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Enantioselective high-performance liquid chromatography determination of methadone enantiomers and its major metabolite in human biological fluids using a new derivatized cyclodextrin-bonded phase

Chuong Pham-Huy[a,*], Nassima Chikhi-Chorfi[a], Hervé Galons[b], Nouredine Sadeg[a], Xavier Laqueille[d], Nicole Aymard[c], France Massicot[a], Jean-Michel Warnet[a], Jean-Roger Claude[a]

[a]*Laboratoire de Toxicologie (EA 207), Faculté de Pharmacie, Université de Paris V, 4, Avenue de l'Observatoire 75006, Paris, France*
[b]*Laboratoire de Chimie Organique, Faculté de Pharmacie, Université de Paris V, 4, Avenue de l'Observatoire 75006, Paris, France*
[c]*Laboratoire de Pharmacologie, Faculté de Pharmacie, Université de Paris V, 4, Avenue de l'Observatoire 75006, Paris, France*
[d]*Service de Toxicologie, Hôpital Sainte-Anne, 1, Rue Cabanis 75014, Paris, France*

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Abstract

The simultaneous determination of methadone (Mtd) enantiomers and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in human urine and serum by enantioselective HPLC using a new Cyclobond I-2000 RSP column is described. After alkaline extraction from urine or serum with estazolam as an internal standard, Mtd enantiomers and its metabolite (EDDP) are separated on the previous column with reversed-mobile phase and detected at 210 nm. Peak resolutions are about 2.0 for Mtd enantiomers. The relative standard deviations (R.S.D.) of Mtd and EDDP standards are between 0.5 and 4.5%. Most drugs of abuse are shown not to interfere with this technique. The method has been applied to study the levels of each Mtd enantiomer and of its racemic metabolite in urine and serum of patients under maintenance treatment for opiate dependence. In urine, *R*-(−)-Mtd levels are always higher (about 2±0.5-fold) than those of *S*-(+)-Mtd and in most cases, metabolite concentrations are greater than those of global Mtd enantiomers. However, the *R*-(−) enantiomer levels of residual drug in serum of some patients were lower than those of its antipode. This method is suitable for pharmacokinetic and toxicological studies of Mtd enantiomers and its major metabolite in biological fluids. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Methadone; 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

1. Introduction

Methadone (Mtd), a central-acting analgesic with high affinity for μ-opiod receptors, has been used to treat opiate dependence and also cancer pain [1–3]. Mtd is a chiral synthetic compound (Fig. 1), mostly used in therapy under racemic mixture of *R*-(−) and *S*-(+) enantiomers. In humans, *R*-(−)-Mtd is about 25–50 fold more potent as an analgesic than its *S*-(+) antipode [4]. Moreover, these differences in

*Corresponding author.

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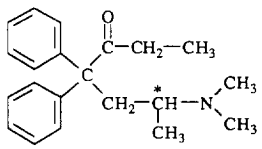
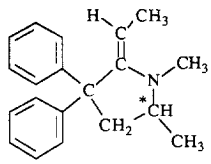
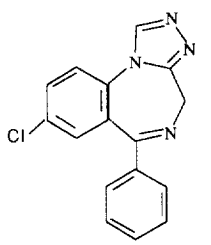


Methadone (Mtd)



2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (Major metabolite)



Estazolam (I.S.)

Fig. 1. Structures of methadone, EDDP metabolite and internal standard.

potency are due not only to the pharmacodynamic effects but also to the stereoselective pharmacotoxicokinetic disposition. Because of inter-individual differences in the pharmacokinetics of Mtd, it is suitable to analyze the respective Mtd enantiomer levels in biological fluids rather than the total Mtd concentrations in order to establish their individual therapeutic profiles and also to prevent their toxic effects.

For the quantification of Mtd in biological fluids, various non-stereoselective assays were cited in the literature, including gas chromatography (GC) with thermoionic detection [5,6] mass spectrometry [7,8], high-performance liquid chromatography (HPLC) [9,10], fluorescence polarization immunoassay (FPIA) [10] and radioimmunoassay (RIA) [11]. Recently, some stereoselective analyses of Mtd enantiomers have been reported in the literature such as enantioselective HPLC assays [12–15] using either the α_1-acid glycoprotein (chiral AGP) column [12–14] or the cyclodextrin β-RSP-bonded phase [15], chiral GC [16] and stereospecific RIA [17].

The aim of this study is to report a technique for the simultaneous analysis of methadone enantiomers and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (Fig. 1) in human biological fluids by enantioselective HPLC utilizing estazolam (Fig. 1) as an internal standard (I.S.) and a new derivatized β-cyclodextrin-bonded phase (Cyclobond-I 2000 RSP column) with reversed-mobile phase. The method has been applied to study the levels of each Mtd enantiomer and of its major racemic metabolite in urine and serum of patients under maintenance treatment for opiate dependence.

2. Experimental

2.1. Chemicals

Racemic methadone (Mtd) hydrochloride was kindly provided by Central Pharmacy of Hospitals (Paris, France). Major primary methadone metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP), estazolam used as an internal standard (I.S.) was purchased from Sigma (St Louis, MO, USA). *S*-(+)-Mtd used for identifying the two peaks of racemic compound was prepared by chemical isolation from racemic Mtd according to the technique previously described [18]. Briefly, racemic Mtd hydrochloride was reacted in hot ethanol with ammonium (+)-bromocamphor-π-sulfonate. The *S*-(+)-Mtd bromocamphor sulfonate formed was precipitated by the addition of water, then converted to its hydrochloride by usual methods. Other chemicals, drugs and solvents were of analytical purity and all obtained from Sigma. Deionized water was purified by Milli Q.UV Plus system (Millipore, Milford, MA, USA).

2.2. Apparatus and chromatographic conditions

The liquid chromatographic system consisted of a Spectra System pump Model P1000, Thermo Separation Products (Fremont, CA, USA), a Waters UV

detector Model 484 (Milford, MA, USA), a Shimadzu CR-6A integrator (Kyoto, Japan) and a Rheodyne injector, Model 7125, fitted with a 50-μl loop. An analytical column (250×4.6 mm I.D.) packed with β-cyclodextrin derivatized with *R,S*-hydroxypropyl ether-bonded phase (new Cyclobond-I-2000 RSP), 5-μm particle diameter was purchased from Advanced Separation Technologies (ASTEC) (Whippany, NJ, USA). A guard column (10×3.2 mm I.D.) packed with the same derivatized β-cyclodextrin bonded phase was connected between the injector and the analytical column.

The reversed-mobile phase was obtained by mixture of acetonitrile, 1% triethylamine acetate buffer (TEAA), pH 4.5, and water (19:8:73, v/v). TEAA buffer was prepared by dilution of 1.0 ml pure triethylamine with about 70 ml deionized water, then adjusted with glacial acetic acid to pH 4.5 and completed to 100 ml with water. Mobile phase was degassed by sonication and pumped at a flow-rate of 0.4 ml/min. Injection volumes of samples and standards were performed with the 50-μl sample loop. The UV detector was set at 210 nm. Column temperature was ambient (18–20°C).

2.3. Preparation of standards

Three stock standard solutions were obtained by dissolving individually racemic Mtd hydrochloride, EDDP perchlorate (metabolite) and estazolam (I.S.) in absolute ethanol at 1.0 mg/ml (free base). Stock solutions were stable for several months at 4°C. Standard calibration solutions were prepared by spiking drug-free human urine or serum with stock standard solutions further diluted to achieve final concentrations between 0.05 and 2.0 μg/ml of racemic Mtd and of EDDP metabolite and stored at −20°C. An I.S. working solution at 10 μg/ml was obtained by dilution of an aliquot of estazolam stock solution with absolute ethanol.

2.4. Extraction procedure

One hundred μl of thawed urine or serum human samples or standard calibration solutions were added into a labelled 16×100 mm centrifuge glass tube, then followed successively by 100 μl of I.S. for urine or 50 μl for serum, 300 μl of 10% anhydrous sodium carbonate in water and 4 ml of hexane. 2-Propanol (0.5 ml) was added in the case of serum sample for avoiding emulsion. The tubes were tightly capped and vortex-mixed for 2 min. After centrifugation for 10 min at 1500 *g*, the upper organic layer was transferred into a 5-ml glass tube and completely evaporated under a stream of nitrogen at room temperature. Mobile phase (100 μl) was added into each tube and vortex-mixed for 30 s for regenerating urine or serum residue extract. A 50-μl aliquot of the extract was then injected onto the column. For serum extract, the reconstituted liquid was centrifuged for 7 min at 3000 *g* or filtered through a micro Millex filter 0.22 μm (Millipore) to separate the precipitate. For low levels of Mtd, sample and other reagent volumes were doubled, except for mobile phase which was maintained at 100 μl.

2.5. Validation of the method

The precision of the assay was tested by comparing the results of two human serum and urine standards and samples determined in a day ($n=6$) and from replicate analysis ($n=6$ days) in order to achieve the intra-day and inter-day relative standard deviations (R.S.D.), respectively. The linearity of standard calibration curves of serum and urine was obtained by performing simple measures of spiked standard samples over 6 days in the range of 0.05–2.0 μg/ml. The validation of the method was also realized by comparing the results of 30 urine samples from five treated patients obtained by an automatic non-enantioselective HPLC method (REMEDI) purchased from Biorad (Hercules, CA, USA), for the determination of global Mtd and EDPP, with those achieved by the present enantioselective HPLC method. The REMEDI method is a multi-column system developed for automated analysis of basis drugs in urine. Two polymeric pre-columns, containing PRP-1 and Aminex A-28, were used to isolate the drugs. A short reversed-phase column, coupled to a silica column, produced the analytical separation. Sample preparation consisted of dilution and centrifugation [19].

The recovery of Mtd, EDDP metabolite (100 and 500 ng/ml of racemic compounds) and I.S. from drug-spiked urine and serum after extraction was determined by comparing peak areas of these com-

pounds from sample extracts with those of corresponding non-extracted standards diluted in mobile phase.

The interference with the assay was determined by injection of different standard common drugs or drugs of abuse onto the column either directly or after alkaline extraction.

2.6. Calculation

The analysis of each Mtd enantiomer and its EDDP metabolite in the sample was based on the peak area ratio of each analyzed compound to the I.S. The ratio values found were reported on a standard calibration curve performed under the same conditions with standard calibration solutions at the range of the concentrations studied.

2.7. Application of the method

To test the applicability of the described method for therapeutic monitoring, five patients under maintenance treatment for opiate dependence received orally a single dose of racemic Mtd solution (from 45 to 90 mg per dose) in the morning. Thirty urine samples from five patients were collected 24 h after drug intake and three serum samples from three others for the determination of residue Mtd were taken 24 h after, i.e. before the next administration of drug. All urine aliquots and serum samples were frozen at −20°C before use.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of drug-free human urine and serum, standard calibration urine and serum, and treated subject urine and serum are shown in Figs. 2 and 3. The first set of peaks, at retention times of about 15.5 and 17 min corresponds to the *R*-(−)- and *S*-(+)-Mtd, respectively. The second set of peaks, at a retention time of about 19 min corresponds to the racemic EDDP (major metabolite) and the last peak (22 min) is an internal standard (I.S.) (estazolam).

The resolution factor, $R_s=2(t_{R2}-t_{R1})/w_1+w_2$, and the selectivity factor, $\alpha=t_{R2}-t_{R0}/t_{R1}-t_{R0}$, of Mtd enantiomers were 2.0 and 1.17, respectively. The peak form of all compounds was symmetric. The

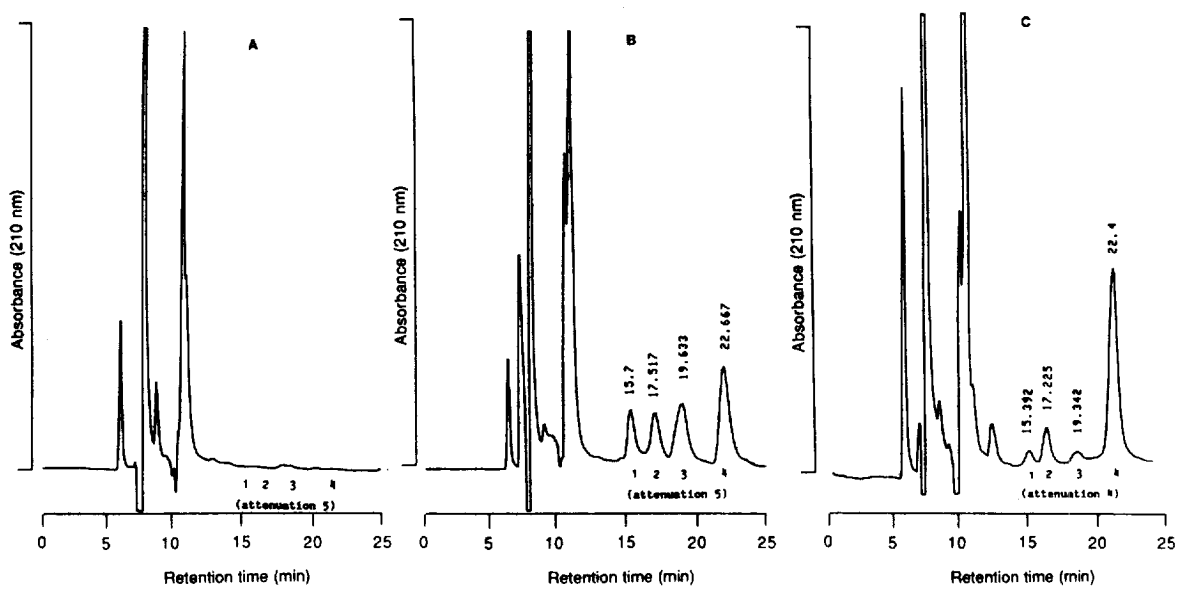


Fig. 2. Chromatograms of methadone enantiomers and EDDP metabolite in human serum after extraction. (A) Blank serum; (B) human serum standard spiked with 100 ng/ml racemic Mtd and EDDP; (C) patient serum after 24 h oral administration of 90 mg racemic Mtd. Peaks: 1 and 2, (*R*)- and (*S*)-Mtd; 3, EDDP; 4, I.S. (estazolam).

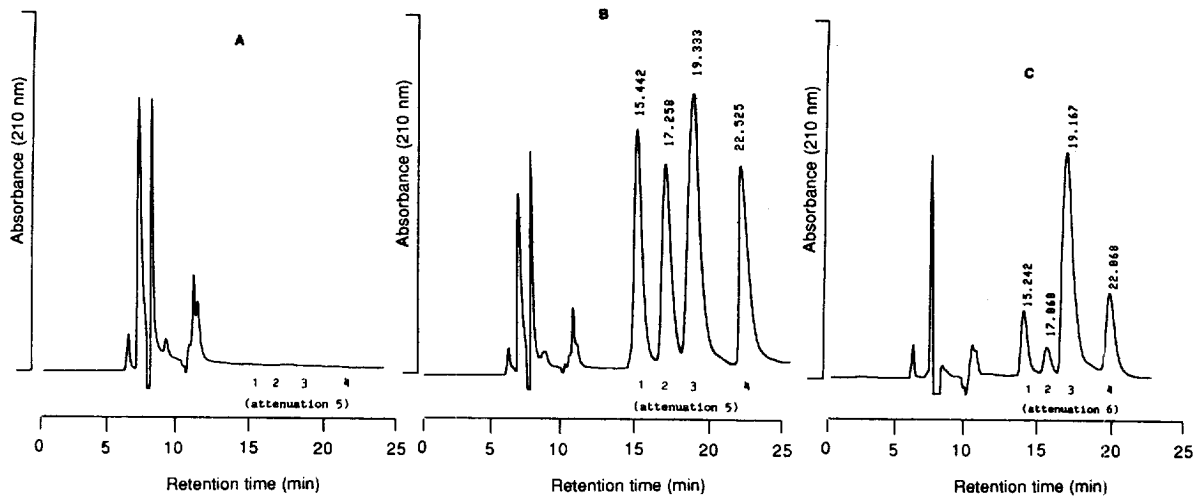


Fig. 3. Chromatograms of methadone enantiomers and EDDP metabolite in human urine after extraction. (A) Blank urine; (B) human urine standard spiked with 500 ng/ml racemic Mtd and EDDP; (C) 24-h urine of a patient treated with 90 mg racemic Mtd per os. Peaks: 1 and 2, (*R*)- and (*S*)-Mtd; 3, EDDP; 4, I.S. (estazolam)

peak area ratio of *R*/*S* standard Mtd enantiomers was 1.00±0.05 ($n=10$).

The relative response factors ($f=C\times A_{\text{I.S.}}/C_{\text{I.S.}}\times A$, where A=area and C=concentration) of Mtd enantiomers and EDDP at 0.1–1.0 μg/ml, and I.S. at 5 and 10 μg/ml, were the same (0.222±0.007).

3.2. Statistical data

The detection limit (signal-to-noise ratio>3) of the assay after extraction was about 1.0 ng/ml for each Mtd enantiomer and 2.0 ng/ml for the racemic EDDP metabolite. The lower limits of quantification after alkaline extraction were about 10 ng/ml for the Mtd enantiomer and 20 ng/ml for the metabolite with an R.S.D. less than 10% and about 5 and 10 ng/ml, respectively, with an R.S.D.<15%.

The standard calibration curves of urine and serum exhibited good linearity for *R*-(−)- and (*S*-(+)-Mtd and EDDP metabolite over the range of concentrations tested (0.05–2.0 μg/ml racemic Mtd and metabolite) with correlation coefficients greater than 0.999 for both enantiomers and EDDP (Table 1).

The intra-day and inter-day R.S.D.s ($n=6$) of two

Table 1
Precision, linearity and recovery of methadone (Mtd) and EDDP metabolite standards ($n=6$)

Compound	Precision (R.S.D.%)				Linearity of calibration standards: 0.05–2.0 μg/ml	Recovery (%)	
	100 ng/ml		500 ng/ml			100 ng/ml	500 ng/ml
	Intra-day	Inter-day	Intra-day	Inter-day			
Serum							
(*R*)-Methadone	1.75	4.52	0.78	3.95	$r=0.9997y=7.14x-0.20$	92.1±2.4	94.6±1.7
(*S*)-Methadone	2.15	4.35	1.07	3.50	$r=0.9998y=6.57x-0.04$	95.4±3.1	91.9±2.8
EDDP	2.96	3.75	1.95	4.25	$r=0.9998y=13.1x+0.02$	89.6±3.8	92.5±2.4
Urine							
(*R*)-Methadone	0.85	1.96	0.51	1.72	$r=0.9996y=7.11x-0.07$	94.5±2.1	95.7±1.8
(*S*)-Methadone	1.32	3.50	0.75	2.10	$r=0.9995y=6.88x-0.10$	93.2±1.7	94.7±3.2
EDDP	1.95	3.75	0.48	2.96	$r=0.9996y=13.6x-0.01$	91.2±3.2	93.1±2.4

urine and serum standards (100 and 500 ng/ml racemic Mtd and EDDP) varied between 0.5–3.0% and 1.7–4.5%, respectively (Table 1). The intra-day R.S.D. of two urine and serum samples from two treated patients varied between 0.5–2.0% and 1.6–3.2%, respectively; the inter-day R.S.D. of the same samples varied between 3.0–4.6% for urine and 3.7–8.8% for serum (Table 2).

The recovery of Mtd enantiomers and metabolite from the two previous urine and serum standards varied between 92 and 95% for Mtd enantiomers, 90 and 93% for EDDP, and that of I.S. was about 80%. We have tried heptane, instead of hexane, for the extraction of Mtd and EDDP from biological samples, and found that this solvent decreased the recovery to lower than 80% for Mtd as well as for the metabolite. Therefore, this could explain the good recovery of our method when compared with another technique which used heptane as a solvent. In the other technique [15], only about 80% of Mtd were found after double extraction. Moreover, the procedure described herein requires only 100–200 μl of sample, contrary to the previous assay [15] which utilized 1 ml of the biological fluid. We have also used diethyl ether as extraction solvent and found that despite the good recovery (about 90–95%) obtained with this solvent, many endogenous compounds of serum and urine interfered with Mtd, EDDP and I.S. peaks. Some authors [9] have used solid-phase extraction (SPE) of samples for the reversed-phase HPLC determination of global Mtd and its two metabolites in serum and found a good recovery (90–99%) for these compounds. However, it is not known if endogenous compounds present in serum and urine extracts obtained from this SPE method interfered with analytes separated by enantioselective HPLC.

A comparison between the levels of racemic Mtd and EDDP metabolite in 30 urine samples from five treated patients determined by the automatic REMEDI method and the concentrations of the sum of Mtd enantiomers and of metabolite in the same samples obtained by this enantioselective HPLC method gave congruent results with a correlation coefficient $r=0.994$ and regression line $y_{\text{REMEDI}}=1.06x_{\text{HPLC}}-0.39$ for Mtd and $r=0.998$ and $y_{\text{REMEDI}}=1.04x_{\text{HPLC}}-0.47$ for EDDP metabolite.

3.3. Choice of chiral column and optimization of mobile phase

We have tested different chiral columns and mobile phases for the simultaneous separation of Mtd enantiomers and EDDP metabolite. A beta-native cyclodextrin-bonded phase (Cyclobond I) either with a polar organic phase (as previously described [20]) or with reversed-phase (TEAA buffer and acetonitrile at different concentrations and pH) was chosen first; subsequently, β-acetylated cyclodextrin (Cyclobond-I-Ac) with the two previous mobile phases and also with the normal-phase mode (hexane-2-propanol) was used. None of the previous columns or phase modes can separate Mtd enantio-

Table 2
Intra-day and Inter-day precision of methadone enantiomers and EDDP metabolite in serum and urine samples of treated patients

Compound	Subject no. 1			Subject no. 2		
	Concentration (μg/ml)	R.S.D.% ($n=6$)		Concentration (μg/ml)	R.S.D.% ($n=6$)	
		Intra-day	Inter-day		Intra-day	Inter-day
Serum						
(*R*)-Methadone	0.014	2.72	8.82	0.015	1.57	7.87
(*S*)-Methadone	0.041	1.82	4.58	0.057	2.78	3.70
EDDP	0.021	2.10	7.32	0.031	3.20	4.75
Urine						
(*R*)-Methadone	3.52	0.55	3.80	2.48	0.95	4.15
(*S*)-Methadone	1.65	1.82	4.56	1.35	2.05	4.63
EDDP	27.10	0.45	2.96	19.40	1.95	3.56

mers. We have also used a Chiralcel OJ column (cellulose Tris–4-methyl-benzoate coated on silica gel purchased from Daicel Co) with normal-phase (hexane–ethanol or 2-propanol and diethyl amine at different compositions, varying from 50 to 90% for hexane and 0.05–0.1% for diethyl amine). This column can separate the two Mtd enantiomers with good resolution (R_s=2.3±0.3); however, the peak of EDDP metabolite was superposed on the first peak of Mtd. Moreover, the EDDP peak showed significant tailing (T=2.3) whatever the composition of the previous normal phase.

For the recently marketed Cyclobond-I-2000 RSP, we have tested different reversed phases constituted of TEAA buffer with final concentrations varying from 0.05 to 1% and with pH from 3.0 to 6.2, and acetonitrile and/or methanol from 10 to 25% and at different flow-rates (0.4–1.0 ml/min). We have observed that, with 1% TEAA buffer, pH 6.0±0.2, the EDDP peak interfered with the second peak of Mtd and the resolution of the last racemic compound was weak (R_s=1.2±0.1). However, when final concentrations of TEAA buffers varied between 0.06 and 0.15% and its pH from 4.1 to 5.0, the simultaneous separation of Mtd enantiomers with EDDP metabolite can occur with good resolution. We have chosen a reversed-mobile phase constituted of 0.08% TEAA buffer, pH 4.5, and 19% acetonitrile with a flow-rate at 0.4 ml/min, because these conditions not only gave the best resolutions of Mtd enantiomers (R_s=2.0) and of the two others compounds (EDDP and I.S.) with good peak symmetry, but also no interferences of a number of drugs of abuse and common drugs with Mtd, metabolite and I.S. were observed (see Section 3.4). However, the EDDP enantiomers cannot be separated by this method, even with two Cyclobond-I-2000 RSP columns coupled in series or with one previous column connected with another Cyclobond-I or I-Ac column. This disparity in the enantioselectivity of the Cyclobond-I-2000 RSP towards Mtd and EDDP molecules could be explained by the evident difference between their chemical structures (Fig. 1). Indeed, the structure of the EDDP metabolite includes in addition a new pyrrolidine ring resulting from an *N*-demethylation followed by a cyclisation of Mtd by cytochrome P-450 in the liver [21]. The presence of this heterocyclic ring could obstruct the formation of inclusion complexes into the cavity of the cyclodextrin molecule, thereby inhibiting the chiral separation of EDDP enantiomers.

According to information supplied by Advanced Separation Technologies (ASTEC, Whippany, USA), the difference between the new Cyclobond-I-2000 columns and the classical β-cyclodextrin (CD) stationary phases is due to the linkage of CDs to a high purity 5-μm silica gel through a spacer arm of optimum chain length by a process which yields a stable, non-hydrolytic, non-nitrogen containing ether bond. Therefore, this second generation of Cyclobond-I products offers increased stability, reproducibility and enantioselectivity compared to the classical β-CD columns. These properties have been demonstrated by the use of the Cyclobond-I-2000 RSP in this work, over a period of about 1 year, without a decrease in resolution of Mtd enantiomers or in the retention times of three compounds. According to some authors [13,14], the chiral AGP column used for the enantioselective determination of Mtd [12–14] has a short lifetime and is, therefore, too expensive.

Concerning the Cyclobond-I-2000 RSP column, the linkage of racemic propylene oxide with hydroxyl groups on the surface of the β-CD results in additional flexible hydrogen bonding groups, extending the interactive potential for sterically hindered or extended chiral centers from the aromatic or included portion on the analyte. Therefore, the choice of CD stationary phases with or without derivatized linkers depends on the potent hydrophilic or hydrophobic interactions between the solutes and the CD stationary phases to enhance chiral affinity, especially in the reversed-phase mode [22].

With this new Cyclobond-I-2000 RSP column and the optimized reversed phase described above, we can simultaneously determine Mtd enantiomers and the major metabolite in biological fluids, contrary to the other enantioselective HPLC techniques [12–15] which can only analyze Mtd isomers and not EDDP. The simultaneous determination of Mtd and metabolite is important in the therapeutic monitoring of treated subjects because the presence of the mother compound is sometimes undetectable in biological fluids. Moreover, the major metabolite levels are in most cases much greater than those of Mtd isomers, especially in urine, EDDP metabolite being consid-

ered as a good marker for the control of the drug intake in treated patients.

3.4. Interferences

Using the described method, most drugs of abuse and common drugs were shown not to interfere with Mtd enantiomers, EDDP metabolite and I.S. either by direct injection of standard solution or after alkaline extraction. All morphinomimetic drugs (morphine, heroin, monoacetylmorphine, codeine, dionine, nalorphine, narcotine) and amphetamine after direct injection of standard solutions had retention times (t_{R_s}) between about 7–9 min. Benzoylecgonine, cocaine and dextropropoxyphene had retention times of, respectively, 14, 14.3 and 14.5 min versus 15.5 min for *R*-(+)-Mtd, 17 min for *S*-(−)-Mtd and 19 min for EDDP. Some benzodiazepines such as racemic oxazepam (t_{R_s} = 27.5 and 41.8 min), racemic lorazepam (t_{R_s} = 33.8 and 36.3 min), clonazepam (t_R = 27.0 min), nitrazepam (t_R = 26.4 min), diazepam (t_{R_s} = 34 min) and some tricyclic derivatives such as imipramine and clomipramine (t_{R_s} > 45 min) did not interfere with this technique. This is also true for caffeine and theophylline which had retention times of, respectively, 9.2 and 8.7 min. All barbiturates, some antiepileptic drugs (phenytoin, valproic acid), salicylic acid and acetylsalicylic acid did not appear on the chromatogram after alkaline extraction. No endogenous interfering peaks were observed with drug-free human serum and urine at the retention times of Mtd enantiomers, EDDP metabolite and I.S. (Figs. 1 and 2). This interference study has demonstrated the specificity of this described method which is an important analytical parameter for the Mtd determination in biological fluids of opiate addicts.

3.5. Application of the method

Some results of *R*-(−)- and *S*-(+)-Mtd and EDDP metabolite levels in urine and serum samples from patients treated with racemic Mtd are depicted in Table 3. The results show that in urine, *R*-(−)-Mtd levels were always higher (about 1.5–2.5-fold) than those of *S*-(+)-Mtd and in most cases, metabolite concentrations were greater than those of global Mtd enantiomers. However, the *R*-(−) enantiomer levels of residual drug in three serum patient samples taken 24 h after single oral administration of drug were lower than those of its antipode. It is the same for serum EDDP metabolite levels which were found to be lower than those of serum global Mtd. These observations confirmed the differences in the stereoselective pharmacokinetic disposition of Mtd enantiomers in humans.

In conclusion, the enantioselective HPLC method described here is applicable to routine therapeutic monitoring and toxico-pharmacokinetic study of Mtd enantiomers and its major metabolite, EDDP, in human serum and urine because of its accuracy, its specificity and its sensitivity. Moreover, the sample volume is small. This enantioselective method will be a suitable tool for exploring the differences between the *R*-(−)- and *S*-(+)-methadone levels in

Table 3
Concentrations of methadone enantiomers and EDDP metabolite in urine and serum of some patients under maintenance treatment

Sample	Rac. Mtd dose (mg)	Concentration (μg/ml) (n = 6)			Ratio	
		(*R*)-Mtd	(*S*)-Mtd	EDDP	(*R*)/(*S*)	EDDP/(*R*)+(*S*)
(1) 24-h urine from patient						
R	90	2.48	1.35	19.4	1.84	5.06
H	85	3.52	1.65	27.1	2.13	5.24
S.T.	85	6.36	2.86	18.1	2.22	1.96
D.T.	50	2.33	1.49	10.3	1.56	2.69
S.	45	1.16	0.45	7.3	2.57	4.53
(2) Serum from patient 24 h after drug intake						
K.F.	50	0.014	0.041	0.021	0.34	0.38
M.P.	50	0.011	0.025	0.017	0.44	0.47
C.V.	60	0.015	0.057	0.031	0.26	0.43

human biological fluids as well as in experimental animal organs.

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