Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1073 (2005) 237–248
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of total and free concentrations of the enantiomers of methadone and its metabolite (2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine) in human plasma by enantioselective liquid chromatography with mass spectrometric detection

Maria Esther Rodriguez-Rosas[a], Juan G. Medrano[a], David H. Epstein[b], Eric T. Moolchan[b], Kenzie L. Preston[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] *Intramural Research Program, Clinical Pharmacology and Therapeutics Research Branch, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA.*

Abstract

A sensitive enantioselective liquid chromatographic assay with mass spectrometric detection (LC–MS) has been validated for the determination of total and free plasma concentrations of (*R*)- and (*S*)-methadone (Met) and (*R*)- and (*S*)-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP, the primary metabolite of Met), using their respective deuterium-labeled compounds as internal standards [(*R*,*S*)-d_3-Met and (*R*,*S*)-d_3-EDDP]. For total drug determinations, 1 ml human plasma was extracted, using a cation-exchange solid-phase extraction cartridge; the eluate was evaporated, reconstituted in the mobile phase, and injected into the LC–MS system. The free fractions of Met and EDDP were determined, using 500 μl of plasma, which were placed in an ultrafiltration device and centrifuged at 2000 × *g* until 250 μl of filtrate was collected. The filtrate was extracted as described above and analyzed. Enantioselective separations were achieved using an α_1-acid glycoprotein chiral stationary phase, a mobile phase composed of acetonitrile–ammonium acetate buffer [10 mM, pH 7.0] (18:82, v/v), a flow rate of 0.9 ml/min at 25 °C. Under these conditions, enantioselective separations were observed for Met ($\alpha = 1.30$) and EDDP ($\alpha = 1.17$) within 15 min. Met, EDDP, [$^2H_3$]-Met and [$^2H_3$]-EDDP were detected using selected ion monitoring at *m*/*z* 310.30, 278.20, 313.30, and 281.20, respectively. Linear relationships between peak height ratio and drug-enantiomer concentrations were obtained for Met in the range 1.0–300.0 ng/ml, and for EDDP from 0.1 to 25.0 ng/ml with correlation coefficients greater than 0.999, where the lower limit of quantification (LLOQ) was 1 ng/ml for Met and 0.1 ng/ml for EDDP. The relative standard deviation (R.S.D.) expressed as R.S.D. for the intra- and inter-day precision of the method were <5.3% and the R.S.D. for accuracy was <5.0%. The method was used to analyze plasma samples obtained from patients enrolled in a Met-maintenance program.

Keywords: (*S*)-Methadone; (*R*)-Methadone; Chiral chromatography; AGP-chiral stationary phase

1. Introduction

Methadone (6-dimethylamino-4,4-dipehnyl-3-heptanone hydrochloride (Met; Fig. 1) is a synthetic opiate used for analgesia in patients with severe pain [1–4] and to treat opioid dependence [5–8]. The primary Met metabolite is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP; Fig. 1), which is formed by cytochrome P4503A (CYP 3A) mediated *N*-demethylation and subsequent spontaneous cyclization [9,10]. Recent studies have suggested that CYP2B6 and CYP2C19 also contribute to this metabolic transformation [11].

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

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.153

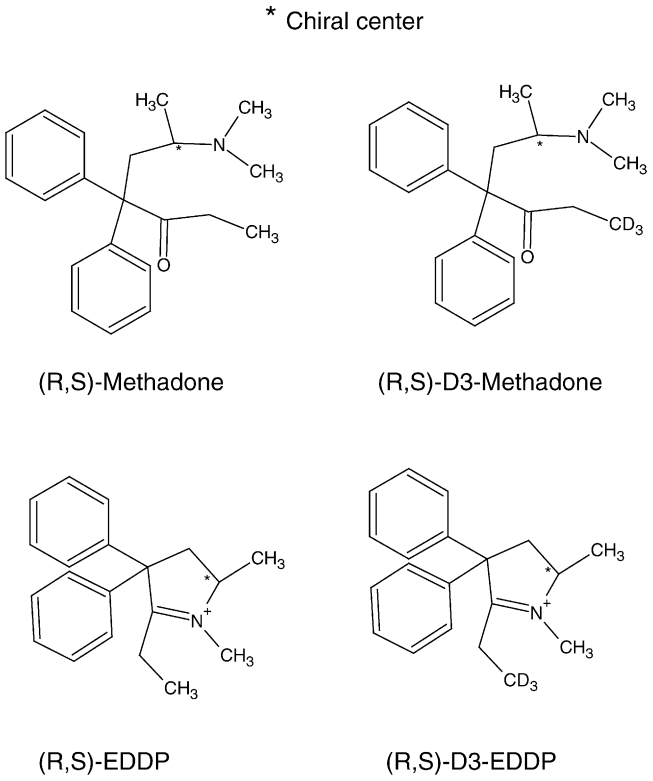

Fig. 1. The chemical structures of Met, EDDP, $^{2}H_3$-Met, and $^{2}H_3$-EDDP.

Met and EDDP are chiral molecules that exist as (+)-(*S*)- and (−)-(*R*)-Met and the corresponding EDDP enantiomers. In every country but Germany, Met is therapeutically administered as a racemic mixture, although (*R*)-Met has a higher affinity than (*S*)-Met for the μ-opioid receptor [12] and the analgesic potency of (*R*)-Met is 50 times greater than that of the (*S*)-enantiomer [13,14].

In addition to enantiospecific pharmacodynamic differences, the Met enantiomers differ in their protein binding and pharmacokinetic profiles. Met binds extensively to α_1-acid glycoprotein (AGP), to the AGP variant orosomucoid 2 (ORM2) and, to a lesser extent, orosomucoid 1 (ORM1) [15,16]. (*S*)-Met is bound more extensively to AGP than (*R*)-Met, 87 and 79%, respectively [17].

The binding of Met to AGP is one of the key factors in the disposition of (*R*)-Met, particularly with regard to its transfer to and from the central and peripheral compartments (k_{12} and k_{21}) and, by extension, to the site of pharmacologic action [10]. Renal excretion of Met is also influenced by its plasma protein binding as evidenced by the significant correlations between the percent unbound in plasma and the renal clearance and plasma half-lives of both enantiomers [10]. It has been suggested that the disposition of the active enantiomer (*R*)-Met, can be partially predicted by CYP3A activity and plasma protein binding to ORM2 [10].

In addition to, or as a result of, the enantioselective differences in protein binding (*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 [18]. There are 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 [19,20]. Some investigators have suggested that due to the extensive inter-individual and intra-individual variability in Met disposition and pharmacodynamics [10,21,22], reliable therapeutic monitoring of Met requires enantioselective bioanalytical techniques. In addition, monitoring should include measurement of both total and free Met concentrations, since it is not clear which, if either, parameter can be used to predict clinical response.

Methods have been reported for analysis of Met by both achiral and chiral high-performance liquid chromatography (HPLC) [13,14,23–32]. The reported enantioselective HPLC assays for Met utilized chiral stationary phases based upon immobilized AGP (AGP-CSP) [27,33–36], native β-cyclodextrin [11,28] and hydroxypropyl-β-cyclodextrin [17,29,33]. Enantioselective capillary electrophoresis methods have also been reported for the quantification of Met enantiomers in hair or urine samples [37,38] as well as gas chromatography [39].

Using an LC–MS method, Veuthey and coworkers [40] quantified the enantiomers of Met, but not those of EDDP. However, Veuthey's laboratory reported non-enantioselective determinations of Met and EDDP in human serum and plasma by LC–MS methods achieving limits of quantification (LOQs) of 10 and 25 ng/ml, respectively [41,42].

The separation and quantification of (*R*)- and (*S*)-EDDP in the presence of Met in human urine by HPLC–UV methods with analysis times of more than 30 min was previously reported [34,35]. These enantioselective separations were also achieved, using an AGP-CSP. The assays were validated and used in the analysis of the urinary concentrations of Met and EDDP enantiomers with lower limits of quantification (LLOQs) for EDDP = 8 ng/ml per enantiomer [34] and 35 ng/ml per enantiomer [35].

The achiral determination of the free concentration of Met has been evaluated using ultrafiltration [17,43]. The determination of free concentrations of Met enantiomers in the drug-free plasma using HPLC with UV detection in eight healthy female volunteers has been reported [17]. However, either no EDDP or unquantifiable concentrations of EDDP were detected in the serum.

We recently reported the development of an enantioselective LC–MS assay for the determination of Met and EDDP enantiomers in saliva [44]. The current report describes the adaptation and validation of this method for the quantification of the total and free concentrations of the enantiomers of Met and EDDP in human plasma. The assay has greater sensitivity than any of the previously reported methods [LLOQ for EDDP was 0.1 ng/ml per enantiomer and the limit of detection (LOD) was 0.01 ng/ml per enantiomer] and a run time of less than 15 min. The method is reproducible and accurate and has been applied to the analysis of plasma samples from patients in a Met-maintenance program.

2. Experimental

2.1. Chemicals and reagents

(+)-(*S*)-Methadone [(*S*)-Met] and (−)-(*R*)-methadone [(*R*)-Met] were provided by The Drug Inventory Supply and Control System of National Institute on Drug Abuse (NIDA, Baltimore, MD, USA); (*R*,*S*)-Met 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*)-[$^2H_3$]-Met, [(*R*,*S*)-Met-d_3], and deuterium-labeled (*R*,*S*)-[$^2H_3$]-EDDP perchlorate [(*R*,*S*)-EDDP-d_3] (100 μg/ml methanol solutions) were purchased from Cerilliant (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 plasma was purchased from Valley Biomedical (Winchester, VA, USA). Extraction cartridges were Oasis MCX 1 ml, 30 mg and Oasis HLB 1 ml, 30 mg (Waters, Milford, MA, USA). Ultrafiltration devices (1 ml, MPS Micropartition Kit) were purchased from Millipore (Billerica, MA, USA).

2.2. Apparatus

The analytical system consisted of a Series 1100 LC/MSD liquid chromatography–mass selective detector (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G1379 A), a quaternary pump (1311 A), a thermostated autosampler (G1329 A), and a thermostated column compartment (G1316 A). The mass selective detector (MSD Quad SL, G1956 B) was equipped with atmospheric pressure ionization electrospray (API-ES, G2908 B) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 2.8 GHz HP Compaq computer (Hewlett-Packard, Palo Alto, CA, USA) running ChemStation software (Rev A.10.01 [1635], 1990–2003, Hewlett-Packard) under Microsoft Windows XP.

Extractions were performed, using a 24-port vacuum manifold, PrepSep from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Chromatographic conditions

Enantioselective separations of (*R*)- and (*S*)-Met, (*R*)- and (*S*)-EDDP, (*R*)- and (*S*)-Met-d_3 and (*R*)- and (*S*)-EDDP-d_3 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 20 μl, and the column temperature was kept at 25 °C.

2.4. Optimization of the mass selective detector (MS) 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), *m*/*z* = 278.20 (EDDP), *m*/*z* = 313.20 (Met-d_3) and *m*/*z* = 281.20 (EDDP-d_3).

The detectability of the Met and EDDP signals was primarily dependent on the MS experimental parameters. The following MS parameters were investigated: fragmentation voltage (50, 60, 70, 80, 90, and 100 V), capillary voltage (700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2500, and 3000 V), nebulizer pressure (20, 30, 40, 50 and 60 psi; 1 psi = 6894.76 Pa), drying gas flow (7, 8, 9, 10, 11, 12, and 13 l/min), and drying gas temperature (300, 310, 320, 330, 340, and 350 °C). The optimized parameters, based on the maximum signal for EDDP, were: fragmentor, 70 V; drying gas flow rate, 11.0 l/min; nebulizer pressure, 30 psi; drying gas temperature, 350 °C and capillary voltage, 1000 V.

2.5. Preparation of stock solutions

Concentrated stock solutions of (*R*,*S*)-Met [40.0 μg/ml as free base], (*R*,*S*)-EDDP [8.0 μg/ml as free base], (*R*,*S*)-Met-d_3 [20.0 μg/ml as free base] and (*R*,*S*)-EDDP-d_3 [10.0 μg/ml as free base] were prepared in methanol, placed in capped polypropylene tubes, wrapped in aluminum foil and stored at −20 °C. Spiked standard solutions for the calibration curve and quality control samples (QCs) were made by serial dilutions with methanol starting with their respective concentrated stock solution. These spiked standards were placed in capped polypropylene tubes, wrapped in aluminum foil, and stored at 4 °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 spiked standard solution containing Met, EDDP, Met-d_3 and EDDP-d_3 to a microcentrifuge tube, evaporating it to dryness in a Speed Vacuum, and spiking it with 1 ml drug-free human plasma. Extraction was then performed as described in Section 2.7.2. In this way, seven-point calibration curves were prepared, one for total (*R*)-Met, total (*S*)-Met, total (*R*)-EDDP, total (*S*)-EDDP, free (*R*)-Met, free (*S*)-Met, free (*R*)-EDDP and free (*S*)-EDDP. The seven-point calibration curve for total Met ranged from 1.0 to 300.0 ng/ml

(1.0, 5.0, 10.0, 50.0, 100.0, 200.0, and 300.0 ng/ml) and for total EDDP from 1 to 25.0 ng/ml (1.0, 2.5, 5.0, 10.0, 15.0, 20.0, and 25.0 ng/ml), using constant concentrations of Met-d_3 [12.5 ng/ml] and EDDP-d_3 [6.2 ng/ml]. The seven-point calibration curve for free Met ranged from 1.0 to 50.0 ng/ml (1.0, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 ng/ml) and for free EDDP from 0.1 to 5.0 ng/ml (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ng/ml), using constant concentrations of Met-d_3 [12.5 ng/ml] and EDDP-d_3 [6.2 ng/ml]. All concentrations are given per enantiomer.

The linearity of each standard curve was determined using the "calibration settings" window in ChemStation (Rev A.10.01 [1635], 1990–2003, Hewlett-Packard) with the weighting function set at "Equal".

The QC standards for total Met were 10.0 ng/ml [low quality control (LQC)], 100.0 ng/ml [medium quality control (MQC)] and 300.0 ng/ml [high quality control (HQC)], while for total EDDP the standards were LQC = 5.0 ng/ml, MQC = 15.0 ng/ml and HQC = 25.0 ng/ml.

The QC standards for free Met were LQC = 10.0 ng/ml, MQC = 30.0 ng/ml and HQC = 50.0 ng/ml, while for free EDDP the standards were LQC = 1.0 ng/ml, MQC = 3.0 ng/ml and HQC = 5.0 ng/ml. All concentrations are given per enantiomer.

2.7. Sample preparation

2.7.1. Collection of plasma from patients

Plasma samples were obtained from Met-maintained outpatients enrolled in a clinical trial of combined behavioral and pharmacologic treatment for opioid and cocaine abuse at the treatment-research clinic at the Intramural Research Program of the National Institute on Drug Abuse (Baltimore, MD, USA).

Plasma collection was scheduled for every 4 weeks for up to 24 weeks after the first day of Met administration. Specimens were collected in 7 ml gray-top (sodium fluoride-containing) Vacutainer tubes. The tubes were then centrifuged at 800 × *g* for 10 min to separate plasma from blood cells, and the plasma layer was transferred into cryotubes, which were stored at −20 °C until thawed for analysis.

2.7.2. Extraction procedure

Samples underwent solid-phase extraction (SPE). In a microcentrifuge tube, an aliquot of 50 μl of the solution, containing the deuterated internal standards Met-d_3 and EDDP-d_3 was evaporated and 1 ml of plasma sample was added, then vortex-mixed for 2 min. An aliquot of 10 μl of a 37% HCl aqueous solution was added, then vortex-mixed for 2 min and centrifuged at 15,000 × *g* (4 °C) for 10 min. Then the sample was transferred to 1 ml SPE cartridges (Oasis cation-exchange cartridges, MCX), and washed with 1 ml of 0.1 M HCl followed by 1 ml methanol. The retained compounds were eluted with 1 ml of a methanolic solution containing 10% (v/v) of a 20% ammonium hydroxide aqueous solution. The eluate was evaporated to dryness in a speed vacuum. The residue was reconstituted in a 100 μl aliquot of mobile phase, vortex-mixed, and transferred to an autosampler vial and a 20 μl aliquot was injected into the LC–MS system.

The free fraction of each enantiomer of Met and EDDP was determined by ultrafiltration and subsequent LC–MS analysis. An aliquot of 500 μl patient plasma was placed in the ultrafiltration device and centrifuged at 2000 × *g* until 250 μl of filtrate was collected. An aliquot of 200 μl of the filtrate was then extracted as described above and analyzed by LC–MS.

2.8. Validation

2.8.1. Matrix effect (ME), recovery (RE), and process efficiency (PE)

The ME was studied by analyzing quality control standards at three levels (LQC, MQC, and HQC) injected directly in mobile phase and comparing the concentration found in this set (set A) with the concentration found of the same analytes spiked after extraction (set B) into five different drug-free plasma pools. The formula used was: ME = set B/set A × 100 [45].

RE was studied by analyzing quality control standards at three levels using five different drug-free plasma pools and comparing the concentration of these analytes before extraction (set C) with another set of the same analytes after extraction (set B). The formula used was: RE = Set C/Set B × 100 [45].

PE was assessed by the formula: PE = (RE × ME)/100 [45]. It was evaluated at three levels (LQC, MQC, and HQC) and is reported as the average value.

Table 1
Identification of the samples analyzed by the present method

Patient no.	Sex	Current Met dose (mg/day)	Week
1	F	70	4
	F	100	8
	F	100	12
	F	100	16
	F	100	20
	F	100	24
2	M	70	4
	M	100	12
	M	100	16
	M	100	20
3	F	70	8
	F	70	12
	F	70	16
4	F	70	4
	F	70	20
	F	70	24
5	F	70	4
	F	70	8
	F	70	16

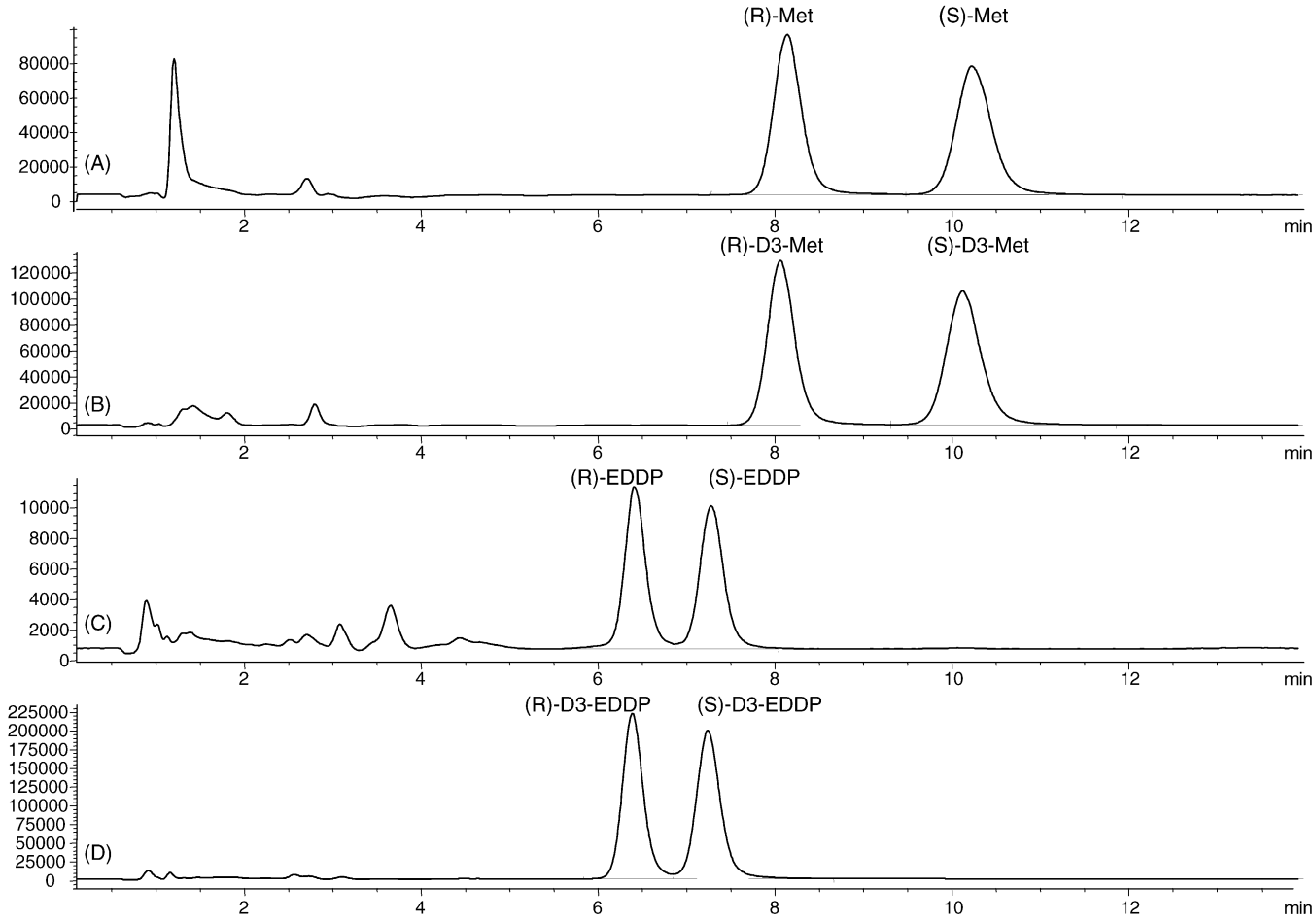

Fig. 2. Representative chromatogram of the low-quality control plasma sample (LQC) containing (*R*,*S*)-Met (10 ng/ml), (*R*,*S*)-EDDP (5 ng/ml), (*R*,*S*)-Met-d_3 (12.5 ng/ml), (*R*,*S*)-EDDP-d_3 (6.2 ng/ml), where the chromatographic trace obtained, using SIM at *m*/*z*: (A) 310.20 (Met); (B) 313.20 (Met-d_3); (C) 278.20 (EDDP); (D) 281.20 (EDDP-d_3).

2.8.2. Intra- and inter-day validation studies

The intra- and inter-day validation studies for precision and accuracy were performed in quintuplicate with QC standards, using five different plasma pools 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*)-Met-d_3, or (*S*)-Met/(*S*)-Met-d_3, or (*R*)-EDDP/(*R*)-EDDP-d_3 or (*S*)-EDDP/(*S*)-EDDP-d_3 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 racemic Met, EDDP, Met-d_3 and EDDP-d_3 after spiking in pooled human plasma.

2.9. Application of the analytical method

The validated method was applied to the analysis of stored plasma samples from a clinical trial. After giving informed consent, patients were stabilized on Met administered orally in liquid suspension, beginning at 30 mg on day 1 and increasing to 70 mg by 10 mg increments over 9 days. Approximately, 5 weeks into treatment, 252 patients were randomly assigned to undergo a dose increase from 70 to 100 mg/day over 5 days or to remain at 70 mg/day. The 19 specimens reported here were taken from four women and one man; their Met doses are shown in the Table 1.

3. Results and discussion

3.1. Chromatographic conditions

The mobile phase composition for the validation and clinical studies were set at acetonitrile–ammonium acetate buffer (10 mM, pH 7.0; 18:82, v/v), as previously reported [44]. Under these conditions, the analysis was completed in 15 min. The relative retentions (*k*) of (*R*)- and (*S*)-Met were 8.75 and 11.38, respectively, and the observed enantioselectivity (α) was 1.30 (Fig. 2A); for (*R*)- and (*S*)-Met-d_3 the *k* values were 8.66 and 11.29, respectively, and the observed α was 1.30

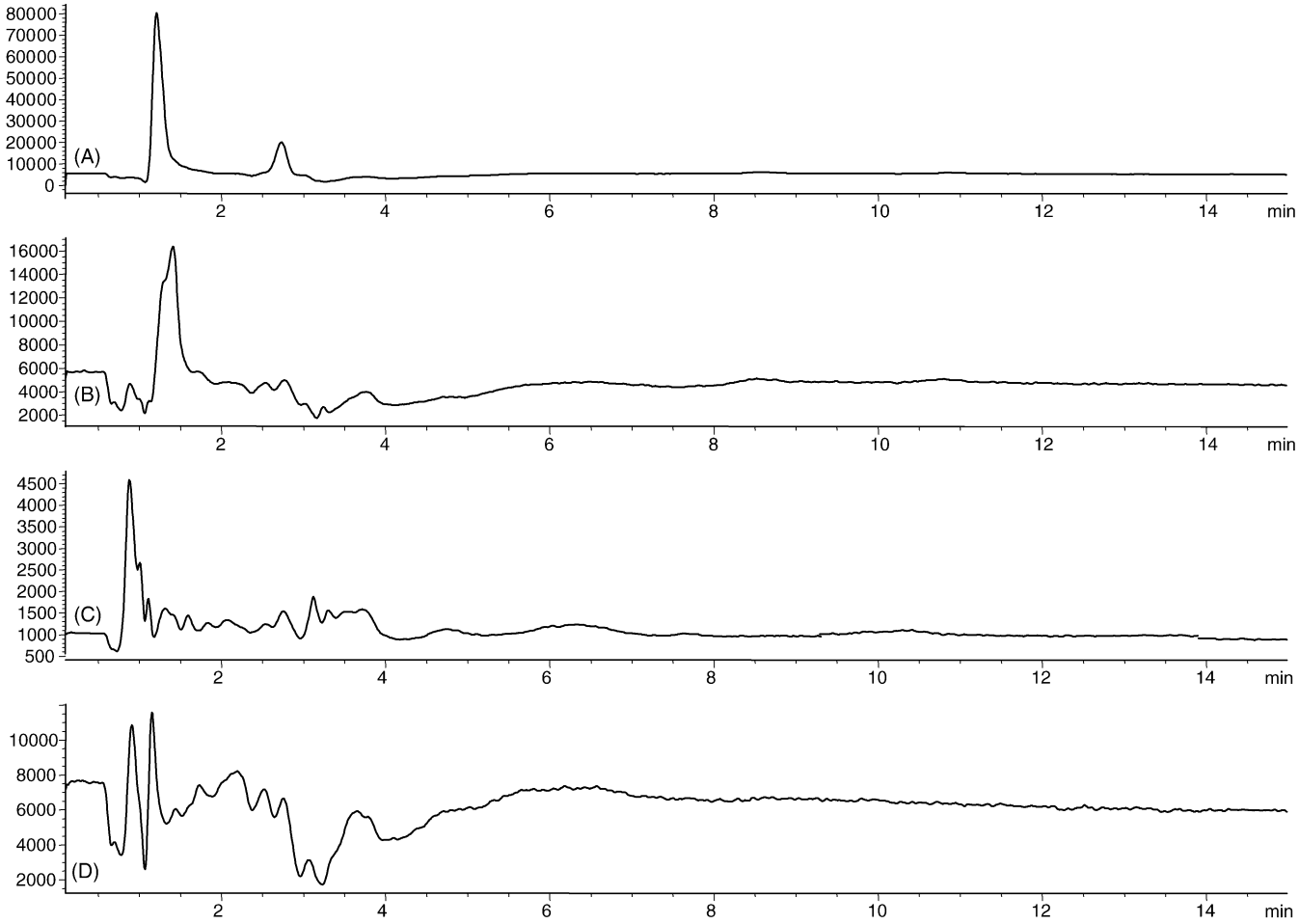

Fig. 3. Representative chromatogram of the blank human plasma, where the chromatographic trace obtained using SIM at *m/z*: (A) 310.20 (Met); (B) 313.20 (Met-d_3); (C) 278.20 (EDDP); (D) 281.20 (EDDP-d_3).

(Fig. 2B). The *k* values for (*R*)- and (*S*)-EDDP were 6.65 and 7.79, respectively, and the observed α was 1.17 (Fig. 2C); for (*R*)- and (*S*)-EDDP-d_3 the *k* values were 6.64 and 7.79, respectively, and the observed α was 1.17 (Fig. 2D). In addition, the analysis of five different drug-free plasma pools at these *m/z* values detected no interfering peaks, a representative chromatogram is presented in Fig. 3.

Under the chromatographic conditions used, (*R*)-Met and (*R*)-EDDP eluted before (*S*)-Met and (*S*)-EDDP. This is consistent with previously reported results obtained on the AGP-CSP [17,25–28,44].

3.2. Optimization of mass spectrometric detection

The chromatograms were monitored, using SIM for Met, EDDP, Met-d_3 and EDDP-d_3. 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. The parameters were optimized for the detection of EDDP and were as follows: fragmentor, 70 V; drying gas flow rate, 11.0 l/min; nebulizer pressure, 30 psi; drying gas temperature, 350 °C and capillary voltage, 1000 V.

3.3. Linearity and detection limits

Calibration curves for total and free concentrations of Met and EDDP were generated by weighted ($1/x$) least squares linear regression. To evaluate the total concentrations of Met and EDDP, linear relationships between peak height ratio and drug-enantiomer concentration of Met in the range 1.0–300.0 ng/ml were described by the following equations: $y = 0.8319x + 0.1692$, $r^2 = 0.9995$ [(*R*)-Met]; $y = 0.8121x + 0.2346$, $r^2 = 0.9999$ [(*S*)-Met]. The linear relationships between peak height ratio and drug-enantiomer concentration of EDDP in the range 1.0–25.0 ng/ml were described by the following

Table 2
Results of the matrix effect (ME), recovery (RE) and process efficiency (PE) on the extraction of Met and EDDP, where for Met: LQC = 10 ng/ml, MQC = 100 ng/ml, HQC = 300 ng/ml; and for EDDP: LQC = 5 ng/ml, MQC = 15 mg/ml, HQC = 25 ng/ml

	Matrix effect (ME)					Recovery (RE)					Process efficiency (PE)			
	Met		EDDP			Met		EDDP			Met		EDDP	
	R	*S*	*R*	*S*		*R*	*S*	*R*	*S*		*R*	*S*	*R*	*S*
LQC														
ME (%)	100.9	102.4	96.7	95.5	RE (%)	99.4	96.1	104.7	104.3	PE (%)	100.0	98.4	101.2	99.6
S.D.	5.6	1.7	2.3	1.7	S.D.	2.4	3.8	2.7	0.8	S.D.	2.2	3.8	1.2	1.6
R.S.D. (%)	5.6	1.7	2.4	1.8	R.S.D. (%)	2.4	4.0	2.6	0.8	R.S.D. (%)	2.2	3.8	1.2	1.6
MQC														
ME (%)	100.6	101.9	99.0	99.6	RE (%)	99.3	100.2	104.1	104.6	PE (%)	99.9	102.1	103.0	104.2
S.D.	0.9	0.6	1.4	1.1	S.D.	1.8	1.1	2.5	3.0	S.D.	1.1	0.9	1.6	1.9
R.S.D. (%)	0.9	0.6	1.4	1.1	R.S.D. (%)	1.8	1.1	2.4	2.8	R.S.D. (%)	1.1	0.9	1.5	1.8
HQC														
ME (%)	102.3	102.4	99.8	99.7	RE (%)	99.2	100.7	104.3	103.8	PE (%)	101.4	103.1	104.1	103.5
S.D.	0.3	1.3	0.5	2.5	S.D.	0.7	2.3	1.4	2.8	S.D.	0.5	1.4	1.3	1.3
R.S.D. (%)	0.3	1.3	0.5	2.5	R.S.D. (%)	0.7	2.3	1.4	2.7	R.S.D. (%)	0.5	1.3	1.2	1.3

All concentrations are per enantiomer.

Table 3
Results from the validation studies for the total concentration of the enantiomers of Met and EDDP in human plasma

	Methadone								EDDP							
	LLOQ (1.0 ng/ml)		LQC (10.0 ng/ml)		MQC (100.0 ng/ml)		HQC (300.0 ng/ml)		LLOQ (1.0 ng/ml)		LQC (5.0 ng/ml)		MQC (15.0 ng/ml)		HQC (25.0 ng/ml)	
	R	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Intra-day																
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Average	1.0	1.0	9.9	9.8	99.8	101.6	298.4	302.3	1.0	1.0	5.0	4.9	15.0	15.0	24.4	24.0
S.D.	0.0	0.0	0.5	0.2	1.1	0.9	1.4	4.0	0.0	0.0	0.1	0.1	0.2	0.3	0.3	0.3
R.S.D. (%)	1.0	1.5	5.3	1.6	1.1	0.9	0.5	1.3	1.0	1.6	1.2	2.0	1.5	1.8	1.2	1.3
Inter-day																
N	7	7	15	15	15	15	15	15	7	7	15	15	15	15	15	15
Average	1.0	1.0	10.2	9.7	100.0	101.7	296.4	299.2	1.0	1.0	5.0	4.9	14.9	14.8	24.2	23.8
S.D.	0.0	0.3	0.4	0.3	1.6	1.4	3.1	5.7	0.0	0.0	0.1	0.1	0.3	0.3	0.4	0.6
R.S.D. (%)	2.3	2.1	4.2	3.1	1.6	1.4	1.0	1.9	2.2	3.1	1.1	1.6	1.7	2.0	1.8	2.3
Accuracy (%)	101.0	99.4	102.1	96.9	100.0	101.7	98.8	99.7	102.0	101.4	99.2	97.8	99.6	98.8	96.6	95.0

Table 4
Results from the validation studies for the free concentration of the enantiomers of Met and EDDP in human plasma

	Methadone								EDDP							
	LLOQ (1.0 ng/ml)		LQC (10.0 ng/ml)		MQC (30.0 ng/ml)		HQC (50.0 ng/ml)		LLOQ (0.1 ng/ml)		LQC (1.0 ng/ml)		MQC (3.0 ng/ml		HQC (5.0 ng/ml)	
	R	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*	*R*	*S*
Intra-day																
N	5	5	15	15	15	15	15	15	5	5	5	5	5	5	5	5
AVERAGE	1.0	1.0	10.3	10.4	29.6	29.8	50.2	50.6	0.1	0.1	1.0	1.0	3.0	3.0	5.1	5.0
S.D.	0.0	0.0	0.1	0.1	0.2	0.3	0.2	0.6	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0
R.S.D. (%)	2.0	3.0	0.6	1.4	0.7	1.1	0.5	1.1	1.0	1.6	4.9	0.0	1.7	1.7	1.8	0.9
Inter-day																
N	7	7	15	15	15	15	15	15	7	7	15	15	15	15	15	15
AVERAGE	1.0	1.0	10.5	10.6	29.7	30.1	50.2	50.7	0.1	0.1	1.0	1.0	3.0	2.9	5.0	5.0
S.D.	0.1	0.1	0.2	0.2	0.3	0.4	0.5	0.4	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1
R.S.D. (%)	6.0	5.3	2.0	1.8	1.0	1.2	0.9	0.9	2.2	3.1	4.9	4.6	1.7	1.7	1.5	1.6
Accuracy (%)	102.2	102.8	104.7	105.5	99.0	100.5	100.4	101.3	102.0	101.4	104.0	104.0	99.0	98.3	100.7	99.6

equations: $y = 0.1101x - 0032$, $r^2 = 0.9980$ [(*R*)-EDDP]; $y = 0.1101x - 0.0082$, $r^2 = 0.9981$ [(*S*)-EDDP]. The data were based on three replicates of a seven-point calibration curve.

To evaluate the free concentrations of Met and EDDP, linear relationships between peak height ratio and drug-enantiomer concentration of Met in the range 1.0–50.0 ng/ml were described by the following equations: $y = 0.8319x + 0.1692$, $r^2 = 0.9995$ [(*R*)-Met]; $y = 0.8121x + 0.2346$, $r^2 = 0.9999$ [(*S*)-Met]. The linear relationships between peak height ratio and drug-enantiomer concentration of EDDP in the range 0.1–5.0 ng/ml were described by the following equations: $y = 0.2572x - 0.0062$, $r^2 = 0.9989$ [(*R*)-EDDP]; $y = 0.2602x - 0.0042$, $r^2 = 0.9993$ [(*S*)-EDDP]. The data were based on three replicates of a seven-point calibration curve.

The lower limit of quantification (LLOQ) is the concentration of the drug in the matrix that can be determined with a high percentage of accuracy (80–120%) [46]. The LLOQ for Met in this study was 1.0 ng/ml per enantiomer, since preliminary analyses of plasma samples indicated that this established LLOQ was preferred for the study, but in fact, it can be as low as 0.2 ng/ml per enantiomer. The LLOQ for EDDP was 0.1 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 0.02 ng/ml per enantiomer and for EDDP 0.01 ng/ml per enantiomer.

3.4. ME, RE, and PE

The MEs of (*R*)- and (*S*)-Met and (*R*)- and (*S*)-EDDP from five different pools of plasma were investigated, using quality control standards at three levels (LQC, MQC and HQC). See results in Table 2. The total average ME for (*R*)-Met was 101.3 ± 3.2%, for (*S*)-Met was 102.3 ± 1.2%, for (*R*)-EDDP was 98.5 ± 2.0% and for (*S*)-EDDP was 98.3 ± 2.7%.

During the development of the extraction method, the SPE cartridges tested were the hydrophilic–lipophilic balance cartridges (Oasis HLB 1 ml/30 mg) and the cation-exchange cartridges (Oasis MCX, 1 ml/30 mg). The HLB cartridges gave low recoveries in the range 50–60% for Met and 60–70% for EDDP. When using the Oasis MCX cartridges it was necessary to optimize the elution solvent. The eluents tested were methanolic solutions containing different concentrations of a 20% ammonium hydroxide aqueous solution to yield final concentrations of 1, 2, 5, 10, 15, and 20% (v/v). The best recoveries were obtained, using the Oasis MCX cartridges and an eluent composed of a methanolic solution containing 10% (v/v) of a 20% ammonium hydroxide aqueous solution. Under these conditions the recoveries ranged from 99.0 ± 3.6 to 104.4 ± 2.1%, Table 2.

Table 5
The total and free concentration of the enantiomers of Met and EDDP in plasma samples from patients in a Met-maintenance program for opioid dependence

Patient no.	Total concentration (ng/ml)						Free concentration (ng/ml)					
	Met			EDDP			Met			EDDP		
	R	*S*	*R/S*	*R*	*S*	*R/S*	*R*	*S*	*R/S*	*R*	*S*	*R/S*
1	108.3	131.0	0.8	1.8	2.7	0.7	12.7	10.2	1.2	0.4	0.6	0.7
	141.2	92.6	1.5	1.5	1.8	0.8	17.3	8.7	2.0	0.4	0.5	0.8
	114.2	141.2	0.8	2.4	3.5	0.7	15.6	12.5	1.2	0.6	0.9	0.7
	105.4	118.1	0.9	2.1	2.9	0.7	15.1	11.6	1.3	0.6	0.8	0.8
	123.1	111.1	1.1	2.9	3.6	0.8	20.9	12.4	1.7	0.7	1.0	0.7
	121.2	163.8	0.7	2.0	2.8	0.7	11.9	9.8	1.2	0.3	0.4	0.8
2	76.3	79.5	1.0	1.2	1.6	0.8	18.0	16.2	1.1	0.2	0.3	0.7
	109.6	114.1	1.0	1.2	1.7	0.7	27.2	17.7	1.5	0.3	0.4	0.8
	116.1	126.0	0.9	1.8	2.4	0.8	13.3	11.5	1.2	0.3	0.4	0.8
	116.3	119.9	1.0	1.9	2.4	0.8	11.4	8.7	1.3	0.3	0.3	1.0
3	106.4	144.1	0.7	1.7	2.6	0.7	10.0	7.9	1.3	0.3	0.4	0.8
	97.9	96.7	1.0	1.6	2.2	0.7	9.6	8.2	1.2	0.3	0.3	1.0
	126.9	129.0	1.0	2.3	3.5	0.7	12.4	8.1	1.5	0.5	0.7	0.7
4	196.0	251.7	0.8	2.3	3.3	0.7	9.8	7.1	1.4	0.2	0.3	0.7
	104.7	127.2	0.8	1.1	1.5	0.7	6.7	5.4	1.2	0.0*	0.1	0.5
	170.7	203.5	0.8	1.6	2.2	0.7	11.2	7.6	1.5	0.2	0.3	0.7
5	81.6	77.4	1.1	1.5	2.2	0.7	12.6	9.8	1.3	0.4	0.5	0.8
	104.8	108.5	1.0	1.7	2.5	0.7	8.7	6.7	1.3	0.4	0.5	0.8
	98.5	77.5	1.3	2.3	2.9	0.8	12.7	9.2	1.4	0.6	0.7	0.9
Average	116.8	127.0	1.0	1.8	2.5	0.7	13.5	10.0	1.4	0.4	0.5	0.8
S.D.	28.1	43.1	0.2	0.5	0.6	0.1	4.8	3.1	0.2	0.2	0.2	0.1

*Estimated value was 0.05, which was below the LLOQ, but above the LOD. See Table 1 for patient and dosing details.

The recoveries of (*R*)- and (*S*)-Met and (*R*)- and (*S*)-EDDP from five different pools of plasma were also investigated using quality control standards at three levels (LQC, MQC and HQC). See results in Table 2. The total average RE for (*R*)-Met was 99.3 ± 4.1%, for (*S*)-Met was 99.0 ± 3.6%, for (*R*)-EDDP was 104.4 ± 2.1% and for (*S*)-EDDP was 104.2 ± 2.2%.

The PE was also evaluated at three levels, LQC, MQC and HQC. See results in Table 2. The average PE for (*R*)-Met was 100.4 ± 1.5%, for (*S*)-Met was 101.2 ± 3.0%, for (*R*)-EDDP was 102.8 ± 1.8%, and for (*S*)-EDDP was 102.3 ± 2.6%.

3.5. Accuracy and precision

Accuracy and precision of the method for the total and the free concentrations of Met and EDDP were evaluated from quintuplicate analysis of each QC standard level (LQC, MQC and HQC) and repeated for 3 days. For the total concentration, the calculated average accuracy was 100.3 ± 3.0% for (*R*)-Met, 99.5 ± 2.9% for (*S*)-Met, 98.4 ± 2.0% for (*R*)-EDDP and 97.1 ± 2.5% for (*S*)-EDDP, Table 3.

For the free concentration, the calculated average accuracy was 101.3 ± 2.9% for (*R*)-Met, 102.4 ± 2.6% for (*S*)-Met, 101.2 ± 3.7% for (*R*)-EDDP and 100.6 ± 4.0% for (*S*)-EDDP, Table 4.

The intra- and inter-day precision of the method for the total and the free concentration were determined as relative standard deviation (%R.S.D.). The results were ≤5.3% for (*R*)-Met, ≤3.1% for (*S*)-Met, ≤4.9% for (*R*)-EDDP and ≤4.6% for (*S*)-EDDP. The results of the validation studies in Tables 3 and 4 demonstrate that the method has acceptable accuracy and precision.

3.6. Stability studies

The Met and EDDP standards were frozen at −20 °C for 4 months, defrosted and analyzed. There was no observable degradation of either analyte. Stability of Met and EDDP

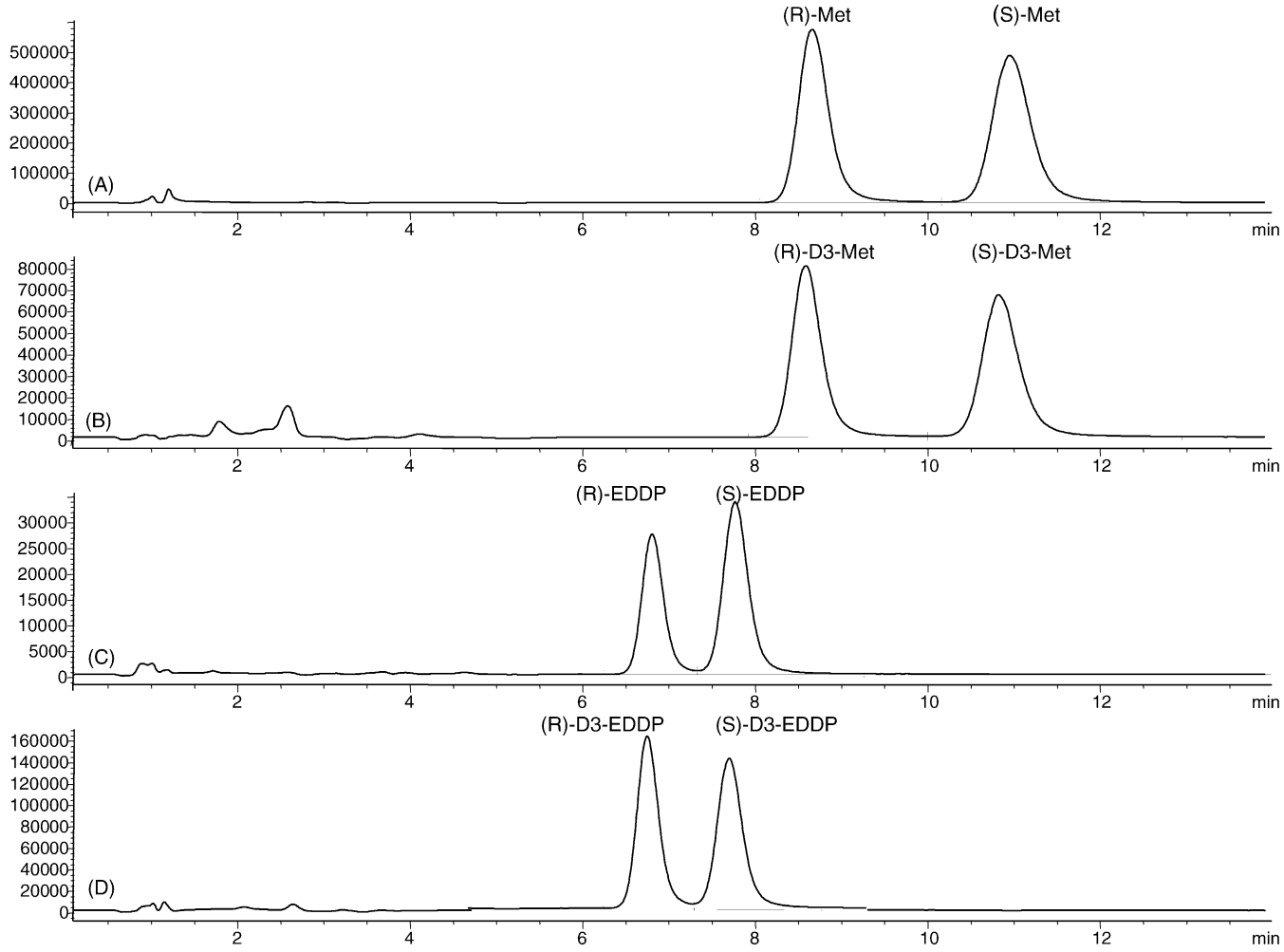

Fig. 4. Representative chromatograms of the analysis of a plasma sample from a patient number 2 at 12 weeks. The total concentrations of Met and EDDP measured were: (*R*)-Met 109.6 ng/ml, (*S*)-Met 114.1 ng/ml, (*R*)-EDDP 1.2 ng/ml and (*S*)-EDDP 1.7 ng/ml per enantiomer where the chromatographic trace obtained using SIM at *m*/*z*: (A) 310.20 (Met); (B) 313.20 (Met-d_3); (C) 278.20 (EDDP); (D) 281.20 (EDDP-d_3).

were determined after three freeze and thaw cycles. The spiked plasma samples at three quality control levels (LQC, MQC, and HQC) were stored at −20 °C for 24 h and thawed unassisted at room temperature ($n = 3$). When completely thawed, the samples were refrozen two more times, then analyzed. There was no observable degradation of either analyte. The LQCs, MQCs and HQCs for Met and EDDP were placed in the autosampler at room temperature and assayed at 0, 4, 8, 12, 16, 20, and 24 h. There was no observable degradation of either analyte during this period.

3.7. Application to clinical samples

The validated method has been applied to the analysis of 19 plasma samples obtained from Met-maintained outpatients, see Table 1 and Section 2.7.1, for details. The results from the analyses are presented in Table 5. Representative chromatograms from the determination of the total serum concentrations of Met and EDDP in one patient are presented in Fig. 4 and the chromatograms from the determination of the free Met and EDDP concentrations in the same sample are presented in Fig. 5. The measured total concentrations were: (*R*)-Met 109.6 ng/ml, (*S*)-Met 114.1 ng/ml, (*R*)-EDDP 1.2 ng/ml, (*S*)-EDDP 1.7 ng/ml, and the measured free concentrations were: (*R*)-Met 27.2 ng/ml, (*S*)-Met 17.7 ng/ml, (*R*)-EDDP 0.3 ng/ml, (*S*)-EDDP 0.4 ng/ml.

Previous studies of the plasma concentrations of (*R*)- and (*S*)-Met have utilized a variety of doses and sampling times [10,15,17–18,23,47–50]. In this study, plasma samples were obtained at steady state after either 70 or 100 mg doses, Table 1. The total plasma concentrations for (*R*)- and (*S*)-Met were consistent with all of the previously reported data obtained in patients who had chronically received the racemic drug.

In studies involving single doses of racemic Met and a small experimental cohort, the observed ratio of (*R*)- to (*S*)-Met (*R*/*S*-Met) was <1.0, although a wide variability in this ratio has been reported. In a larger sampling ($n = 45$), the median *R*/*S*-Met ratio was calculated as 1.02 with a range

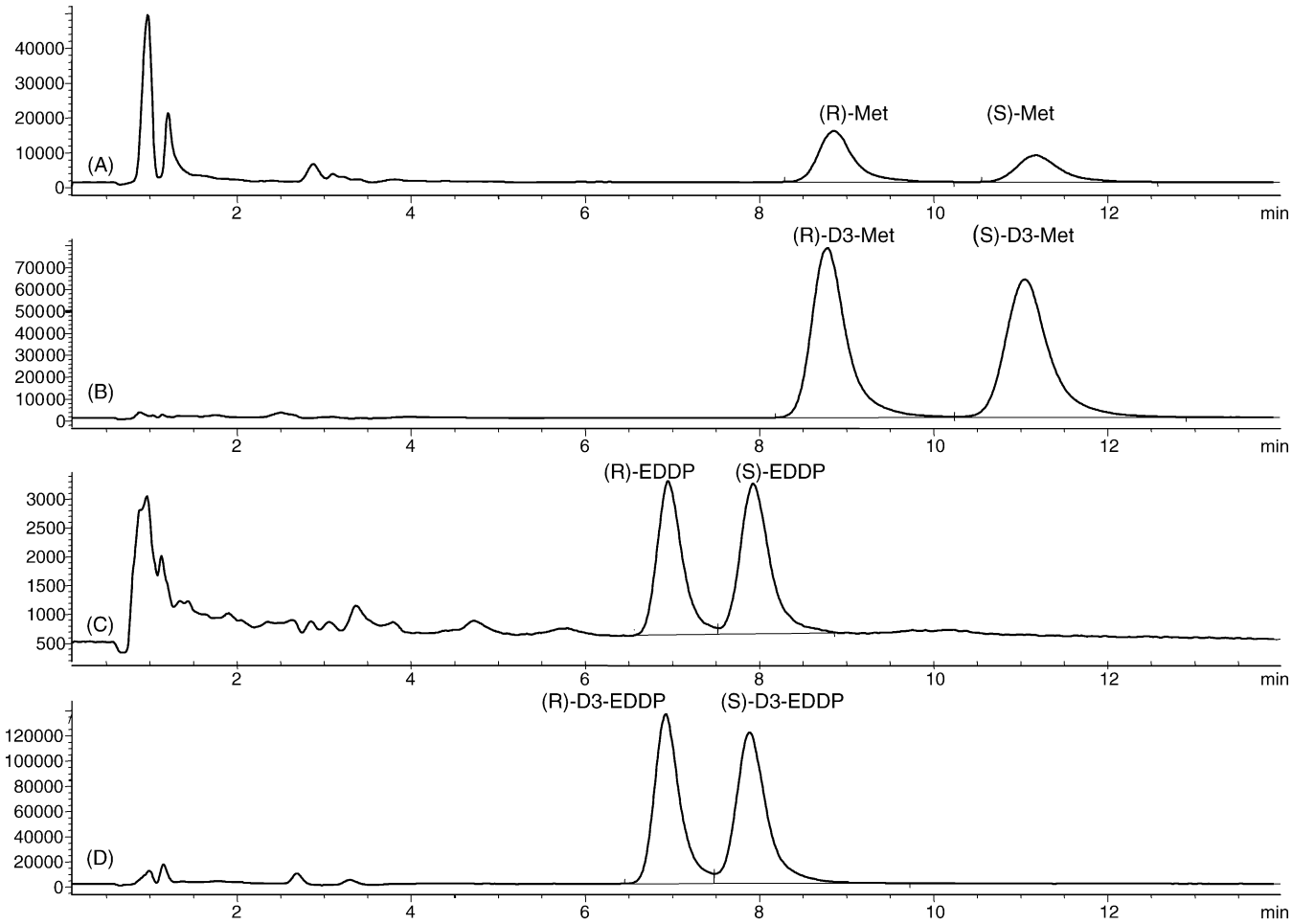

Fig. 5. Representative chromatograms of the analysis of a plasma sample obtained from the same patient number 2 at 12 weeks. The free concentrations of Met and EDDP measured were: (*R*)-Met 27.2 ng/ml, (*S*)-Met 17.7 ng/ml, (*R*)-EDDP 0.3 ng/ml and (*S*)-EDDP 0.4 ng/ml per enantiomer where the chromatographic trace obtained using SIM at *m*/*z*: (A) 310.20 (Met); (B) 313.20 (Met-d_3); (C) 278.20 (EDDP); (D) 281.20 (EDDP-d_3).

0.57–1.89 [23]. In this study, the average *R*/*S*-Met ratio was 1.0 ± 0.2 with a range 0.7–1.5. Thus, the results are consistent with the previous observations of significant inter-individual variations in the total *R*/*S*-Met ratios.

Previous studies have reported the free concentrations of (*R*)- and (*S*)-Met as percent of the total Met concentrations [10,15,18,49]. When 29 healthy volunteers were studied, the calculated free fractions were $12.44 \pm 1.53\%$ for (*R*)-Met and $9.24 \pm 1.61\%$ for (*S*)-Met [49]. Similar results were obtained in another study with 45 healthy volunteers, where the measured free fractions were $14.2 \pm 3.2\%$ for (*R*)-Met and $10.0 \pm 2.9\%$ for (*S*)-Met [15]. The results from this study are consistent with above data as the calculated free fractions were $12.2 \pm 5.2\%$ for (*R*)-Met and $8.8 \pm 4.3\%$ for (*S*)-Met.

There are no previous studies reporting the free plasma concentrations of (*R*)- or (*S*)-EDDP, primarily due to the lack of a sensitive analytical method. In this study, the total (*R*)-EDDP plasma concentrations ranged from 1.1 to 2.9 ng/ml and from 1.5 to 3.6 ng/ml for (*S*)-EDDP, Table 5. The total *R*/*S*-EDDP ratio was 0.7 ± 0.1 indicating a clear enantioselectivity in the production of this metabolite. The same trend was observed for the free concentrations where (*R*)-EDDP concentrations ranged from 0.05 (estimated) to 0.7 ng/ml and 0.1 to 1.0 ng/ml for (*S*)-EDDP. The free *R*/*S*-EDDP ratio was 0.8 ± 0.1.

4. Conclusions

The bioanalytical assay reported in this manuscript is a simple, sensitive and reproducible method for the enantioselective determination of the total and free concentrations of Met and EDDP in human plasma. The assay has greater sensitivity than previously reported methods and a run time of less than 15 min.

The development of a rapid and accurate method for the determination of both total and free plasma concentrations of Met and EDDP enantiomers is an important step towards developing useful therapeutic monitoring of patients in Met-maintenance programs. A wide inter-patient variability in the response to Met treatment has been associated with variations in total plasma levels of Met [46]. However, no consistent correlation has been established.

Preliminary data from this study demonstrated no statistically significant correlation between total and free plasma concentrations for either (*R*)- or (*S*)-Met. Thus, if free (*R*)-Met is the key source of the observed pharmacological activity, monitoring the total plasma concentration cannot provide an accurate clinical assessment. This suggests that the determination of free plasma concentrations of (*R*)- and (*S*)-Met could be crucial for establishing a pharmacodynamic relationship between drug concentration and clinical response, which will be addressed through further analyses of plasma samples.

Acknowledgements

The authors wish to thank Mr. Dat Phan, Mr. Stephen Bollinger, and Mr. Brooke Miller of Agilent Technologies Inc., for their kind advice in mass spectrometry.

References

[1] C.E. Inturrisi, K. Verebely, Clin. Pharmacol. Ther. 13 (1972) 633.
[2] W.H. Horns, M. Rado, A. Goldstein, Clin. Pharmacol. Ther. 17 (1975) 636.
[3] J. Holmstrand, E. Anggard, L.M. Gunne, Clin. Pharmacol. Ther. 23 (1978) 175.
[4] G.K. Gourlay, R.J. Willis, P.R. Wilson, Anesthesiology 61 (1984) 19.
[5] P. Beauverie, E. Khan, B. Ghaleh, M. Van de Vyver, N. Poisson, C. Jacquot, Ann. Med. Interne. 145 (1994) 15.
[6] G. Bertschy, M. Martin, Med. Hyg. 52 (1994) 2178.
[7] J.J. Deglon, Med. Hyg. 39 (1981) 2798.
[8] J.J. Deglon, Med. Hyg. 39 (1981) 2901.
[9] H.R. Sullivan, S.L. Due, J. Med. Chem. 8 (1973) 909.
[10] D.W. Boulton, P. Arnaud, C.L. DeVane, Clin. Pharmacol. Ther. 70 (2001) 48.
[11] J.G. Gerber, R.J. Rhodes, J. Gal, Chirality 16 (2004) 36.
[12] B.A. Judson, W.H. Horns, A. Goldstein, Clin. Pharmacol. Ther. 20 (1976) 445.
[13] H.F. Fraser, H. Isbell, Bull. Narc. 14 (1962) 25.
[14] N.B. Eddy, H. Halbach, O.J. Braeden, Bull. WHO 14 (1967) 353.
[15] C.B. Eap, C. Cuendet, P. Baumann, Clin. Pharmacol. Ther. 47 (1990) 338.
[16] M.J. Garrido, C. Aguirre, I.F. Troconiz, M. Marot, M. Valle, M.K. Zamacoma, Int. J. Clin. Pharmacol. Ther. 38 (2000) 35.
[17] D.W. Boulton, C.L. Devane, Chirality 12 (2000) 681.
[18] K. Kristensen, T. Blemmer, H.R. Angelo, L.L. Christrup, N.E. Drenck, S.N. Rasmussen, P. Sjogren, Ther. Drug Monit. 18 (1996) 221.
[19] M.J. Kreek, D.L. Hachey, P.D. Klein, Life Sci. 24 (1979) 925.
[20] K. Nakamura, D.L. Hachey, M.J. Kreek, C.S. Irving, P.D. Klein, J. Pharm. Sci. 71 (1982) 40.
[21] K. Wolff, A. Rostami-Hodjegan, S. Shires, A.W. Hay, M. Feely, R. Calvert, Br. J. Clin. Pharmacol. 44 (1997) 325.
[22] K. Wolff, M. Sanderson, A.M. Hay, D. Raistrick, Clin. Chem. 37 (1991) 205.
[23] S. Rudaz, D. Ortelli, M. Gex-Fabry, J.-J. Deglon, L.P. Balant, J.-L. Veuthey, Chirality 11 (1999) 487.
[24] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobsson, J. Chromatogr. 570 (1991) 198.
[25] P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele, P. Mangin, J. Forensic Sci. 42 (1997) 291.
[26] P. Kintz, A. Tracqui, C. Marzullo, A. Darreye, F. Tremeau, P. Greth, B. Ludes, Ther. Drug Monit. 20 (1998) 35.
[27] K. Kristensen, H.R. Angelo, T. Blemmer, J. Chromatogr. A 666 (1994) 283.
[28] R.L.G. Norris, P.J. Ravenscroft, S.M. Pond, J. Chromatogr. B 661 (1994) 346.
[29] 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.
[30] S. Rudaz, J.-L. Veuthey, J. Pharm. Biomed. Anal. 14 (1996) 1271.
[31] N. Schmidt, K. Brune, G. Geisslinger, J. Chromatogr. 583 (1992) 195.
[32] T.L. Pierce, A.G.W. Murray, W. Hope, J. Chromatogr. Sci. 30 (1992) 443.
[33] S. Rudaz, J.-L. Veuthey, Chirality 11 (1999) 319.

[34] H.R. Angelo, N. Beck, K. Kristensen, J. Chromatogr. B 724 (1999) 35.
[35] D.J.R. Foster, A.A. Somogyi, F. Bochner, J. Chromatogr. B 744 (2000) 165.
[36] D. Ortelli, S. Rudaz, A.-F. Chevalley, A. Mino, J.-J. Deglon, L. Balant, J.-L. Veuthey, J. Chromatogr. A 871 (2000) 163.
[37] M. Frost, H. Kohler, G. Blaschke, Electrophoresis 18 (1997) 1026.
[38] M. Lanz, W. Thormann, Electrophoresis 17 (1996) 1945.
[39] K. Kristensen, H.R. Angelo, Chirality 4 (1992) 263.
[40] S. Souverain, Ch. Eap, J.-L. Veuthey, S. Rudaz, Clin. Chem. Lab. Med. 41 (2003) 1615.
[41] D. Ortelli, S. Rudaz, S. Souverain, J.-L. Veuthey, J. Sep. Sci. 25 (2002) 222.
[42] S. Souverain, S. Rudaz, D. Ortelli, E. Varesio, J.-L. Veuthey, J. Chromatogr. B 784 (2003) 117.
[43] J.N. Wilkins, A. Ashofteh, D. Setoda, W.S. Wheatley, H. Huigen, W. Ling, Ther. Drug Monit. 19 (1997) 83.
[44] M.E. Rodriguez-Rosas, K.L. Preston, D.H. Epstein, E.T. Moolchan, I.W. Wainer, J. Chromatogr. B 796 (2003) 355.
[45] B.K. Matuszewski, Proceedings of the 14th International PBA Symposium, Orlando, FL, 14 September 2003.
[46] Guidance for Industry. Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, May 2001.
[47] K.R. Dyer, J.M. White, D.J.R. Foster, F. Bochner, A. Menelaou, A.A. Somogyi, J. Clin. Psychopharm. 21 (2002) 78.
[48] C.B. Eap, M. Bourquin, J.-L. Martin, J. Spagnoli, S. Livoti, K. Powell, P. Baumann, J.-L. Deglon, Drug Alcohol Depend. 61 (2000) 47.
[49] M.K. Romach, K.M. Piafsky, J.G. Abel, V. Khouw, E.M. Sellers, Clin. Pharmacol. Ther. 29 (1981) 211.
[50] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobsson, J. Chromatogr. B 570 (1991) 198.