ELSEVIER

Quantitative determination of the enantiomers of methadone and its metabolite (EDDP) in human saliva by enantioselective liquid chromatography with mass spectrometric detection

Maria Esther Rodriguez Rosas [a], Kenzie L. Preston [b], David H. Epstein [b],
Eric T. Moolchan [b], Irving W. Wainer [a,*]

[a] *Bioanalytical and Drug Discovery Unit, Gerontology Research Center, National Institute on Aging, National Institutes of Health,
5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA*
[b] *Clinical Pharmacology and Therapeutics Research Branch, Intramural Research Program,
National Institute on Drug Abuse, National Institute of Health, Baltimore, MD, USA*

Abstract

A sensitive enantioselective liquid chromatographic assay with mass spectrometric detection (LC–MS) has been developed and validated for the simultaneous determination of saliva concentrations of (R)- and (S)-methadone (Met) and (R)- and (S)-2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP, a primary metabolite of Met). Saliva specimens were collected using Salivette devices (Sarsedt), and centrifuged; collected saliva was then spiked with deuterated internal standards, D3-Met and D3-EDDP, and directly injected into the LC–MS. Enantioselective separations were achieved on a liquid chromatographic chiral stationary phase (CSP) based upon immobilized α_1-acid glycoprotein (AGP) using a mobile phase composed of acetonitrile: ammonium acetate buffer (10 mM, pH 7.0) in a ratio of 18:82 (v/v), a flow rate of 0.9 ml/min and a temperature of 25 °C. Under these conditions, enantioselective separations were observed for methadone ($\alpha = 1.30$) and EDDP ($\alpha = 1.17$) within 15 min. Met, EDDP, D3-Met and D3-EDDP were detected using selected ion monitoring at m/z 310.20, 278.20, 313.20 and 281.20, respectively. Linear relationships between peak height ratio and drug-enantiomer concentrations were obtained for methadone in the range of 5.0–600.0 ng/ml, and for EDDP from 0.5 to 15.0 ng/ml per enantiomer with correlation coefficients better than 0.9994, where lower limit of quantification (LLOQ) for Met was 5 ng/ml and for EDDP 0.5 ng/ml. Acceptable intra- and inter-day precision of the method (CVs $< 4.0\%$) and accuracy (CVs $< 4.0\%$) were obtained. These findings demonstrate the accuracy and precision of the method used to successfully analyze saliva obtained from patients enrolled in a methadone-maintenance program.
Published by Elsevier B.V.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Methadone; 2-Ethylene-1,5-dimethyl-3,3-diphenyl-pyrrolidine

1. Introduction

Methadone (6-dimethylamino-4,4-dipehnyl-3-heptanone hydrochloride, Met, Fig. 1) is a synthetic opioid agonist used therapeutically in both programs for opioid dependence and for analgesia in patients

---

* Corresponding author. Tel.: +1-410-558-8498;
fax: +1-410-558-8409.
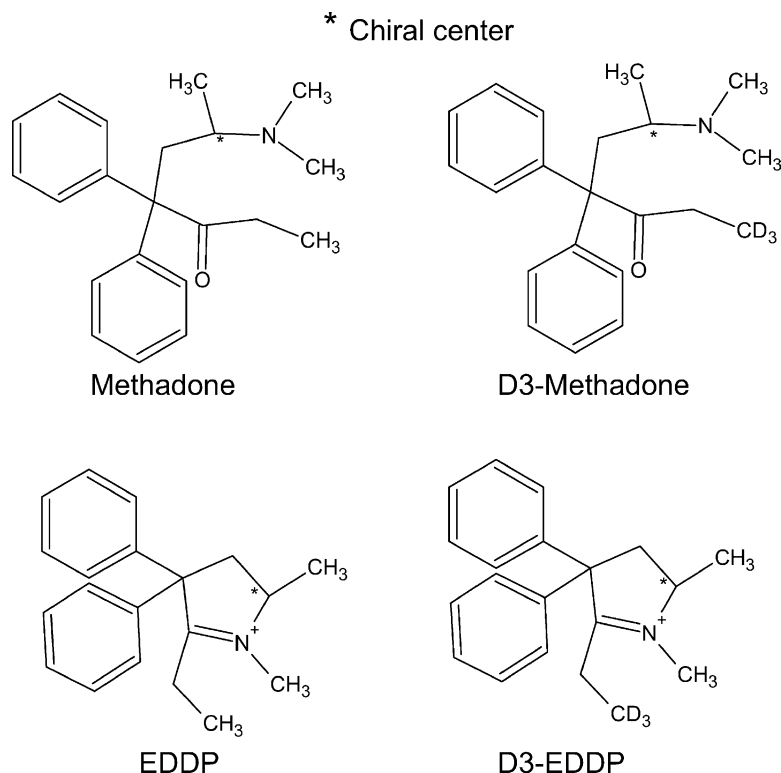*E-mail address:* wainerir@grc.nia.nih.gov (I.W. Wainer).

Fig. 1. The chemical structures of methadone, EDDP, D3-methadone and D3-EDDP.

with severe pain [1]. Met is a chiral molecule, that exists in (+)-(S)-Met and (−)-(R)-Met forms. The primary Met metabolite is the chiral compound 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP), in which (−)-(S)-EDDP arises from (S)-Met and (+)-(R)-EDDP arises from (R)-Met [2].

Met is therapeutically administered as a racemic mixture, i.e. a 50:50 mixture of its enantiomers, although (R)-Met has a higher affinity than (S)-Met for the μ-opioid receptor [1] and the analgesic potency of (R)-Met is 50 times greater than that of the (S)-enantiomer [3,4]. There is also a significant difference in the plasma protein binding of the Met enantiomers, with (S)-Met bound more extensively to α_1-acid glycoprotein (AGP) than (R)-Met, 87–79%, respectively [5].

In addition to pharmacodynamic and protein binding differences between the enantiomers, there are also enantioselective differences in the pharmacokinetic profiles. (R)-Met has a significantly longer elimination half-life ($t_{1/2}\beta$) than (S)-Met as well as

a larger total volume of distribution [6]. However, there are also significant inter-individual differences in these parameters; for example, in narcotic addicts in maintenance programs, $t_{1/2}\beta$ for (R)-Met ranged from 37.9 to 58.9 h and the $t_{1/2}\beta$ for (S)-Met ranged from 28.1 to 41.3 h [7,8]. Due to the enantioselective and inter-individual differences in Met disposition, therapeutic monitoring of this agent may require the use of enantioselective bioanalytical techniques.

Both non-chiral and chiral high-performance liquid chromatography (HPLC) methods have been reported for the bioanalytical analysis of Met [3,4,9–18]. The reported enantioselective HPLC assays for Met utilized chiral stationary phases (CSP) based upon immobilized α_1-acid glycoprotein (AGP–CSP) [13,19–22], native β-cyclodextrin [14] and hydroxypropyl-β-cyclodextrin [5,15,19]. Enantioselective capillary electrophoresis methods have also been reported for the quantification of methadone enantiomers in hair or urine samples [23,24] as well as gas chromatography [25].

Although most therapeutic drug monitoring assays are based upon plasma or urine, saliva has been investigated as an alternative matrix. Saliva is a clean matrix, consisting of 98% water and a protein concentration of about 0.3 g/100 ml [26]. In addition, saliva sample collection is non-invasive and painless, readily available and requires no specially trained personnel.

An enantioselective liquid chromatography–mass spectrometry (LC–MS) assay has been reported for the quantification of Met in saliva using an AGP–CSP [22]. The method was rapid (analysis time of <10 min) and a lower limit of quantification (LLOQ) of 5 ng/ml for each enantiomer was reported. The method was validated for the determination of (*R*)-/(*S*)-Met ratios in plasma and saliva and applied to the analysis of 28 samples from 28 heroin addicts undergoing Met treatment.

The LC–MS method developed by Veuthey and co-workers [22] did not simultaneously determine the saliva concentrations of EDDP enantiomers. However, Veuthey's laboratory has reported non-enantioselective determinations of Met and EDDP in human plasma and serum by LC–MS methods achieving LOQ's of 10 and 25 ng/ml, respectively [27,28].

The separation and quantification of (*R*)-EDDP and (*S*)-EDDP in the presence of Met has been previously reported [20,21]. These enantioselective separations were also achieved using an AGP–CSP and UV detection. The assays were validated and used in the analysis of the urinary concentrations of Met and EDDP enantiomers.

This report describes the development and validation of a LC–MS method to quantify Met and EDDP enantiomers in saliva. The assay was based upon previously reported methods that utilized the AGP–CSP [20–22]. The method is reproducible and accurate and was applied to the analysis of saliva samples from polysubstance-using patients enrolled in a methadone-maintenance program.

2. Experimental

2.1. Chemicals and reagents

(+)-(*S*)-Methadone [(*S*)-Met] and (−)-(*R*)-methadone [(*R*)-Met], were provided by The Drug Inven-

tory Supply and Control System of National Institute on Drug Abuse (NIDA, Baltimore, MD, USA); (*R*,*S*)-methadone hydrochloride [(*R*,*S*)-Met], was purchased from Sigma–Aldrich (St. Louis, MO, USA); (*R*,*S*)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium perchlorate [(*R*,*S*)-EDDP] (1.0 mg/ml methanol solution); deuterium labeled (*R*,*S*)-D3-methadone [(*R*,*S*)-D3-Met], and deuterium labeled (*R*,*S*)-D3-EDDP perchlorate [(*R*,*S*)-D3-EDDP] (100 μg/ml methanol solutions); were purchased from Cerilliant Corporation (Austin, TX, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC reagent grade ammonium acetate was obtained from J.T. Baker (Phillippsburg, NJ, USA). Ultra-pure water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). Pooled drug-free human saliva was obtained from healthy volunteers.

2.2. Apparatus

The analytical system consisted of a Series 1100 Liquid Chromatography/Mass Selective Detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G1322 A), a binary pump (1312 A), an autosampler (G1313 A), a thermostated column compartment (G1316 A); a mass selective detector, MSD (G1946 B) equipped with atmospheric pressure ionization electrospray (API-ES) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 250 MHz Kayak XA computer (Hewlett-Packard, Palo Alto, CA, USA) running ChemStation software (Rev A.08.03 [847], 1990–2000, Hewlett-Packard).

2.3. Chromatographic conditions

The enantioselective separations of (*R*)- and (*S*)-Met, (*R*)- and (*S*)-EDDP, (*R*)- and (*S*)-D3-Met and (*R*)- and (*S*)-D3-EDDP were accomplished using a chiral stationary phase based upon immobilized α_1-acid glycoprotein (chiral–AGP) from Advanced Separation Technologies (Whippany, NJ, USA). A chiral–AGP guard column (10 mm × 2.0 mm i.d., 5 μm) and a chiral–AGP analytical column (100 mm × 4.0 mm i.d., 5 μm) were used in series. The mobile phase consisted of acetonitrile: ammonium acetate buffer (10 mM, pH

7.0 (adjusted with 2.0% aqueous ammonium hydroxide), 18:82 (v/v)). The flow rate was 0.9 ml/min, the injection volume was 40 µl and the column temperature was kept at 25 °C.

2.4. Optimization of the mass selective detector (MSD) parameters

Mass spectra were recorded using a full scan in positive ion mode, with a scan range from m/z 100 to 600. Single ion monitoring (SIM) was used to quantify the target compounds. The chromatograms were monitored at $m/z = 310.20$ (Met), 278.20 (EDDP), 313.20 (D3-Met) and 281.20 (D3-EDDP).

The sensitivity of the Met and EDDP signals were primarily dependent on the MSD experimental parameters. In order to identify the optimized condition, the following MSD parameters were investigated: fragmentation voltage (50, 55, 60, 65 and 70 V), capillary voltage (1000, 1250, 1500, 1750, 2000 and 2500 V), nebulizer pressure (10, 20, 30, 40, and 50 psig) and drying gas flow (5,7,9,11,13 l/min) and drying gas temperature (200, 250, 300 and 350 °C). The optimized parameters were as follows: fragmentor, 60 V; drying gas flow-rate, 7.0 l/min; nebulizer pressure, 20 psig; drying gas temperature, 350 °C and capillary voltage, 1250 V.

2.5. Preparation of stock solutions

Concentrated stock solutions of (*R,S*)-Met (10.0 µg/ml as free base), (*R,S*)-EDDP (1.0 µg/ml as free base), (*R,S*)-D3-Met (10.0 µg/ml as free base) and (*R,S*)-D3-EDDP (5.0 µg/ml as free base) were prepared in water, placed in capped polypropylene tubes, wrapped in aluminum foil and stored at −80 °C. Spiking standard solutions for the calibration curve and quality control samples (QCs) were made by serial dilutions with water starting with their respective concentrated stock solution. These spiking standards were placed in capped polypropylene tubes, wrapped in aluminum foil and stored at −80 °C.

2.6. Preparation of calibration curve and quality control standards

The determinations of Met and EDDP were based on the internal standard method, using their respective deuterium labeled compounds as internal standards. Calibration and QC standards were prepared daily by adding 50 µl of the corresponding spiking standard solution containing Met, EDDP, D3-Met and D3-EDDP to 200 µl drug-free saliva. The 8-point calibration curve for Met ranged from 5.0 to 600.0 ng/ml (5.0, 50.0, 100.0, 200.0, 300.0, 400.0, 500.0, 600.0 ng/ml) and for EDDP from 0.5 to 15.0 ng/ml (0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 ng/ml) using constant concentrations of D3-Met (50.0 ng/ml) and D3-EDDP (25.0 ng/ml). The linearity of the standard curves were determined using the "calibration settings window" running in the ChemStation software (Rev A.08.03 [847], 1990–2000, Hewlett-Packard) with the weighting function set at equal.

The QC standards for Met were 50.0 ng/ml {low quality control (LQC)}, 300.0 ng/ml {medium quality control (MQC)} and 600.0 ng/ml {high quality control (HQC)} while for EDDP were LQC = 1.0, MQC = 7.5 and HQC = 15.0 ng/ml. All the concentrations are given per enantiomer.

2.7. Sample preparation

2.7.1. Collection of saliva from patients
Saliva was obtained from methadone-maintained outpatients enrolled in a clinical trial on combined behavioral and pharmacologic treatment for polydrug abuse at Archway, the treatment-research clinic at the National Institute on Drug Abuse Intramural Research Program (Baltimore, MD, USA). Specimens were collected with Salivette devices (Sarstedt, Newton, NC, USA); a cylindrical cotton swab was placed under the tongue or between the gum and cheek of the patient for 3–4 min or until saturated with saliva. The swab was placed back into the Salivette insert and firmly capped with the stopper.

2.7.2. Recovery of saliva from Salivette device
At the end of each dosing period (i.e. within 2.5 h), all swabs were centrifuged at $1200 \times g$ (4 °C) for 5 min. During this centrifugation step, the saliva was collected in the centrifuge tube, and the tube was re-capped and frozen at −20 °C until analysis.

2.7.3. Saliva sample preparation
After thawing, a 500 µl aliquot of saliva was transferred to a new microcentrifuge tube and then cen-

trifuged at $15,000 \times g$ (4 °C) for 15 min. A 200 µl aliquot of the supernatant was transferred to a clean tube and 50 µl of the solution containing the deuterated internal standards D3-Met and D3-EDDP was added, the resulting solution was vortex-mixed for 2 min, transferred to a 300 µl autosampler vial and 40 µl was injected onto the LC–MSD system.

2.8. Validation

The intra- and inter-day validation studies for precision and accuracy were performed in quintuplicate with QC standards at concentrations specified in Section 2.6. The analyses were carried out over a period of 3 days for the inter-day validation. The curves were constructed by plotting the peak height ratio (*R*)-Met/(*R*)-D3-Met, or (*S*)-Met/(*S*)-D3-Met, or (*R*)-EDDP/(*R*)-D3-EDDP or (*S*)-EDDP/(*S*)-D3-EDDP against its concentration.

Accuracy was determined by comparing the observed concentrations of the QC standards calculated from the calibration curve to their nominal concentrations.

The specificity of the method for each analyte was examined by individually screening Met, EDDP, D3-Met and D3-EDDP after spiking in pooled human saliva.

2.9. Application of the analytical method

The validated method was applied to the analysis of human saliva obtained from methadone-maintained outpatients at Archway Clinic, as described above. After giving informed consent, the patients were stabilized on methadone administered orally in a liquid suspension, beginning at 30 mg on Day 1 and increasing to 70 mg by 10 mg increments over 7 days. Approximately 5 weeks into treatment, 252 patients were randomly assigned to undergo a dose increase from 70 to 100 mg per day over 5 days or to remain at 70 mg per day. The five specimens reported here were taken from four men and one woman who had been maintained on methadone for 103–164 days (mean ± S.E.M. = 118.2 ± 25.8). Two of the patients had been on 70 mg per day for 95 and 158 days, respectively; the other three patients had been on 100 mg per day for 65 days (two patients) or 67 days (one patient).

Saliva collection occurred immediately before the daily dose of methadone was administered; thus, each specimen reported here was taken approximately 24 h after the previous dose of methadone (with a possible range of 16.5–31.5 h due to clinic dosing hours).

3. Results and discussion

3.1. Optimization of the chromatographic separation

Enantioselective separations on an immobilized α_1-acid glycoprotein chiral stationary phase (AGP–CSP) are affected by the buffer concentration, the type and concentration of organic modifiers and the pH of the mobile phase [29]. Each of these parameters was systematically studied in the development of the enantioselective separation. Temperature also plays a role in separations on a CSP. However, in this study, the temperature was maintained at 25 °C and this parameter was not adjusted.

3.1.1. Selection of the buffer

The previously reported enantioseleicitve LC–MS assay for Met in saliva used 10 mM ammonium acetate as the primary buffer [22]. In this study, ammonium acetate was also selected as the buffer because of its compatibility in LC–MS applications, and buffer concentrations of 10–20 mM were investigated. There was no significant influence of buffer concentration on the retentions or enantioselective separations of the Met and EDDP enantiomers. At 10 mM, the retention times and enantioselectivity (expressed as α) for (*R*)-Met and (*S*)-Met were 6.7 and 8.9 min, respectively ($\alpha = 1.41$) and for (*R*)-EDDP and (*S*)-EDDP 5.0 and 6.2 min, respectively ($\alpha = 1.32$). At 20 mM, the retention times and enantioselectivity for (*R*)-Met and (*S*)-Met were 6.0 and 8.0 min, respectively ($\alpha = 1.43$) and for (*R*)-EDDP and (*S*)-EDDP 4.5 and 5.6 min, respectively ($\alpha = 1.34$). Based upon these results, a 10 mM concentration of ammonium acetate was chosen for the study.

3.1.2. Selection of the organic modifier

Uncharged modifiers are often essential for enantioselective separations on the AGP–CSP and these

Table 1
The effect of the organic modifier on the enantioselective separations of methadone [(R)-Met, (S)-Met] and EDDP [(R)-EDDP, (S)-EDDP]; where the mobile phase composition was ammonium acetate buffer (10 mM, pH 7.0): modifier (80:20 (v/v)); k: retention factor; α: enantioselectivity

Organic modifier	(R)-Met (k)	(S)-Met (k)	Met (α)	(R)-EDDP (k)	(S)-EDDP (k)	EDDP (α)
1-Propanol	6.04	7.25	1.20	5.00	5.00	1.00
2-Propanol	6.00	7.22	1.20	5.00	5.00	1.00
Methanol	6.16	7.33	1.19	5.12	5.12	1.00
Acetonitrile	7.43	9.39	1.26	5.69	6.32	1.11
Ethanol	16.17	23.91	1.48	12.20	22.55	1.85

modifiers affect both retention and enantioselectivity [29]. The uncharged modifiers most often used with the AGP–CSP are hydrogen bond donor/acceptor such as simple alcohols, e.g. 1-propanol, 2-propanol and ethanol, and hydrogen bond acceptor/dipole such as acetonitrile.

The addition of an uncharged modifier usually reduces retention and increases efficiency often at the expense of enantioselectivity [29–32]. However, instead of reducing the enantioselectivity, the addition of an uncharged modifier can increase an observed chiral separation or induce one. For example, the enantiomers of methylphenobarbital are not resolved on the AGP–CSP when the mobile phase is composed of only phosphate buffer, while the addition of 2% 2-propanol to the mobile phase produces a baseline enantiomeric separation [31]. Similarly, a mobile phase containing phosphate buffer modified with 1-propanol will not produce an enantioselective separation of verapamil, while the replacement of 1-propanol with acetonitrile produces a complete chiral resolution [32].

In this study, the uncharged modifiers methanol, ethanol, 1-propanol, 2-propanol and acetonitrile were screened for their effect on retention and enantioselectivity. The mobile phase composition was ammonium acetate (10 mM, pH 7.0): modifier at a set composition of 80:20 (v/v). Baseline separations of (R)-Met and (S)-Met were achieved with all of the modifiers, with the largest retentions and enantioselectivity produced by the addition of ethanol (Table 1). It is of interest to notice that there was very little difference in the effect of the addition of methanol, 1- and 2-propanol or acetonitrile on retention or enantioselectivity, while the presence of ethanol in the mobile phase produced, on average, a 150% increase in k_R, a 200% increase in k_S and a 25% increase in the observed enantioselectivity (α) relative to the results obtained with the other

modifiers (Table 1). These results cannot be attributed to differences in elutropic strength of the modifiers or differences in the mechanism of interaction with the immobilized AGP. While the same effect, i.e. increase in k and α, has been previously reported with a substitution of ethanol for 2-propanol [33], to our knowledge, the source of this effect has not been elucidated.

When methanol, 1-propanol or 2-propanol was used as the mobile phase modifier, there were no observable enatioselective separations of (R)- and (S)-EDDP (Table 1). The use of acetonitrile as the modifier produced a slight increase in retention and a significant enantioselective separation of the EDDP enantiomers, $\alpha = 1.11$ (Table 1). When ethanol was used as the mobile phase modifier, the effects relative to acetonitrile were an increase of over 110% for k_R and over 250% for k_S while α rose from 1.11 to 1.85. Similar effects produced by the addition of ethanol or acetonitrile on enantioselectivity and retention have been observed in the enantioselective separation of alprenolol and oxprenolol [33] and MDL 73,450 [34]. As with the effect on the enantioselective resolution of Met, to our knowledge, the source of this effect has not been elucidated.

Acetonitrile was chosen as the mobile phase modifier since it produced enantioselective separations of both Met and EDDP with shorter retention times than ethanol.

3.1.3. Optimization of acetonitrile composition

The mobile phase concentration of acetonitrile was varied between 14 and 22% in intervals of 2%. At all percentages of acetonitrile, baseline separations of Met enantiomers were achieved with α ranging from 1.40 (14%) to 1.25 (22%) and the enantiomers of EDDP were also resolved with α values ranging from 1.30 (14%) to 1.11 (22%) (Table 2). How-

Table 2

The effect of the percentage of acetonitrile in the mobile phase on the enantioselective separations of methadone [(R)-Met, (S)-Met] and EDDP [(R)-EDDP, (S)-EDDP]; where the mobile phase composition was ammonium acetate buffer (10 mM, pH 7.0): acetonitrile; k: retention factor; α: enantioselectivity

Acetonitrile in mobile phase (%)	(R)-Met (k)	(S)-Met (k)	Met (α)	(R)-EDDP (k)	(S)-EDDP (k)	EDDP (α)
14.0	14.21	19.86	1.40	9.84	12.79	1.30
16.0	10.23	13.76	1.34	7.40	9.08	1.23
18.0	8.23	10.88	1.32	6.23	7.43	1.19
20.0	5.98	7.63	1.28	4.73	5.39	1.14
22.0	4.77	5.94	1.25	3.93	4.35	1.11

Table 3

The effect of the pH of the ammonium acetate buffer in the mobile phase (10 mM buffer:acetonitrile, 82:18 (v/v)) on the enantioselective separations of methadone [(R)-Met, (S)-Met] and EDDP [(R)-EDDP, (S)-EDDP]; where α: enantioselectivity; Rs: enantioselective resolution

pH	Met (α)	Met (Rs)	EDDP (α)	EDDP (Rs)
5.0	1.29	0.94	1.00	1.00
5.5	1.26	0.64	1.00	1.00
6.0	1.31	2.36	1.20	1.47
6.5	1.42	2.51	1.31	1.92
7.0	1.41	2.63	1.32	2.00
7.5	1.68	4.46	1.44	2.88

3.1.4. Optimization of buffer pH

In general, when cationic solutes are chromatographed on the AGP–CSP, increasing the pH of the mobile phase results in an increase in retention and enantioselectivity [29–31,34]. However, this is not the case for all cationic solutes. Hermansson [31] has observed that for disopyramide, a decrease in mobile phase pH increased the observed α, and Schill et al. [35] observed that the effect of pH on enantioselective separations is highly dependent upon the mobile phase composition, in particular the presence of charged mobile phase modifiers.

In this study, the mobile phase composition was fixed at 82:18 ammonium acetate buffer (10 mM): acetonitrile and the pH of the ammonium acetate buffer was varied over the range 5.0–7.5, in intervals of 0.5 units. The resulting effects on the retentions and enantioselective separations of Met and EDDP were determined and are presented in Table 3 and Fig. 2.

When racemic Met was the solute, there was a discontinuous effect of pH on k, α and R_S. The retention

ever, when the acetonitrile composition was >20%, there was no baseline separation of the EDDP enantiomers. The mobile phase composition of acetonitrile:buffer which gave acceptable enantioselective separations in the shortest time was 18:82, and this composition was used in the remaining optimization studies.
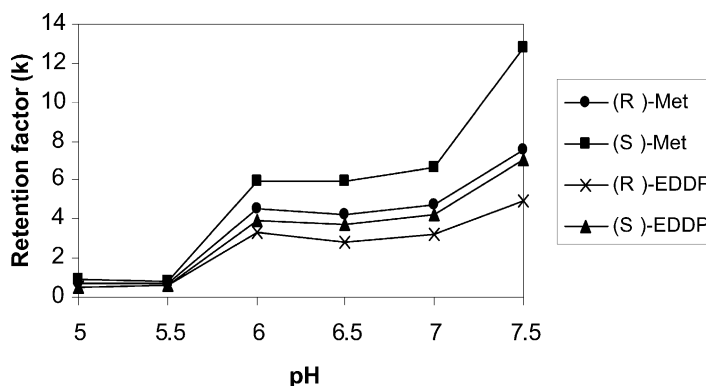

Fig. 2. The effect of the pH of the ammonium acetate component of the mobile phase on the chromatographic retention, expressed as retention factor (k), of the enantiomers of methadone and EDDP.

of both enantiomers increased between pH 5.0 and 6.0, appeared to plateau between 6.0 and 7.0 and increased again when the pH was raised to 7.5 (Fig. 2). The enantioselectivity (α) increased from 1.3 to 1.4 as the mobile phase pH was raised from 5.0 to 7.0 and then jumped to 1.68 when the pH was increased to 7.5. The efficiency of the separation, expressed as the resolution factor R_S, was poor below pH 6.0, i.e. <1.0, significantly increased between pH 6.0 and 7.0, 2.36–2.63 and jumped to 4.46 when the mobile phase pH was increased to 7.5. Similar results were obtained for the retention, enantioselective separation and resolution of (R)-EDDP and (S)-EDDP. However, between pH 5.0 and 5.5 there was no observable enantioselectivity (Table 3, Fig. 2).

The effect of mobile phase pH on the chromatographic separations of Met and EDDP enantiomers reflects the fact that the binding of cationic drugs to non-immobilized AGP is due to a combination of hydrophobic and electrostatic interactions [36,37]. In this study, the pH of the mobile phase remained below the established pK_a values of Met (8.6) and EDDP (10.4) [21]. Thus, the discontinuities in retention, enantioselectivity and resolution cannot be due to substantive changes in the ionization of the solutes.

The binding capacity of AGP also appears to depend, in part, upon conformational changes in the protein. A reversible conformational change in immobilized AGP has been reported between pH 5.0 and 7.0 in association with the enantioselective separation of the anti-malarial agents chloroquine, mefloquine and enpiroline [38] and between pH 4.0 and 7.0 in association with the sorption of acetonitrile [39]. Thus, results from this study are consistent with pH-induced conformational changes in the AGP molecule, which alter the protein's binding affinity and efficiency.

3.1.5. Optimized chromatographic conditions

On the basis of these studies, the pH of the buffer was set at 7.0, which is between the limits of working pHs (4–7) for this type of AGP–CSP column [40]. Therefore, the mobile phase composition for the validation and clinical study were set at acetonitrile:ammonium acetate buffer (10 mM, pH 7.0), 18:82 (v/v). Under these conditions, the analysis was completed in 15 min. The relative retention (k) of (R)-Met and (S)-Met were 8.75 and 11.38, respectively and the observed enantioselectivity (α) was

1.30 (Fig. 3A); for (R)-D3-Met and (S)-D3-Met the k_s were 8.66 and 11.29, respectively, and the observed α was 1.30 (Fig. 3B). The k_s for (R)-EDDP and (S)-EDDP were 6.65 and 7.79, respectively, and the observed α was 1.17 (Fig. 3C); for (R)-D3-EDDP and (S)-D3-EDDP the k_s were 6.64 and 7.79, respectively, and the observed α was 1.17 (Fig. 3D). In addition, the analysis of drug-free saliva at these m/z values detected no interfering peaks, a representative trace obtained at m/z 310.2 (Met) is presented in Fig. 3E.

The enantiomeric elution orders for Met and EDDP were established by chromatographing the single enantiomers. Aqueous solutions (50 ng/ml) of (R)-Met and (S)-Met were prepared and independently injected into the LC–MS and the elution order determined. The EDDP enantiomers were not commercially available, and had to be prepared from the individual Met enantiomers. In a previous study of the analysis of Met using GC-MS, a heat-induced conversion of Met into EDDP was observed [2]. Therefore, aqueous solutions (5 µg/ml) of (R)-Met or (S)-Met were placed at 150 °C for 6 h and the resulting solution was directly injected into the LC–MS. The result from the conversion of (R)-Met to (R)-EDDP is presented in Fig. 4 and was used to establish the enantiomeric elution order for EDDP.

For both Met and EDDP, the enantiomeric elution order was (R), (S). These results are consistent with the previously reported results obtained on the AGP–CSP [13,19–22]. The data also reflect the fact that the plasma protein binding of (S)-Met is greater than that of the (R)-enantiomer [5]. Thus, since Met predominately binds to AGP, the chromatographic results can be assumed to accurately reflect the relative binding of Met enantiomers to AGP.

The extent and enantioselectivity of the binding of EDDP to AGP has not been established. However, the chromatographic results suggest that EDDP is bound to AGP to a lesser extent under the chromatographic conditions used, i.e. the retention times of the EDDP enantiomers were less than those of the Met enantiomers, but with the same relative enantioselectivity, i.e. (S)-EDDP is bound to a greater extent than (R)-EDDP.

3.1.6. Optimization of mass spectrometric detection

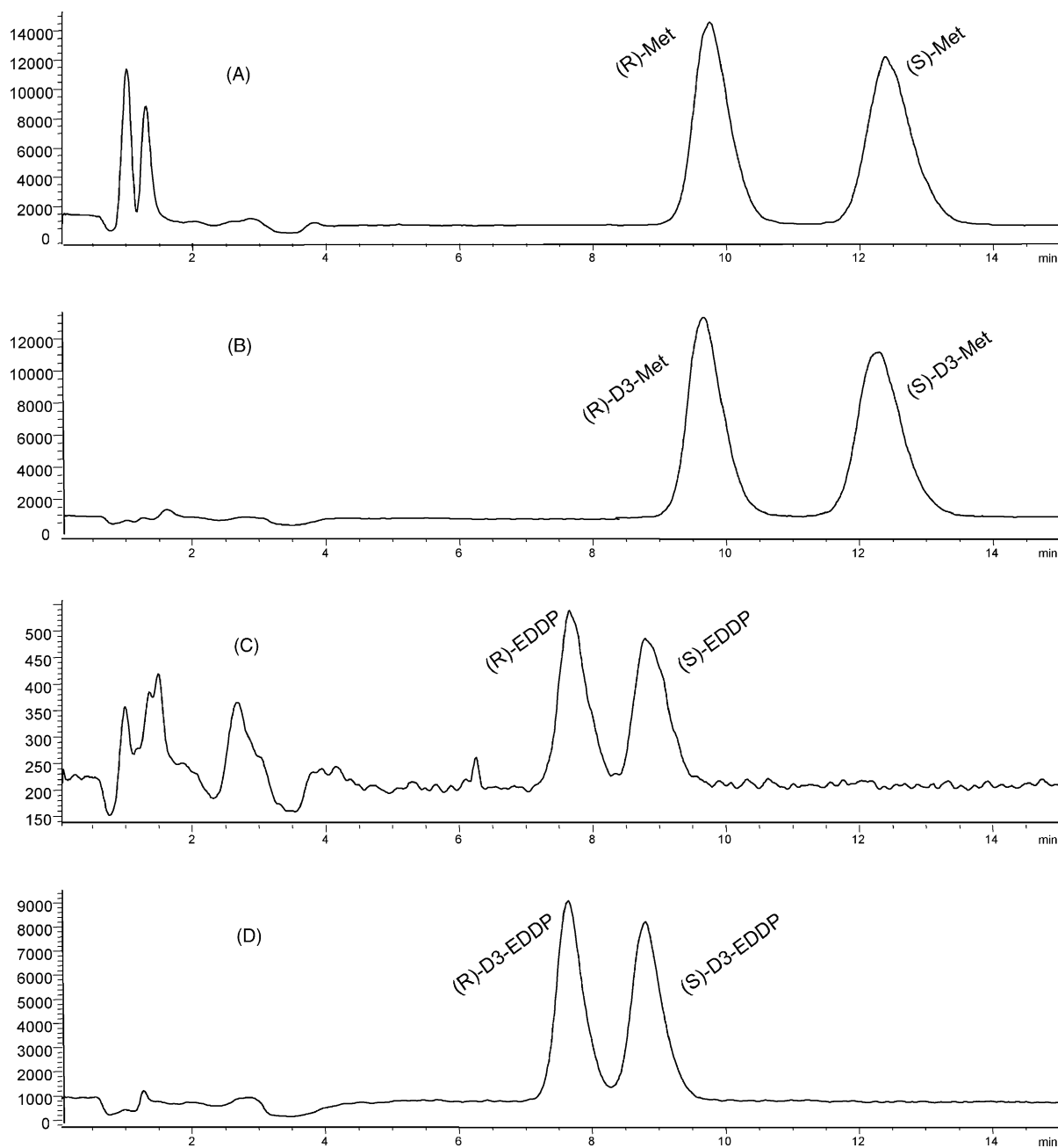The chromatograms were monitored using single ion monitoring for Met, EDDP, D3-Met and

Fig. 3. Representative chromatogram of the low quality control saliva sample (LQC) containing (*R*,*S*)-Met (50 ng/ml); (*R*,*S*)-EDDP (1 ng/ml); (*R*,*S*)-D3-Met (50 ng/ml); (*R*,*S*)-D3-EDDP (25 ng/ml); where: (A) the chromatographic trace obtained using single ion monitoring at $m/z = 310.20$ (Met); (B) the chromatographic trace obtained using single ion monitoring at $m/z = 313.20$ (D3-Met); (C) the chromatographic trace obtained using single ion monitoring at $m/z = 278.20$ (EDDP); (D) the chromatographic trace obtained using single ion monitoring at $m/z = 281.20$ (D3-EDDP); and (E) the chromatographic trace of a drug-free saliva sample obtained using single ion monitoring at $m/z = 310.20$ (Met).
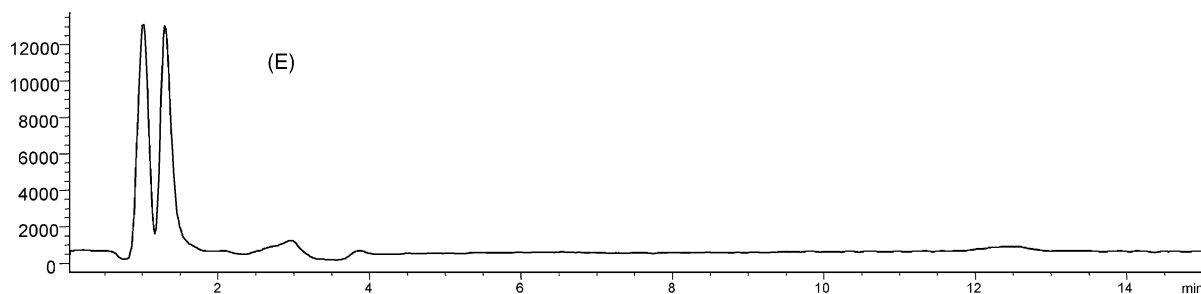
Fig. 3. (*Continued*).

D3-EDDP. Each compound was injected individually; a full scan mass spectra was obtained, and the signals were monitored at each of the specific *m/z* values. The specific ion data were collected on four separate channels and analyzed. The results of these studies demonstrated that there were no overlaps in the mass spectra of the compounds at the *m/z* values chosen for the monitoring.

The purpose of the optimization of the mass selective detector parameters was to find the optimal nebulisation conditions of the sample solution and ionization of the analytes. Based on the maximum response (peak height) the optimized parameters were as follows: fragmentor, 60 V; drying gas flow-rate, 7.0 l/min; nebulizer pressure, 20 psig; drying gas temperature, 350 °C and capillary voltage, 1250 V.

3.2. Linearity and detection limits

Calibration curves were generated by weighted ($1/x$) least squares linear regression. The linear relationships between peak height ratio and drug-enantiomer concentrations for Met in the range of 5.0–600.0 ng/ml were described by the following equations: $y = 0.9536x + 0.1248$, $r^2 = 0.9998$ {(R)-Met}; $y = 0.9335x + 0.0747$, $r^2 = 0.9997$ {(S)-Met}. The linear relationships between peak height ratio and drug-enantiomer concentrations for EDDP in the range of 0.5–15.0 ng/ml were described by the following equations: $y = 0.9896x − 0.0024$, $r^2 = 0.9992$ {(R)-EDDP}; $y = 0.9959x–0.0018$, $r^2 = 0.9997$ {(S)-EDDP}. The data were based on three replicates of an 8-point calibration curve.

The lower limit of quantification is the concentration of the drug in the matrix that can be determined with a high percentage of accuracy (80–120%). The LLOQ for Met was 5.00 ng/ml per enantiomer and for EDDP was 0.50 ng/ml per enantiomer. In contrast, the lower limit of detection (LOD) was defined as the concentration of the compound at which the signal versus noise ratio (S/N) was equal to 3. LOD value for Met was 0.10 ng/ml per enantiomer and for EDDP 0.25 ng/ml per enantiomer. While the LOD established for Met indicates that the LLOQ for Met could have been reduced by at least a factor of 10, preliminary analyses of saliva samples indicated that the established LLOQ was preferred for the study.

3.3. Accuracy and precision

Accuracy and precision of the method for Met and EDDP were evaluated from quintuplicate analysis of each QC standard level (LQC, MQC and HQC) and repeated for 3 days. The calculated average accuracy was 100.4% for (R)-Met, 101.6% for (S)-Met, 103.0% for (R)-EDDP and 102.4% for (S)-EDDP (Tables 4 and 5).

The intra- and inter-day precision of the method was determined as coefficient of variance (CV(%)). The results were ≤4.0% for (R)-Met, ≤3.7% for (S)-Met, ≤3.0% for (R)-EDDP and ≤3.9% for (S)-EDDP. The results of the validation studies demonstrate that the method has acceptable accuracy and precision.

3.4. Recoveries

The recoveries of (R)- and (S)-Met and (R)- and (S)-EDDP from saliva were investigated using the low quality control standards and high quality control standards. For (R)- and (S)-Met, the average recovery from the LQC was 96.7 ± 0.0 and $96.3 \pm 0.0\%$, respectively, and the recoveries from the HCQ were 99.2 ± 0.2 and $99.5 \pm 0.3\%$, respectively, where
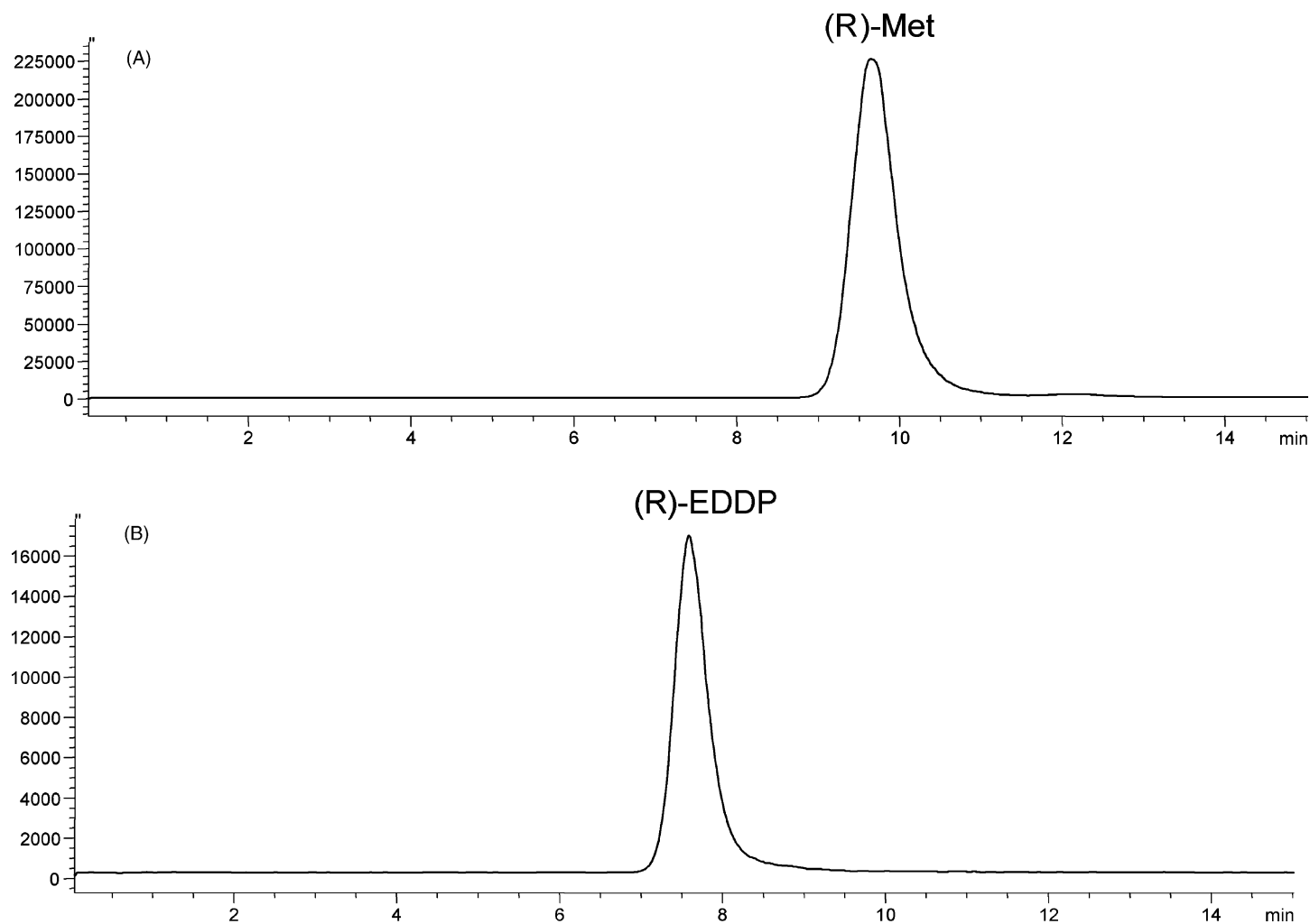
Fig. 4. Representative chromatogram of the heat-induced conversion of (R)-Met to (R)-EDDP, where (A) the (R)-Met concentration remaining after heating; (B) the (R)-EDDP produced by the heating.

Table 4
Results from the validation studies for methadone

	LLOQ (5.0 ng/ml)		LQC (50.0 ng/ml)		MQC (300.0 ng/ml)		HQC (600.0 ng/ml)	
	R	S	R	S	R	S	R	S
Intra-day								
N	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mean	5.1	5.0	50.2	53.4	304.8	307.9	603.5	611.0
S.D.	0.2	0.3	1.7	1.6	3.9	5.8	8.1	8.4
CV (%)	4.4	6.7	3.3	2.9	1.3	1.9	1.3	1.4
Inter-day								
N	7.0	7.0	15.0	15.0	15.0	15.0	15.0	15.0
Mean	5.0	5.0	50.3	52.4	301.2	305.6	602.1	601.6
S.D.	0.2	0.3	2.0	2.0	8.5	6.0	19.2	12.1
CV (%)	4.0	5.7	4.0	3.8	2.8	2.0	3.2	2.0
Accuracy (%)	100.3	99.5	100.5	104.7	100.4	101.9	100.4	100.3
Recovery (%)			96.7	96.3	98.7	97.9	99.2	99.5

$n = 3$ for each determination. For (R)- and (S)-EDDP, the average recovery from the LQC was 95.1 ± 0.0 and $94.8 \pm 0.0\%$, respectively, and the recoveries from the HCQ were 100.0 ± 0.0 and $100.0 \pm 0.0\%$, respectively, where $n = 3$ for each determination.

3.5. Stability studies

The Met and EDDP standards were frozen at $-80\,°C$ for 4 months, defrosted and analyzed. There was no observable degradation of either analyte. The spiked saliva samples used in the preparation of the Met and EDDP standard curves were assayed, frozen

at $-20\,°C$ for 13 h, defrosted and assayed. There was no observable degradation of either analyte. The LQCs for Met and EDDP were placed in the autosampler at room temperature and assayed at 0, 3, 15, 18, 20 and 24 h. There was no observable degradation of either analyte during this period.

4. Application to clinical samples

The validated method was applied to the analysis of human saliva specimens obtained from patients in methadone maintenance. A representative

Table 5
Results from the validation studies for EDDP

	LLOQ (0.5 ng/ml)		LQC (1.0 ng/ml)		MQC (7.5 ng/ml)		HQC (15.0 ng/ml)	
	R	S	R	S	R	S	R	S
Intra-day								
N	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mean	0.5	0.5	1.1	1.0	7.5	7.4	14.8	15.3
S.D.	0.0	0.0	0.0	0.0	0.2	0.1	0.2	0.4
CV (%)	7.8	5.5	2.2	3.6	2.6	1.9	1.0	2.8
Inter-day								
N	7.0	7.0	15.0	15.0	15.0	15.0	15.0	15.0
Mean	0.5	0.5	1.1	1.1	7.4	7.4	15.0	14.9
S.D.	0.1	0.1	0.0	0.0	0.2	0.2	0.3	0.4
CV (%)	8.7	9.0	3.0	3.9	2.7	2.4	2.1	2.8
Accuracy (%)	105.4	105.1	108.2	105.9	98.4	99.2	99.8	99.5
Recovery (%)			95.1	94.8	96.6	99.6	100.0	100.0

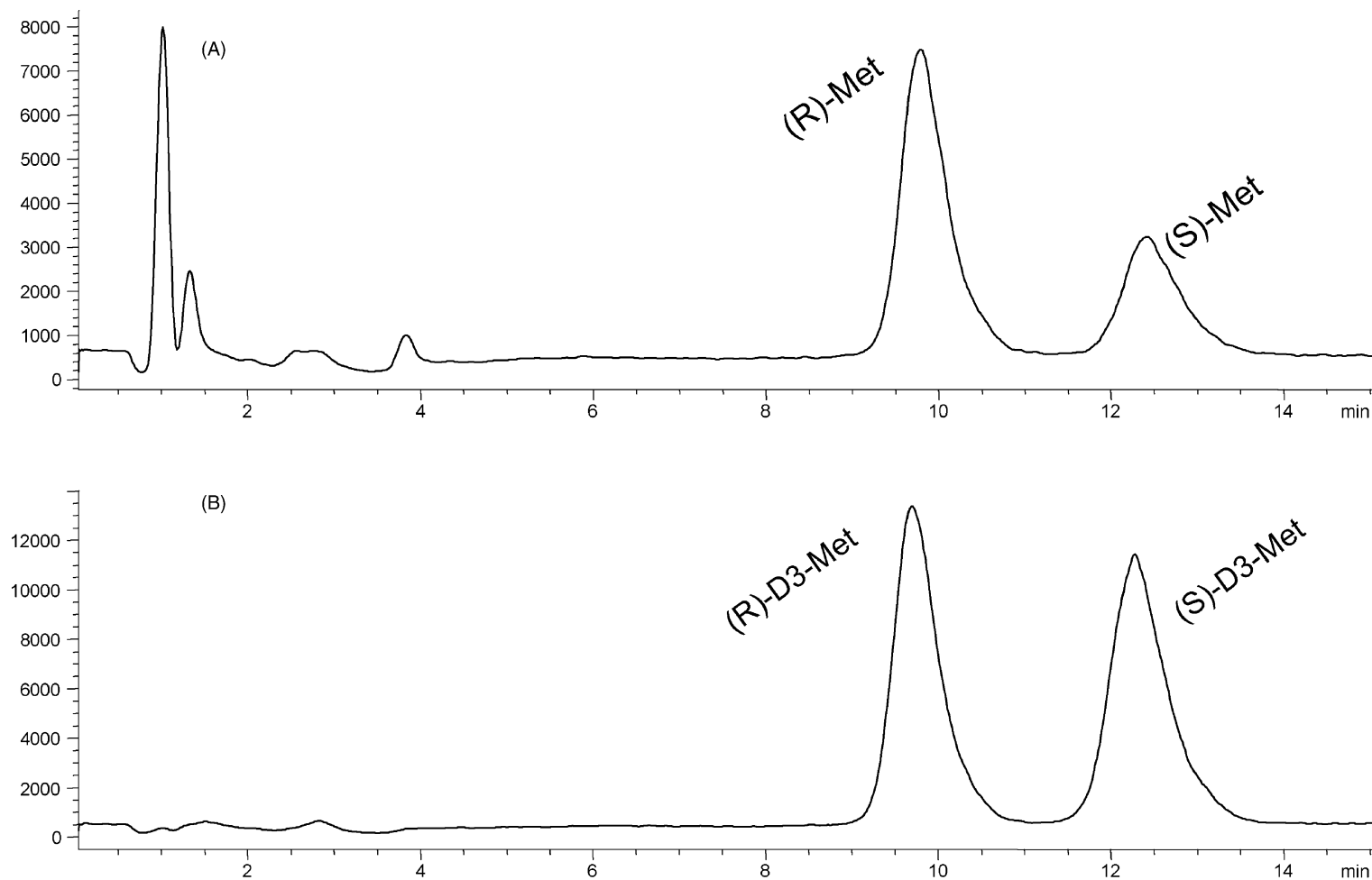M.E. Rodríguez Rosas et al. / J. Chromatogr. B 796 (2003) 355–370

367

Fig. 5. Representative chromatograms of the analysis of a saliva sample collected before subsequent dosing from a patient who had received methadone (70 mg per day) for 158 days. The measured concentrations were: (R)-Met 29.0 ng/ml, (S)-Met 13.7 ng/ml, (R)-EDDP 0.7 ng/ml and (S)-EDDP 0.6 ng/ml per enantiomer, where: (A) the chromatographic trace obtained using single ion monitoring at $m/z = 310.20$ (Met); (B) the chromatographic trace obtained using single ion monitoring at $m/z = 313.20$ (D3-Met); (C) the chromatographic trace obtained using single ion monitoring at $m/z = 278.20$ (EDDP); (D) the chromatographic trace obtained using single ion monitoring at $m/z = 281.20$ (D3-EDDP).
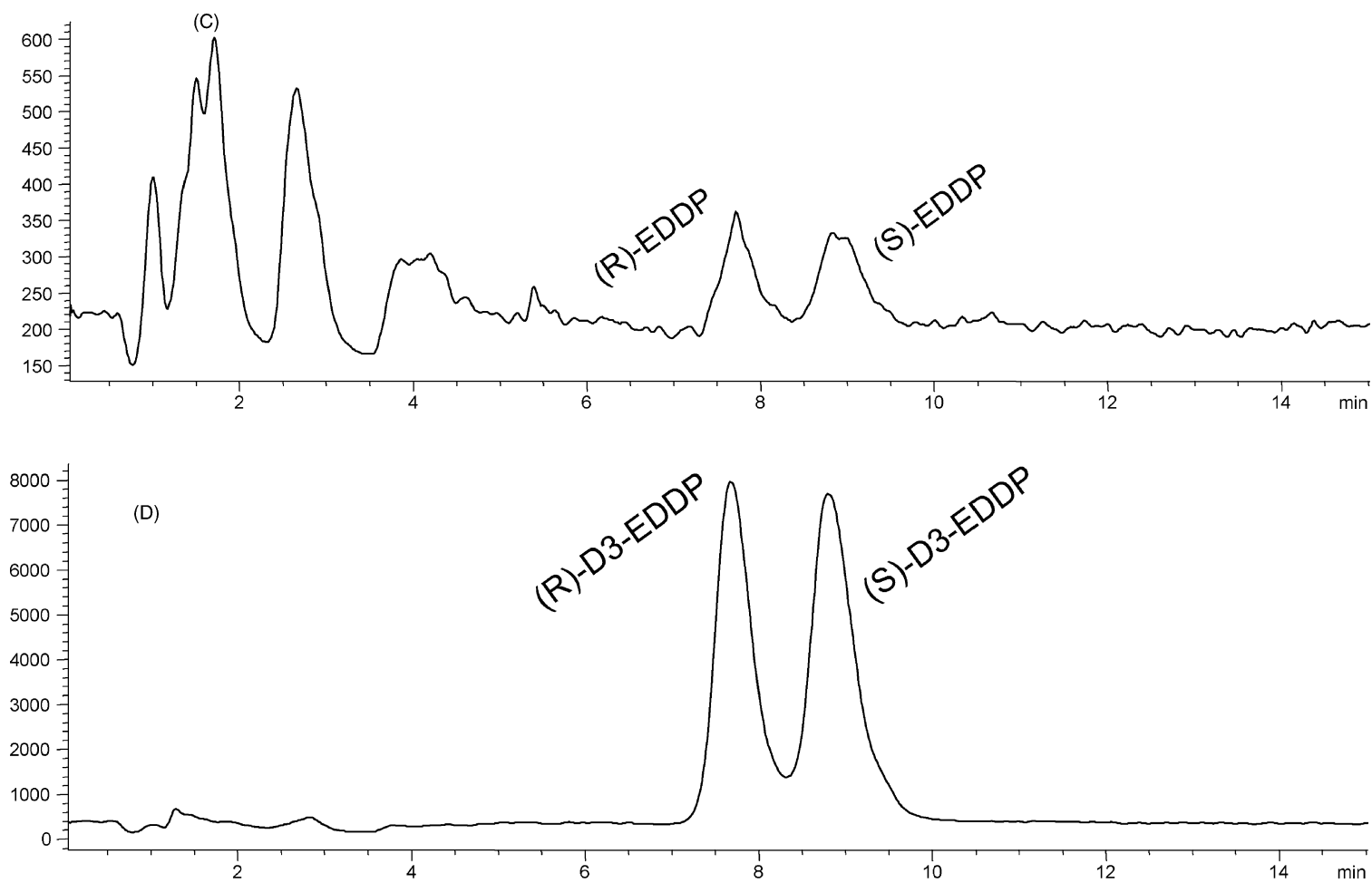
Fig. 5. (*Continued*).

Table 6
The concentration of the enantiomers of methadone (Met) and 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP) in saliva samples from patients in a methadone-maintenance program for polydrug abuse

Patient	Met dose (mg/day)	(*R*)-Met (ng/ml)	(*S*)-Met (ng/ml)	*R/S* Met (ng/ml)	(*R*)-EDDP (ng/ml)	(*S*)-EDDP (ng/ml)	*R/S* EDDP (ng/ml)
1	100	191.3	117.0	1.64	0.5	0.6	0.83
2	100	18.9	8.7	2.17	0.3[a]	0.4[a]	0.75
3	100	189.3	144.9	1.31	1.2	1.4	0.86
4	70	28.9	13.7	2.11	0.7	0.6	1.17
5	70	448.8	224.8	2.00	3.2	3.0	1.07

[a] Measured levels were below the LLOQ, but above the LOD.

chromatogram of a sample collected before dosing from a patient who had received methadone (70 mg per day) for 158 days is presented in Fig. 5.

Preliminary results obtained from 5 patients from a 252 patient study receiving either 100 mg per day or 70 mg per day are presented in Table 6. The *R/S* ratio for Met ranged between 1.3 and 2.2 regardless of the dose. This range is consistent with the previously reported range of 1–9 [23]. There was also a wide variation in the total concentration of Met after both the 100 and 70 mg per day doses, which is also consistent with the previously reported high inter-individual variability [23,41].

The large inter-individual variability in Met disposition has been associated with the formation of EDDP, which is mediated predominately by cytochrome P450 3A4 (CYP3A4) with the possible involvement of CYP 2C9 and CYP 2C19 [41]. Inter-individual differences in the expression of these CYP isoforms may have contributed to the observed variations.

The CYP mediated conversion of Met to EDDP has been described as "not markedly stereoselective" [41]. However, the analyses of urine samples from patients on methadone-maintenance therapy offer a different, albeit conflicting picture [20,21,24]. In two of the studies, the *R/S* ratio for EDDP was <1.0 [20,21] while in the third this ratio was >1.0 [24], although the authors of the third paper did not definitively identify the enantiomeric ratio. If the third observation is indeed valid, the source of the difference in enantioselectivity cannot be readily determined from these publications.

The preliminary data from the initial five patients does not reveal any significant enantioselective excess of (*R*)-EDDP or (*S*)-EDDP. However, it is of interest to note that the *R/S* ratio for the patients receiving 100 mg per day was <1.0 while for the patients receiving 70 mg per day the *R/S* ratio was >1.0 (Table 6). The significance of this difference and its relationship to the administered dose can only be determined from the analyses of the entire study population. These results will be published elsewhere.

5. Conclusions

The data from this study demonstrate that a rapid and validated method has been developed for the determination of Met and EDDP enantiomers in saliva. While initial reports of enantioselective HPLC assays for Met utilizing the AGP–CSP have mentioned a rapid deterioration of the CSP [13,19], in this study over 150 spiked standards and patient samples could be analyzed without a significant change in the chromatographic results. This method was successfully applied to the analysis of saliva specimens from patients in a methadone-maintenance program.

References

[1] B.A. Judson, W.H. Horns, A. Goldstein, Clin. Pharmacol. Ther. 20 (1976) 445.
[2] F.R. Galloway, N.F. Bellet, J. Anal. Toxicol. 23 (1999) 615.
[3] H.F. Fraser, H. Isbell, Bull. Narc. 14 (1962) 25.
[4] N.B. Eddy, H. Halbach, O.J. Braeden, Bull. WHO 14 (1967) 353.
[5] D.W. Boulton, C.L. Devane, Chirality 12 (2000) 681.

[6] K. Kristensen, T. Blemmer, H.R. Angelo, L.L. Christrup, N.E. Drenck, S.N. Rasmussen, P. Sjogren, Ther. Drug Monit. 18 (1996) 221.

[7] M.J. Kreek, D.L. Hachey, P.D. Klein, Life Sci. 24 (1979) 925.

[8] K. Nakamura, D.L. Hachey, M.J. Kreek, C.S. Irving, P.D. Klein, J. Pharm. Sci. 71 (1982) 40.

[9] S. Rudaz, D. Ortelli, M. Gex-Fabry, J.-J. Deglon, L.P. Balant, J.-L. Veuthey, Chirality 11 (1999) 487.

[10] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobsson, J. Chromatogr. 570 (1991) 198.

[11] P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele, P. Mangin, J. Forensic Sci. 42 (1997) 291.

[12] P. Kintz, A. Tracqui, C. Marzullo, A. Darreye, F. Tremeau, P. Greth, B. Ludes, Ther. Drug Monit. 20 (1998) 35.

[13] K. Kristensen, H.R. Angelo, T. Blemmer, J. Chromatogr. A 666 (1994) 283.

[14] R.L.G. Norris, P.J. Ravenscroft, S.M. Pond, J. Chromatogr. B 661 (1994) 346.

[15] C. Pham-Huy, N. Chikhi-Chorfi, H. Galons, N. Sadeg, X. Laqueille, N. Aymard, F. Massicot, J.-M. Warnet, J.-R. Claude, J. Chromatogr. B 700 (1997) 155.

[16] S. Rudaz, J.-L. Veuthey, J. Pharm. Biomed. Anal. 14 (1996) 1271.

[17] N. Schmidt, K. Brune, G. Geisslinger, J. Chromatogr. 583 (1992) 195.

[18] T.L. Pierce, A.G.W. Murray, W. Hope, J. Chromatogr. Sci. 30 (1992) 443.

[19] S. Rudaz, J.-L. Veuthey, Chirality 11 (1999) 319.

[20] H.R. Angelo, N. Beck, K. Kristensen, J. Chromatogr. B 724 (1999) 35.

[21] D.J.R. Foster, A.A. Somogyi, F. Bochner, J. Chromatogr. B 744 (2000) 165.

[22] D. Ortelli, S. Rudaz, A.-F. Chevalley, A. Mino, J.-J. Deglon, L. Balant, J.-L. Veuthey, J. Chromatogr. A 871 (2000) 163.

[23] M. Frost, H. Kohler, G. Blaschke, Electrophoresis 18 (1997) 1026.

[24] M. Lanz, W. Thormann, Electrophoresis 17 (1996) 1945.

[25] K. Kristensen, H.R. Angelo, Chirality 4 (1992) 263.

[26] K.M. Hold, D. de Boer, J. Zuidema, R.A.A. Maes, Int. J. Drug Test. 1 (1996) 1.

[27] D. Ortelli, S. Rudaz, S. Souverain, J.-L. Veuthey, J. Separation Sci. 25 (2002) 222.

[28] S. Souverain, S. Rudaz, D. Ortelli, E. Varesio, J.-L. Veuthey, J. Chromatogr. B 784 (2003) 117.

[29] I.W. Wainer, in: I.W. Wainer (Ed.), Drug Stereochemistry: Analytical Methods and Pharmacology, Marcel-Dekker, NY, 1993, pp. 167–168.

[30] J. Hermansson, J. Chromatogr. 269 (1983) 71.

[31] J. Hermansson, Trends Anal. Chem. 8 (1989) 143.

[32] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 271.

[33] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 285.

[34] B.M. Bunton, T.M. Walker, J. Chromatogr. A 699 (1995) 389.

[35] G. Schill, I.W. Wainer, S.A. Barkan, J. Chromatogr. 365 (1986) 73.

[36] R. Kaliszan, A. Nasal, M. Turowski, J. Chromatogr. A 722 (1996) 25.

[37] T. Fournier, N. Medjoubi-N, D. Porquet, Biochim. Biophys. Acta 1482 (2000) 157.

[38] A.-F. Aubry, F. Gimenez, R. Farinotti, I.W. Wainer, Chirality 4 (1992) 30.

[39] K. Gyimesi-Forras, G. Szasz, G. Bathory, G. Meszaros, A. Gergely, Chirality 15 (2003) 377.

[40] Instruction Manual Chiral-AGP, ChromTech AB.

[41] K.M. Rentsch, J. Biochem. Biophys. Methods 54 (2002) 1.