{Vt_{eof}} \quad (2)$$

where μ_{ep} is the electrophoretic mobility of the analyte, μ_{ap} is the apparent mobility, μ_{eof} is the electroosmotic mobility, l is the effective length to the detector, L_{tot} is the total length of the capillary, V is the applied voltage, t and t_{eof} are the migration time of the analyte and the migration time of the neutral marker (mesityl oxide), respectively.

3. Results and discussion

3.1. Optimization of chiral separations

In order to obtain baseline enatioseparations by CE, different CDs, including DM-β-CD, HP-β-CD, HS-γ-CD and HS-β-CD; some of them previously been used for the enantioseparation of MTD and/or EDDP were tested for their capacity as chiral selectors for the purpose of our study. Preliminary evaluations of each CD were performed using a 50 mM phosphate buffer at pH 2.5. The concentration of DM-β-CD was 1 mM, while the concentration of HS-β-CD and HS-γ-CD were adjusted to 0.2% (w/v). Only HS-γ-CD was able separate all enantiomers simultaneously. Thus, the optimization to find final operational conditions was performed using HS-γ-CD as the chiral selector. The optimization was focused on main parameters affecting the separation, i.e., pH of the buffer, concentration of the HS-γ-CD or capillary temperature.

The influence of the pH on the enantioresolution was examined between 4 and 6 (Fig. 1a). At higher pH, the enantioresolution decreased which may due to the increase of the electroosmotic flow. Best enantioresolutions were observed at pH 4.5, as at pH 4 appeared a peak tailing affecting negatively the peak resolutions.

The effect of the electric potential on the enantioresolution was analysed in the range of 15–30 kV. A loss of peak resolutions was observed above 20 kV. This may be due the increased electric field, reducing the migration time of the enantiomers and in parallel, also the time available for complexation with the CDs [37]. Therefore, a voltage of 20 kV was chosen for further experiments.

Capillary temperatures ranging from 15 to 35 °C were examined. Best enantioresolutions for MTD (R_s = 2.79) and for EDDP (R_s = 1.60) were observed at 20 °C and they began to decline significantly at 25 °C. At 15 °C, the run time was 4 min longer

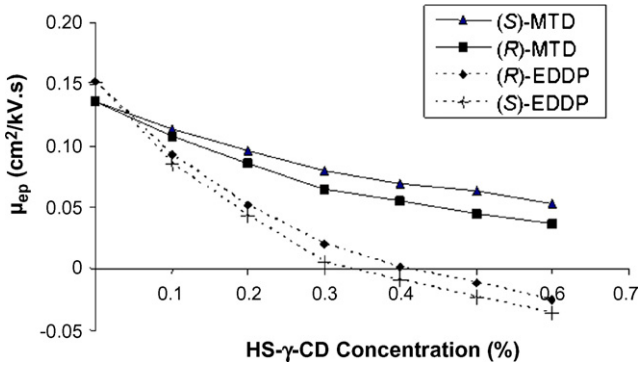

Fig. 2. Evolution of the electrophoretic mobility of the enantiomers of MTD and EDDP in function of the HS-γ-CD concentration, ranging from 0 to 0.6% (w/v) using a 50 mM phosphate buffer at pH 4.5; other experimental conditions as in Fig. 1.

Table 1
Linearity, limits of detection and quantification

Compound	Linearity (ng/mL)	Regression line		R^2	LOD (ng/mL)	LLOQ (ng/mL)
		Slope	Intercept			
(*S*)-MTD	8.1–625	0.0093 ± 0.0007	−0.2430 ± 0.2476	0.991	2.4	8.1
(*R*)-MTD	8.1–620	0.0096 ± 0.0007	−0.2310 ± 0.2506	0.992	2.4	8.1
(*R*)-EDDP	7.6–500	0.0057 ± 0.0003	−0.0475 ± 0.0624	0.995	2.3	7.6
(*S*)-EDDP	7.6–500	0.0060 ± 0.0004	−0.0123 ± 0.0760	0.994	2.3	7.6

compared to the analysis time at 20 °C (6 min) and a tailing phenomenon began to degrade the separation.

The effect of the CD concentration on the enantioresolutions was investigated by testing the concentrations of HS-γ-CD from 0.1 to 0.6% (w/v) (Fig. 1b). An almost baseline resolution was observed for both analytes with the lowest concentration of HS-γ-CD tested (0.1%), demonstrating the high resolution power of this chiral selector. The enantioresolutions regularly increased with the CD concentration until 0.6% (w/v), where the peak tailing influences negatively the enantioselective separation of MTD. As presented in Fig. 2, the effective mobility of the enantiomers decreases as the CD concentration increases and it becomes even negative for the stronger complexed EDDP enantiomers. Thus, at concentrations of HS-γ-CD lower than 0.3%, the enantiomers of MTD and EDDP migrated as cations in front of the EOF marker [35]. However, at concentrations higher than 0.3% (w/v), the EOF migrated between the peaks of MTD and EDDP and the acquisition times were significantly increased. Optimal CD concentration for the final method was chosen in order to provide good resolutions ($R_s > 1.5$) and short analysis time with low CD consumptions. Thus, further enantioselective separations of MTD and EDDP were performed at a concentration of 0.2% (w/v) of HS-γ-CD.

3.2. Application to oral fluid testing

Fig. 3 shows a typical blank oral fluid specimen containing only the IS and obtained after analysis by CE. Fig. 4 was obtained after the analysis of a blank oral fluid spiked with a racemic mixture of MTD and EDDP. Both analytes were separated into their enantiomers using the final optimized conditions. The enantiomeric elution order for MTD was investigated by injecting an OF specimen spiked with (*R*)-MTD and (*S*)-MTD in a ratio of 10:1. Due to the lack of commercial pure standards for EDDP enantiomers, (*R*)-EDDP was prepared from (*R*)-MTD using a procedure described previously by Rosas et al. [3]. An aqueous solution of (*R*)-MTD (10 ng/μL) was placed at 150 °C for 6 h and the resulting residue, dissolved in a mixture of 50 μL MeOH:H_2O (50:50, v/v), was analysed by CE/UV. For MTD, the enantiomeric elution order was $S < R$, while the preferentially bonded (*S*)-EDDP migrated later than the (*R*)-EDDP.

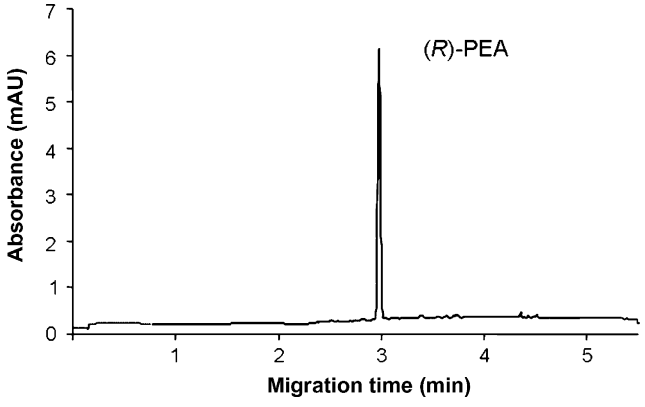

Fig. 3. Electropherogram of an oral fluid sample spiked with 10 ng/mL (*R*)-PEA, used as internal standard (IS), obtained under optimized conditions: fused-silica capillary 375 μm o.d., 50 μm i.d., 40.2 cm total length (effective length 32.8 cm); 50 mM phosphate buffer, pH 4.5, 0.2% HS-γ-CD (w/v); applied voltage, 20 kV; temperature: 20 °C, UV detection at 200 nm.

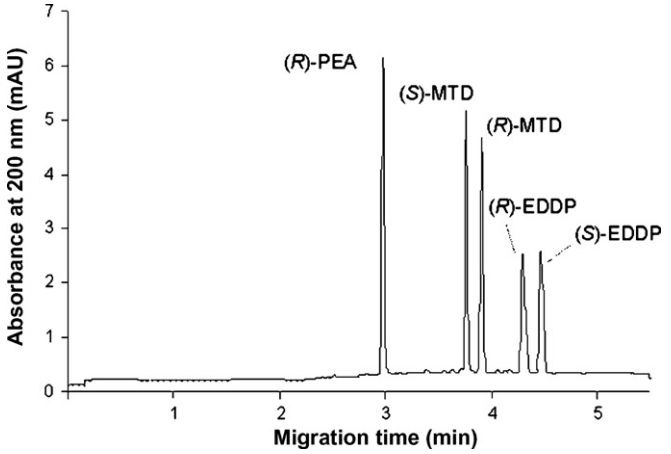

Fig. 4. Electropherogram of an oral fluid sample spiked with 50 ng/mL of (*R*,*S*)-MTD, (*R*,*S*)-EDDP and (*R*)-PEA (IS) using the same optimized conditions as in Fig. 3.

Table 2
Analytes recoveries

Concentration (ng/mL)	MTD		EDDP	
	S	*R*	*R*	*S*
250				
Mean (%)	92.9	92.1	81.0	84.7
S.D.[a]	9.9	10.1	8.5	6.8
125				
Mean (%)	81.1	80.8	89.9	90.8
S.D.[a]	7.6	9.9	9.4	5.8
40				
Mean (%)	89.9	90.7	77.4	81.7
S.D.[a]	10.5	9.8	1.9	0.8

[a] S.D.: standard deviation.

Table 3
Intra- and inter-assay precision and accuracy data

Compound	Concentration (ng/mL)	Intra-day ($n=10$)		Inter-day ($n=5$)	
		Precision (R.S.D.[a], %)	Accuracy (bias, %)	Precison (R.S.D., %)	Accuracy (bias,%)
(*S*)-MTD	50	5.4	8.6	7.3	8.9
	250	9.5	0.2	8.9	3.2
(*R*)-MTD	50	4.9	6.8	6.8	8.8
	250	9.3	1.4	9.0	4.1
(*R*)-EDDP	50	6.1	1.9	8.4	8.4
	250	10.0	5.1	8.1	5.0
(*S*)-EDDP	50	6.8	7.5	7.1	8.6
	250	7.6	9.5	8.8	7.5

[a] R.S.D.: relative standard deviation.

As several drugs are usually found in OF from patients on a MTD maintenance programme, a OF was spiked with some drugs in order to exclude possible interferences with the analytes. The enantiomers of MTD, EDDP and the IS did not co-migrate with any of the following tested substances: cocaine, ecgonine methylester, benzoylecgonine, morphine, monoacetylmorphine, codeine, diazepam, temazepam, norazepam, oxazepam and lorazepam, diphenylhydramine, and amphetamine type stimulants (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyethylamphetamine, 3,4-methylenedioxymethamphetamine).

3.3. Method validation for oral fluid testing

The data of the method validation are summarised at Tables 1–3. The calibration curves were generated using a weighted ($1/x$) least-square regression model. Standard curve plots were linear in the range of 8.1–625 ng/mL and 7.6–500 ng/mL for MTD and EDDP enantiomers, respectively. The LOD and LLOQ of MTD enantiomers were 2.4 ng/mL and 8.1 ng/mL, respectively. For EDDP enantiomers, LOD limits were 2.3 ng/mL and the LLOQ values were 7.6 ng/mL. The analytical recoveries of MTD and EDDP enantiomers determined at three different concentrations ranged from 77.4 to 92.9% and they were considered adequate for the purpose of the study. The calculated precisions and accuracies were always lower than 10.0%, which demonstrate that the method has acceptable accuracy and precision (Table 3).

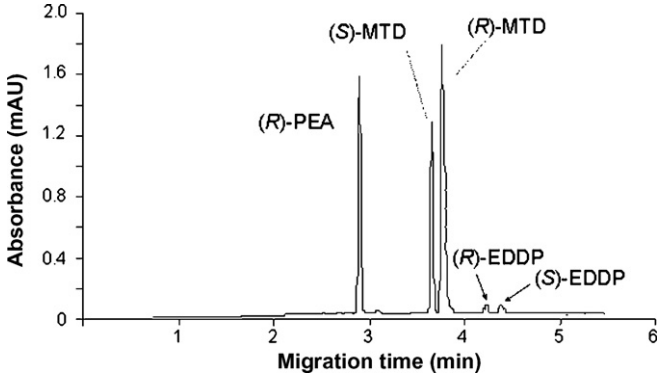

Fig. 5. Representative electropherogram of an oral fluid (No. = 44) obtained before the daily administration from a patient undergoing a MTD maintenance programme; same operating conditions as in Fig. 3.

3.4. Application to clinical patients

The validated method was applied to 60 OF specimens obtained from patients undergoing a MTD maintenance treatment. Fig. 5 shows a typical electropherogram of an oral fluid obtained from a patient. All OF analysed were positive for both enantiomers of MTD and the concentrations ranged from 18.6 to 619.5 ng/mL for (*S*)-MTD and from 28.4 to 622.5 ng/mL for (*R*)-MTD (Table 4). The *R*/*S* ratios varied between 1.00 and 3.13. These results are consisted with previously reported data, as the ratios determined in saliva are representative of the free fraction of MTD in blood only [4]. Furthermore, a significant correlation

Table 4
Concentrations and enantiomeric ratios of MTD and EDDP enantiomers in oral fluid from patients undergoing a methadone maintenance programme

No.	MTD dose (mg)	(*S*)-MTD (ng/mL)	(*R*)-MTD (ng/mL)	*R*/*S* ratio	(*R*)-EDDP (ng/mL)	(*S*)-EDDP (ng/mL)	*R*/*S* ratio
1	30	69.2	120.7	1.74	nd[a]	nd	–
2	ni[b]	77.1	120.7	1.57	nd	nd	–
3	25	27.8	33.2	1.19	nd	nd	–
4	ni	39.6	58.4	1.48	nd	nd	–
5	ni	33.7	40.9	1.21	nd	nd	–
6	30	55.4	87.6	1.58	nd	nd	–
7	ni	116.6	184.9	1.59	13.3	19.1	0.70
8	55	159.9	365.8	2.29	nd	nd	–
9	20	37.6	54.6	1.45	nd	nd	–
10	30	195.0	194.6	1.00	nd	nd	–
11	30	39.6	76.0	1.92	nd	nd	–

Table 4 (*Continued*)

No.	MTD dose (mg)	(*S*)-MTD (ng/mL)	(*R*)-MTD (ng/mL)	*R/S* ratio	(*R*)-EDDP (ng/mL)	(*S*)-EDDP (ng/mL)	*R/S* ratio
12	3	47.6	53.7	1.13	nd	nd	–
13	15	65.2	83.7	1.28	nd	nd	–
14	80	146.0	373.6	2.56	nd	nd	–
15	55	274.3	430.0	1.57	nd	nd	–
16	25	31.6	44.8	1.42	nd	nd	–
17	20	29.7	48.7	1.64	nd	nd	–
18	ni	65.2	120.7	1.85	nd	nd	–
19	80	49.5	126.5	2.56	nd	nd	–
20	15	232.9	247.1	1.06	nd	nd	–
21	30	71.2	112.9	1.59	nd	nd	–
22	30	97.1	180.4	1.86	nd	nd	–
23	80	163.4	167.0	1.02	nd	nd	–
24	65	493.3	496.1	1.01	13.6	17.5	0.78
25	60	47.5	93.5	1.96	nd	nd	–
26	ni	51.4	60.4	1.18	nd	nd	–
27	80	79.0	146.0	1.85	nd	nd	–
28	30	157.9	326.9	2.07	nd	nd	–
29	25	38.0	41.2	1.08	nd	nd	–
30	60	116.5	175.2	1.50	nd	nd	–
31	ni	71.2	97.4	1.37	nd	nd	–
32	ni	93.0	173.5	1.87	nd	nd	–
33	ni	163.0	263.1	1.61	nd	nd	–
34	15	50.0	57.7	1.15	nd	nd	–
35	30	80.2	200.1	2.49	nd	nd	–
36	20	33.4	34.5	1.03	nd	nd	–
37	15	61.9	67.2	1.08	nd	nd	–
38	30	139.4	254.9	1.83	nd	nd	–
39	80	111.4	213.0	1.91	15.9	22.4	–
40	ni	619.5	622.5	1.00	13.9	18.6	0.75
41	15	236.4	250.8	1.06	nd	nd	–
42	60	45.2	66.2	1.46	nd	nd	–
43	30	18.6	28.4	1.53	nd	nd	–
44	ni	321.6	338.6	1.05	14.4	19.6	0.74
45	ni	31.4	75.9	2.42	nd	nd	–
46	ni	169.8	317.2	1.87	13.6	18.5	0.74
47	30	356.9	505.7	1.42	17.7	22.4	0.79
48	15	41.0	46.9	1.14	nd	nd	–
49	75	89.2	279.1	3.13	nd	nd	–
50	20	60.5	79.8	1.32	nd	nd	–
51	ni	37.4	37.3	1.00	nd	nd	–
52	30	89.1	129.4	1.45	9.0	10.9	0.83
53	ni	61.9	72.6	1.17	nd	nd	–
54	ni	125.5	249.3	1.99	nd	nd	–
55	ni	60.5	63.2	1.04	nd	nd	–
56	15	103.8	134.9	1.30	10.8	11.6	0.93
57	20	45.2	63.3	1.40	nd	nd	–
58	ni	47.6	55.2	1.16	nd	nd	–
59	140	227.0	464.4	2.05	16.1	17.2	0.94
60	65	132.3	247.1	1.87	nd	nd	–

[a] nd = non detected.
[b] ni = non indicated.

(Spearman rank-test) was found between the administered dose and MTD total concentration ($r = 0.55$, $P = 0.0001$). The EDDP enantiomers were also quantified in 10 OF specimens and the enantiomeric concentrations ranged from 9.0 to 17.7 ng/mL for (*R*)-EDDP and from 10.9 to 22.4 ng/mL for (*S*)-EDDP. Contrary to a previous study where no clear predominance for any EDDP enantiomer was observed after the analysis of 5 OF specimen, the data of this study revealed *R*/*S* ratios ranging from 0.70 to 0.94 with an enantioselective excess of (*S*)-EDDP. The same trend was also observed for free plasma concentrations of EDDP [2].

4. Conclusions

A rapid and a validated method has been developed for the enantioselective quantification of MTD and of its major metabolite EDDP in OF. This method was successfully applied to the determination of enantiomeric ratios of MTD and EDDP in 60 specimens obtained from patients enrolled in a MTD maintenance programme. Our data pointed out that higher concentrations of (*R*)-MTD and the enantioselective excess of (*S*)-EDDP in OF may reflect the free fraction of MTD and EDDP

enantiomers in plasma. The enantiomeric concentrations may give interesting indications about the MTD metabolism variability of a patient as some cytochromes have been shown to be stereoselective for MTD.

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