ELSEVIER

Journal of Chromatography B, 744 (2000) 165–176

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Stereoselective quantification of methadone and its major oxidative metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, in human urine using high-performance liquid chromatography

David J.R. Foster[a,*], Andrew A. Somogyi[a,b], Felix Bochner[a,b]

[a]*Department of Clinical and Experimental Pharmacology, University of Adelaide, Adelaide 5005, Australia*
[b]*Department of Clinical Pharmacology, Royal Adelaide Hospital, Adelaide 5000, Australia*

Received 12 October 1999; received in revised form 13 April 2000; accepted 17 April 2000

Abstract

A stereoselective HPLC assay was developed for the quantification of the enantiomers of methadone and its major oxidative metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in human urine. The compounds were quantified in a single assay following liquid–liquid extraction and stereoselective HPLC with UV detection. Calibration curve concentrations ranged from 0.125 to 12.5 μM for each enantiomer. Assay performance was assessed using quality control samples, and the inter- and intra-assay bias (<10%) and precision (<15%) were acceptable for all compounds. The assay was successfully used to quantitate the enantiomers of methadone and EDDP in urine samples obtained from subjects receiving methadone maintenance therapy.

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

1. Introduction

Methadone is the most widely used pharmacological agent for the treatment of opioid dependence [1]. *rac*-Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) is a chiral molecule (Fig. 1) comprising *R*- and *S*- enantiomeric forms. *R*-Methadone has a higher affinity at μ and δ opioid receptors [2] and prevents the occurrence of opioid withdrawal symptoms, while *S*-methadone is ineffective [3].

Methadone is eliminated from the body primarily by metabolism. The formation of the quantitatively major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP, Fig. 1), is catalysed mainly by CYP3A4 [4–7]. After oral dosing, unchanged methadone and EDDP are found in similar amounts in the urine of humans and account for up to 50% of the dose [8–10]. Lesser amounts of EDDP have also been recovered in the faeces of methadone maintenance patients and account for 6% [11] to 18% [12] of the daily dose during chronic treatment, while methadone was found to account for less than 1% [11,12].

While there are several HPLC methods available for the quantification of the individual methadone enantiomers in biological fluids [13–20], few methods are available for the stereoselective quantifica-

*Corresponding author. Tel.: +61-8-8303-5985; fax: +61-8-8224-0685.

E-mail address: dfoster@medicine.adelaide.edu.au (D.J.R. Foster)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00246-2

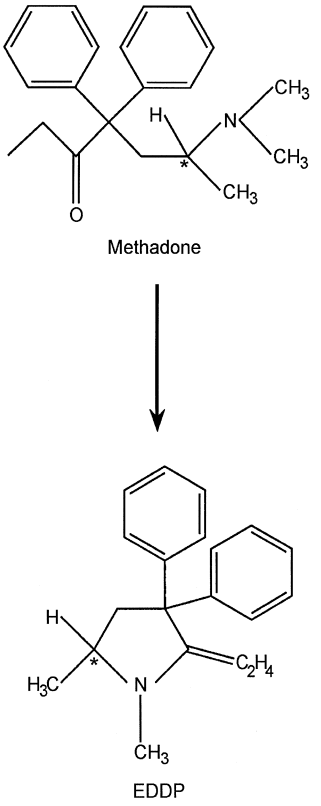

Fig. 1. Metabolic pathway showing N-demethylation of methadone to EDDP. *, Indicates a chiral centre.

tion of the major metabolite, EDDP. Frost et al. used capillary electrophoresis (CE) with a cyclodextrin chiral selector for the simultaneous quantification of methadone and EDDP enantiomers in serum, urine and hair [21]. Similarly, Lanz and Thormann developed a CE method, and applied this to quantitate the enantiomers of methadone and EDDP in urine samples from methadone maintenance patients [22]. Recently, Ramseier et al. applied a similar CE assay to allow for the identification of the enantiomers of methadone and EDDP, and other commonly abused drugs, in urine samples [23]. Other investigators have used CE to quantitate the enantiomers of methadone, but not EDDP [24]. Although CE methods do provide a relatively simple and inexpensive alternative to HPLC, many laboratories do not yet use this technique routinely. In contrast, the use of a chiral column in a conventional HPLC system is more accessible to many laboratories, such as our own. Kintz et al. quantitated the enantiomers of methadone and EDDP in hair using a liquid chromatography–mass spectrometry (LC–MS) technique [25]. Although LC–MS assays are sensitive, they are expensive and complex. Using stereoselective HPLC, Pham-Huy et al. were able to simultaneously quantify methadone and EDDP. However, this method only resolved the methadone enantiomers but not those of EDDP [26]. Recently Angelo et al. reported a method for the simultaneous quantification of the enantiomers of methadone and EDDP in urine using stereoselective HPLC [27]. This method used a non-chiral analytical column coupled in series with a chiral analytical column to effect resolution.

The aim of the present study was to develop a simple, sensitive and robust HPLC method for the quantification of the individual enantiomers of methadone and its primary metabolite, to allow a better understanding of the renal elimination and metabolism of methadone and EDDP. A comparison was also made with an achiral assay for methadone and EDDP, and a chiral assay for the individual enantiomers of methadone only.

2. Experimental

2.1. Chemicals

rac-Methadone as the hydrochloride salt, *R*- and *S*-methadone as the free bases, and *R*- and *S*-EDDP as the perchlorate salts were obtained from the National Institute of Drug Abuse (Rockville, MD, USA). *rac*-EDDP as the hydroiodide salt was purchased from Alltech (State College, PA, USA). 3-Methoxymorphinan as the hydrobromide salt was a kind gift from Roche (Sydney, Australia). Diazepam was a kind gift from Professor J. Miners (Department of Clinical Pharmacology, Flinders Medical Centre, Adelaide, Australia). Morphine as the hydrochloride salt was from McFarlane Smith (Edinburgh, UK). Dextromoramide as the tartrate salt was provided by Faulding (Adelaide, Australia). HPLC grade acetonitrile, methanol, triethylamine and dimethyloctylamine were from BDH (Poole, UK). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

2.2. Instrumentation and chromatography conditions

2.2.1. Chromatography conditions for the quantification of R-methadone, S-methadone, R-EDDP and S-EDDP in human urine

The HPLC system comprised a LC-10AT pump (Shimadzu, Kyoto, Japan), a Sil-10A autoinjector (Shimadzu) and a SPD-M10A photo-diode array detection system (Shimadzu) set at 210 nm. The system was controlled using CLASS-LC10 software (version 1, Shimadzu) running under Windows 3.11 (Microsoft, WA, USA) on a 486 DX IBM compatible computer. The analytical column was a Chiral AGP column (100×4.0 mm, 5 μm; Chromtech, Hägersten, Sweden) with a Chiral AGP pre-column (10× 3.0 mm, 5 μm; Chromtech). Optimal separation of the compounds of interest was achieved with a mobile phase of 20 m*M* NaH_2PO_4 in water containing 2 m*M* dimethyloctylamine and 9% acetonitrile final pH adjusted to 5.5 with *o*-phosphoric acid and pumped through the system at 0.4 ml/min at room temperature.

2.2.2. Chromatography conditions for the quantification of R-methadone and S-methadone in human urine

The HPLC system was identical to the system described above. The analytical column was a Cyclobond I 2000 RSP column (250×4.6 mm, Astec, Whippany, NJ, USA) with a Cyclobond I 2000 RSP pre-column (20×4.0 mm, Astec). Optimal separation of the compounds of interest was achieved with a mobile phase of methanol–acetonitrile–1% triethylamine (9:11:80, v/v) in water with the final pH adjusted to 6.0 with *o*-phosphoric acid and pumped through the system at 1.0 ml/min at room temperature.

2.2.3. Chromatography conditions for the quantification of rac-methadone and rac-EDDP in human urine

The chromatography conditions have been previously described [7]. Concentrations of *rac*-methadone and *rac*-EDDP obtained by this assay were used to compare the sum of *R*- and *S*-methadone or *R*- and *S*-EDDP, respectively, in urine obtained with the stereoselective assays.

2.3. Sample preparation

2.3.1. Quantification of R-methadone, S-methadone, R-EDDP and S-EDDP and rac-methadone and rac-EDDP in human urine

Urine samples (0.5 ml) and internal standard (I.S.; 50 μl 10 μ*M* dextromoramide in water) were aliquoted into 10-ml screw capped borosilicate glass tubes, alkalinized (0.4 ml 0.1 *M* Na_2CO_3, pH 10) and extracted with 5 ml of 100% hexane for 20 min on a rotary mixer. Samples were then centrifuged (2000 *g*, 10 min) and the organic phase transferred to a clean 5-ml borosilicate glass tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted in 0.5 ml mobile phase, and separate 100-μl aliquots injected onto the chromatography systems for analysis of *R*- and *S*-methadone and *R*- and *S*-EDDP and *rac*-methadone and *rac*-EDDP. The extraction procedure used 100% hexane as the extraction solvent as the use of diethyl ether–hexane (30:70, v/v) (see Section 2.3.2) produced interfering peaks in the chromatography.

2.3.2. Quantification of R-methadone and S-methadone in human urine

Urine samples (1 ml) and the I.S. (100 μl 5 μg/ml 3-methoxymorphinan in water) were aliquoted into 10-ml tapered bottom plastic tubes, alkalinized (0.4 ml 0.1 *M* Na_2CO_3, pH 10) and extracted with 6 ml of diethyl ether–hexane (30:70, v/v) for 20 min on a rotary mixer. Samples were then centrifuged (2000 *g*, 10 min) and the organic phase transferred to a clean 10-ml tapered bottom plastic tube containing 0.25 ml 5 m*M* HCl and vortexed for 1 min. Samples were then centrifuged (2000 *g*, 10 min) and the organic phase aspirated to waste and 100 μl of the 5 m*M* HCl was injected onto the chromatography system. The extraction procedure used diethyl ether–hexane (30:70, v/v) as the extraction solvent as this gave greater extraction efficiencies for methadone (Tables 2 and 3) compared to 100% hexane. Evaporation of the organic solvent to dryness and injection of the reconstituted residue produced interfering peaks in the chromatography. A back extraction into 5 m*M* HCl was incorporated as this prevented the interference, while maintaining a high extraction efficiency for methadone and the I.S. (Table 3).

2.4. Calibration, precision, accuracy, and extraction efficiency

Retention times of the individual enantiomers of methadone and EDDP were confirmed by direct injection of aqueous solutions of enantiomerically pure compounds. Rac-methadone and rac-EDDP were used to prepare calibration standards for all assays. Replicate injections ($n=8$) of aqueous solutions spiked with *rac*-EDDP and *rac*-methadone produced mean±SD *R*/*S* enantiomer peak area ratios of 0.99±0.02 and 1.01±0.04 for EDDP and methadone, respectively, using the chromatographic system for the simultaneous quantification of *R*- and *S*-methadone and *R*- and *S*-EDDP. These data indicate that the racemic mixtures comprised equal amounts of the individual enantiomers.

For the simultaneous quantification of *R*- and *S*-methadone and *R*- and *S*-EDDP, calibration curves consisting of seven standards over the concentration range 0.125–12.5 μ*M* (0.25–25 μ*M* for *rac*-methadone and *rac*-EDDP) of *R*- and *S*-methadone and *R*- and *S*-EDDP were constructed in blank urine using *rac*-methadone and *rac*-EDDP. Low, medium and high quality control (QC) samples were also prepared in duplicate, with final concentrations of 0.4, 1.0 and 6.3 μ*M* for *R*- and *S*-EDDP, and 0.4, 1.0 and 8 μ*M* for *R*- and *S*-methadone.

For the quantification of *R*- and *S*-methadone, calibration curves consisting of eight standards over the concentration range 0.14–5.7 μ*M* of *R*- and *S*-methadone were constructed in blank urine using *rac*-methadone. Low, medium and high quality control samples were also prepared in duplicate, with final concentrations of 0.32, 1.15 and 3.2 μ*M* for *R*- and *S*-methadone.

The robustness of the analytical methods was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay accuracy and precision were determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on several different assay days.

Extraction efficiency was analysed at each QC concentration and for the I.S. for all assays. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

Peak areas of each compound of interest were converted into peak area ratios using the peak area of the I.S. Linear regression analysis, weighted $1/y^2$, of peak area ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

2.5. Patients

Ethical approval was obtained from the Royal Adelaide Hospital Research Ethics Committee to conduct this study. The patients had been enrolled in the South Australian Public Methadone Maintenance Program for at least 6 months and had not had a methadone dose change for at least 2 months. There were nine males and one female whose body masses ranged from 60 to 91 kg (mean±SD; 73±10 kg) and were aged from 21 to 45 years (36±8 years). Their once daily *rac*-methadone dose ranged from 7.5 to 130 mg/day, which corresponded to 0.1–1.9 mg/kg/day (0.81±0.54 mg/kg/day). Patients were excluded from the study if they were pregnant or had positive HIV serology. Each patient was admitted to the inpatient facility of the maintenance program 1 h before their scheduled daily *rac*-methadone dose and remained in the unit for the subsequent 24 h. Methadone was administered as a syrup under supervision of the study personnel, and a 24-h pooled urine sample was obtained over the subsequent inter-dosing interval. Patient samples that were above the upper limit of quantification were diluted in blank urine before re-analysis. Urinalysis of patient urine samples and self-reported medications indicated that the following drugs were concurrently taken by the patients: benzodiazepines (four patients), cannabinoids (five patients), opioids other than methadone (three patients), sympathomimetic amines (one patient).

2.6. Data analysis

Accuracy was calculated as the mean (calculated concentration/nominal concentration)·100% for each individual sample, and the residual standard deviation of the mean (RSD) was taken as the precision. As a validation procedure, ordinary least products linear regression analysis [28,29] was used to compare the concentrations of *R*- and *S*-methadone

obtained with the two stereoselective assays, the sum of *R*- and *S*-methadone concentrations with *rac*-methadone concentrations, and the sum of *R*- and *S*-EDDP concentrations with *rac*-EDDP concentrations using EXCEL v7.0a (Microsoft). Linear regression analysis was performed using GRAPHPAD PRISM v2.01 (GraphPad Software, CA, USA).

3. Results and discussion

For the simultaneous quantification of *R*- and *S*-methadone and *R*- and *S*-EDDP, chromatograms resulting from the extraction of a blank urine sample, a low calibration standard and a patient sample are shown in Fig. 2. The chromatogram obtained from the patient sample contained four peaks with identical retention times to *R*- and *S*-methadone and *R*- and *S*-EDDP as in the calibration standard. Retention times of the enantiomers of methadone and EDDP were confirmed by separate injection of the individual enantiomers. Under the chromatography conditions described, the retention times of *R*-EDDP, *S*-EDDP, *R*-methadone, *S*-methadone and the I.S. (dextromoramide) were 10, 11.5, 13, 16 and 27 min, respectively, with a total runtime of 35 min. Under these conditions all compounds of interest were adequately resolved. No decrease in resolution was observed after over 200 injections. There were no interfering peaks in the chromatography in several blank urine samples, and in the patient urine samples. Direct injection of morphine and diazepam solutions (drugs which are commonly used by former heroin addicts, especially morphine as it is also a metabolite of heroin) did not produce interfering peaks.

Similar results were obtained for the analysis of *R*- and *S*-methadone alone. Under the chromatography conditions described, the retention times of *R*- and *S*-methadone and the I.S. (3-methoxymorphinan) were 8.3, 9.6 and 21 min, respectively, with a total runtime of 30 min. Under these conditions all compounds of interest were baseline resolved. EDDP was not resolved from the *R*-methadone peak under these chromatography conditions. Recently Rudaz and Veuthey [19] reported similar inability to resolve EDDP from methadone with the Cyclobond I 2000 RSP column. However, EDDP is not back-extracted into the 5 m*M* HCl, and did not interfere with the analysis of *R*- and *S*-methadone in this assay. No decrease in resolution was observed after over 400 injections. There were no interfering peaks in the chromatography in several blank urine samples, and in the patient urine samples.

Calibration curves for *R*- and *S*-methadone and *R*- and *S*-EDDP were linear over the 0.125–12.5 μ*M* concentration range for each enantiomer, with mean r^2 values ($n=4$ assays) greater than 0.994 (Table 1). The inter-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard (0.125 μ*M*) are shown in Table 1. Similarly, intra-assay accuracy and precision at the three QC concentrations are shown in Table 2. Inter- and intra-assay accuracy and precision data for the assay of *R*- and *S*-methadone, and *rac*-methadone and *rac*-EDDP are presented in Tables 3 and 4, respectively.

Extraction efficiency was analysed using the intra-assay replicate low, medium and high QC samples for *R*- and *S*-methadone, *R*- and *S*-EDDP and for the I.S. (Table 2). Similar results were obtained for the assay of *R*- and *S*-methadone (Table 3) and *rac*-methadone and *rac*-EDDP (Table 4). The extraction efficiencies demonstrated no concentration dependency, and were similar between enantiomers. EDDP showed a somewhat lower recovery than methadone and the I.S.. This may be due to its substantially higher pK_a (10.4 vs. 8.6, respectively) [30] when compared to methadone. However, the calibration curves were linear, inter- and intra-assay validation data were acceptable, and the LOQ of the assay was well below the lowest concentration observed in the patient samples. The extraction efficiency of methadone, EDDP and the I.S. was slightly greater for the racemic assay when compared to the corresponding values obtained with the stereoselective assay. This result may be due to the slightly longer reconstitution time, as the aliquot taken for injection onto the racemic assay HPLC system was sampled several minutes after the aliquot taken for the stereoselective assay.

Concentrations of the enantiomers of EDDP, and methadone, were summed and compared to the concentrations obtained with the non-chiral assay. The concentrations of *R*- and *S*-methadone obtained with the two stereoselective assays were also com-

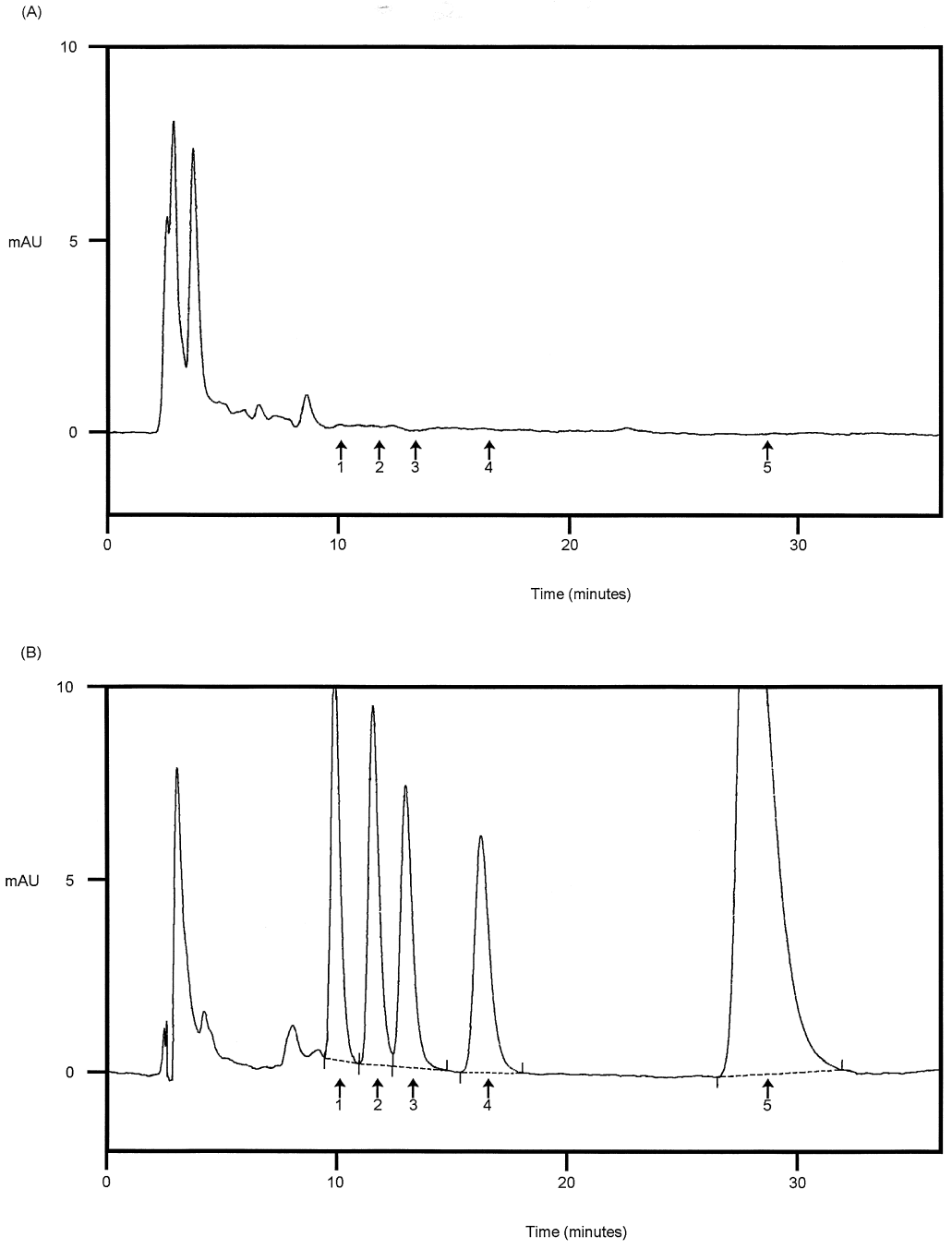

Fig. 2. Representative chromatograms assayed for the simultaneous quantitation of *R*- and *S*-methadone and *R*- and *S*-EDDP from a blank urine sample (A); calibration standard containing 0.5 μ*M* *R*- and *S*-EDDP and *R*- and *S*-methadone (B); and a patient's urine sample containing 1.26 μ*M* *R*-EDDP, 1.82 μ*M* *S*-EDDP, 1.11 μ*M* *R*-methadone and 0.68 μ*M* *S*-methadone (C) – see next page. 1 = *R*-EDDP; 2 = *S*-EDDP; 3 = *R*-methadone; 4 = *S*-methadone; 5 = dextromoramide (I.S.).

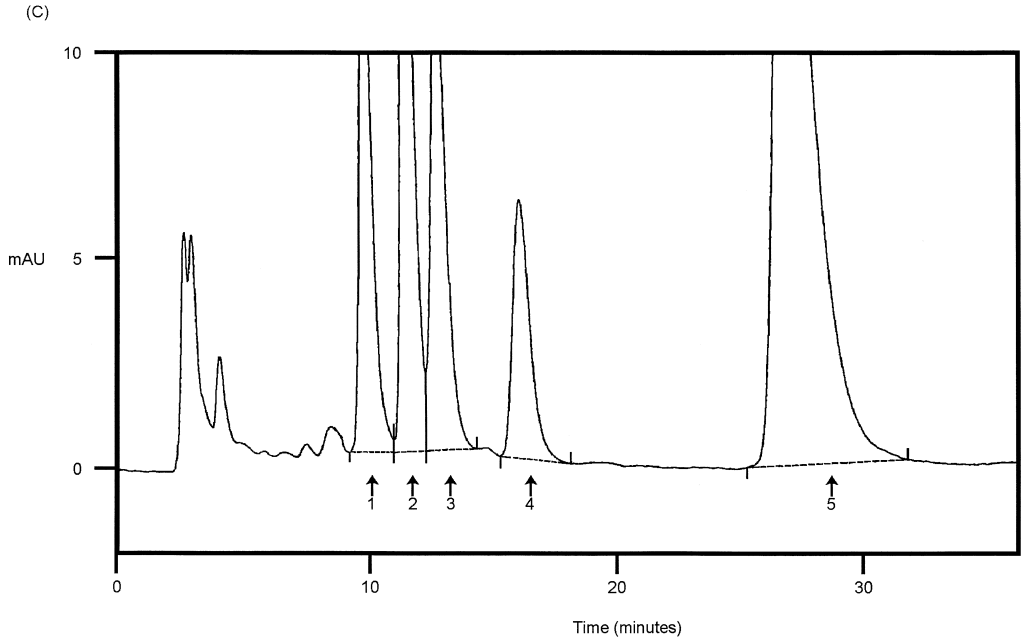

Fig. 2. (*continued*)

pared, allowing a thorough validation of the assays. Comparisons between assays were made using ordinary least products linear regression analysis [28,29]. This technique is sensitive to both fixed and proportional bias, unlike conventional linear-regression analysis, as it does not assume that one axis is error-free [28,29]. The analyses yielded strong and significant correlations ($r^2 > 0.996$, $P < 0.05$) for all comparisons. The 95% confidence intervals (CI) of the slope included 1 for all comparisons, indicating no proportional bias, and the 95% CI of the intercepts included 0 for all comparisons, indicating no fixed bias. These analyses demonstrated an excellent performance of all HPLC assays, and indicate that it is unlikely that there was interference by other licit and illicit drugs. By using ordinary least products linear regression analysis to compare the concentrations of methadone and EDDP found in urine samples using three chromatography systems with distinct selectivities, we have demonstrated our assay to be adequately selective.

A representative chromatogram from one subject's urine sample and analysed for the simultaneous determination of *R*- and *S*-methadone and *R*- and *S*-EDDP is shown in Fig. 2 with comparison chromatograms obtained from a blank urine sample, and a low calibration standard. Analysis of the ten subjects' urine samples revealed a marked difference in the concentrations of the enantiomers of methadone and EDDP. Concentrations ranged from 0.5 to 25.1 μM for *R*-EDDP, 0.8 to 36.3 μM for *S*-EDDP, 1.6 to 51.9 μM for *R*-methadone and 0.94 to 31.3 μM for *S*-methadone. Urinary *R*/*S* concentration ratios (mean$\pm$SD) for methadone and EDDP were 1.64 ± 0.19 and 0.69 ± 0.04, respectively, indicating that the metabolism of methadone to EDDP is likely to be stereoselective, in close agreement with Kristensen et al. [27] for both methadone and EDDP, and Lanz and Thormann [22] for methadone. In contrast, Lanz and Thormann [22] reported finding higher concentrations of *R*-EDDP compared to *S*-EDDP in the urine samples obtained from methadone maintenance subjects. These results demonstrate that important characteristics of drug metabolism would fail to be observed using non-chiral analytical techniques, highlighting the need for stereochemical considerations when drawing conclusions about the metabolism of compounds.

This assay for the simultaneous quantification of the enantiomers of methadone and EDDP compares

Table 1
Inter-assay accuracy and precision of the lowest calibration standard (LOQ), QC samples and linear regression analysis for *R*- and *S*-EDDP and *R*- and *S*-methadone ($n=4$ assays)[a]

	Nominal Concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 (SD)	Mean slope (SD)
R-EDDP				0.995 (0.004)	0.245 (0.066)
LOQ	0.125	97.6	5.2		
LQC	0.4	97.4	7.0		
MQC	1.0	104.4	8.8		
HQC	3.25	96.7	4.6		
S-EDDP				0.994 (0.004)	0.235 (0.065)
LOQ	0.125	102.6	4.6		
LQC	0.4	98.2	13.4		
MQC	1.0	103.3	8.5		
HQC	3.25	96.9	6.1		
R-Methadone				0.998 (0.002)	0.302 (0.068)
LOQ	0.125	103.6	0.6		
LQC	0.4	97.2	7.4		
MQC	1.0	98.4	8.1		
HQC	4.0	100.0	5.3		
S-Methadone				0.998 (0.002)	0.297 (0.067)
LOQ	0.125	102.3	2.3		
LQC	0.4	101.8	4.3		
MQC	1.0	99.1	6.5		
HQC	4.0	98.6	4.6		

[a] LOQ, limit of quantification; LQC, low quality control sample; MQC, medium quality control sample; HQC, high quality control sample.

favourably with the CE methods of Lanz and Thormann [22], Frost et al. [21], and that of Ramseier et al. [23] adaptation of the Lanz and Thormann method. Liquid–liquid [21,23] or solid-phase extractions [22] of urine samples at pH>9, with subsequent evaporation of the organic solvent to dryness, and reconstitution of the residue, were employed in the CE assays. Lanz and Thormann attempted direct injection of urine samples, but this was unsuccessful as less than half of the samples tested produced acceptable electropherograms [22]. Our assay employed a liquid–liquid extraction of alkalinized samples. In comparison to the present assay, the published CE methods [21–23] offer no advantages in terms of sample preparation.

The assay range employed by Frost et al. [21] for both methadone and EDDP (10–2500 ng/ml each enantiomer) from a 1-ml urine sample, was similar to our method (methadone: 39–3900 ng/ml each enantiomer; EDDP: 35–3500 ng/ml each enantiomer) whereas Lanz and Thormann [22] employed a much higher concentration range (methadone: 1.5–26.7 μg/ml each enantiomer; EDDP: 1.1–21.1 μg/ml each enantiomer). Limits of detection for each enantiomer were reported to be 10 ng/ml [21] and approximately 100 ng/ml [22] for both methadone and EDDP from a 1-ml urine sample. Ramseier et al. did not report a calibration range, and the limit of detection of the assay was ill defined [23]. The calibration curve concentration range employed in the present assay spanned the range of concentrations measured in the majority of patient samples, and is comparable to that reported in the CE methods. No samples contained concentrations of either methadone and/or EDDP below the limit of quantification.

Intra-day precision (RSD) values were reported to be <10% by Lanz et al. [22], and <6% (methadone) and <2% (EDDP) but only assessed at 1000 ng/ml by Ramseier et al. [23]. Frost et al. [21] reported intra-day RSD at 50, 500 and 2500 ng/ml urine for each enantiomer to be <12% at the higher con-

Table 2
Intra-assay accuracy and precision of QC samples for *R*- and *S*-EDDP and *R*- and *S*-methadone ($n=6$ replicate samples)[a]

	Nominal Concentration (μM)	Accuracy (%)	Precision (%)	Extraction efficiency % (SD)
R-EDDP				
LQC	0.4	104.5	6.8	71.3 (8.8)
MQC	1.0	101.9	9.0	72.6 (8.9)
HQC	3.25	95.0	11.0	72.4 (5.9)
S-EDDP				
LQC	0.4	105.1	4.6	73.3 (6.1)
MQC	1.0	102.3	8.9	73.9 (9.0)
HQC	3.25	96.5	10.3	72.8 (7.1)
R-Methadone				
LQC	0.4	97.8	1.7	85.1 (3.3)
MQC	1.0	100.6	3.6	93.0 (4.0)
HQC	4.0	94.8	2.6	89.1 (3.9)
S-Methadone				
LQC	0.4	97.5	2.0	88.2 (3.6)
MQC	1.0	100.7	2.2	95.6 (2.6)
HQC	4.0	94.0	2.1	90.2 (3.4)
Dextromoramide	(I.S.)			79.1 (2.4)

[a] LQC, low quality control sample; MQC, medium quality control sample; HQC, high quality control sample.

centrations, but increased up to 17% at 50 ng/ml. Mean concentrations were within 5% of the nominal concentration at the higher concentrations, but increased to 12–24% at 50 ng/ml. Accuracy data were not presented in the other two assay papers [22,23], while inter-day precision or accuracy data were not presented in any of the three papers [21–23]. In comparison, the present assay demonstrated excellent inaccuracy ($<5\%$) and good precision ($<13\%$ RSD) even at the limit of quantification. This was maintained for both intra- and inter-assay analysis. In comparison to the published CE methods, our assay offers better precision and accuracy over a similar concentration range.

Separation of the methadone and EDDP enantiomers was demonstrated to be adequate in all three CE assays [21–23]. Separation of the second EDDP peak and first methadone peak was less successful for Lanz et al. [22] and Ramseier et al. [23], although Lanz et al. were able to achieve baseline separation of all compounds by increasing capillary length to 100 cm with resultant long runtimes (>40 min) [22]. In contrast, Frost et al. [21] obtained excellent separation of the methadone and EDDP enantiomers while maintaining complete baseline separation of the second EDDP peak and first methadone peak. In comparison, our assay demonstrated adequate resolution of all compounds of interest but with runtimes of 35 min. Ramseier et al. [23] demonstrated excellent specificity of their assay for the methadone and EDDP enantiomers, as no interference was seen with drugs of abuse and their metabolites (13 compounds), as did Frost et al. [21] (18 compounds). Lanz and Thormann did not address this issue [22]. Although not as thoroughly investigated, the present assay demonstrated adequate specificity.

It is difficult to compare the cost of our HPLC method with that of the CE methods. Similar sample preparation is required for both types of methods and analysis times are similar. However, it is likely that the initial set up of our validated assay would be more readily achieved as many laboratories already have a conventional HLPC system and would only require a specialised chromatography column. In contrast, the ongoing costs of the CE methods are likely to be much less expensive compared to our chiral HPLC method, as the columns employed are

Table 3
Inter- and intra-assay accuracy and precision of QC samples for *R*- and *S*-methadone[a]

	Nominal Concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 (SD)	Mean Slope (SD)
Inter-assay ($n=5$)					
R-Methadone				0.996 (0.003)	0.0012 (0.0002)
LOQ	0.14	101.1	1.1		
LQC	0.32	97.0	8.4		
MQC	1.15	96.8	6.7		
HQC	3.2	95.0	7.6		
S-Methadone				0.998 (0.003)	0.0014 (0.0002)
LOQ	0.14	102.6	4.6		
LQC	0.32	99.8	7.6		
MQC	1.15	96.6	7.1		
HQC	3.2	96.2	8.0		
Intra-assay ($n=10$)				Extraction efficiency	
R-Methadone				% (SD)	
LQC	0.32	103.5	4.3	96.9 (5.5)	
MQC	1.15	96.3	4.3	104.0 (2.3)	
HQC	3.2	103.5	5.5	104.9 (1.9)	
S-Methadone					
LQC	0.32	102.0	5.8	90.7 (8.1)	
MQC	1.15	96.9	4.8	105.2 (2.9)	
HQC	3.2	96.1	4.8	107.6 (2.9)	
3-Methoxymorphinan	(I.S.)			109.0 (3.0)	

[a] LOQ, limit of quantification; LQC, low quality control sample; MQC, medium quality control sample; HQC, high quality control sample.

cheaper than chiral HPLC columns, more easily maintained, and consume much less running/rinsing solutions compared to HPLC mobile phase consumption.

During the preparation of this manuscript, Angelo et al. [27] reported a method for the simultaneous quantification of the enantiomers of methadone and EDDP in urine using stereoselective HPLC [27]. Rudaz and Veuthey [19] also reported their detailed investigations with methadone using chiral HPLC. While they [19] noted that EDDP did not interfere with methadone using the Chiral AGP column, they did not attempt to quantitate EDDP. Angelo et al.'s [27] method used a non-chiral C_8 (30×2 mm) analytical column coupled in series with the same chiral analytical column (Chiral AGP) used in the present assay. They reported that the use of the non-chiral C_8 column provided better selectivity than the non-chiral analytical cyano column (10×3 mm) previously reported by them [16]. In both of these assays, they also used a non-chiral precolumn, and did not use a Chiral AGP precolumn [16,27]. In our hands, a short cyano analytical column (50×4.6 mm) retained methadone and EDDP for a long time, resulting in unacceptable peak broadening using mobile phases containing low concentrations (<15%) of acetonitrile, even when the Chiral AGP column manufacturer's (Chromtech) highest mobile phase flow-rate (0.9 ml/min) was used. We found that using an appropriate mobile phase in combination with a low mobile phase flow-rate, it was possible to resolve the enantiomers of both methadone and EDDP simultaneously using a Chiral AGP column in conjunction with a Chiral AGP precolumn, without the need for a non-chiral analytical column, or a non-chiral precolumn, connected

Table 4
Inter- and intra-assay accuracy and precision of QC samples for *rac*-EDDP and *rac*-methadone[a]

	Nominal Concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 (SD)	Mean Slope (SD)
Inter-assay ($n=4$)					
rac-EDDP				0.996 (0.003)	0.245 (0.063)
LOQ	0.25	97.2	1.6		
LQC	0.8	96.7	7.9		
MQC	2.0	100.9	5.4		
HQC	6.5	95.2	4.0		
rac-Methadone				0.998 (0.002)	0.308 (0.077)
LOQ	0.25	99.9	0.8		
LQC	0.8	101.2	3.4		
MQC	2.0	99.0	7.4		
HQC	8.0	98.9	2.8		
Intra-assay ($n=6$)				Extraction efficiency	
rac-EDDP				% (SD)	
LQC	0.8	103.0	4.8	75.9 (6.0)	
MQC	2.0	105.3	8.3	81.3 (8.6)	
HQC	6.5	96.8	11.3	75.9 (11.5)	
rac-Methadone					
LQC	0.8	101.0	3.2	97.4 (3.2)	
MQC	2.0	105.9	3.0	98.6 (3.6)	
HQC	8.0	96.1	2.9	103.2 (2.4)	
Dextromoramide	(I.S.)			83.6 (2.1)	

[a] LOQ, limit of quantification; LQC, low quality control sample; MQC, medium quality control sample; HQC, high quality control sample.

in series with the Chiral AGP column. The use of a lower mobile phase flow-rate (0.4 ml/min) compared to that used by Angelo et al. [27] (0.9 ml/min), while still obtaining very similar runtimes, results in considerably less mobile phase usage. The use of a non-chiral analytical column was also reported to extend the working life of the Chiral AGP column [16]. We noticed no decrease in resolution of the Chiral AGP column after more than 200 injections. However, our assay utilised a Chiral AGP precolumn to protect the analytical column, which was not attempted by Kristensen et al. [16].

In summary, we present a simple, accurate and precise assay method to investigate the role of stereoselectivity in the pharmacokinetics and metabolism of methadone. This assay offers a more appropriate concentration range than previous conventional HPLC methods, lower mobile phase consumption and avoids the complication of using both a non-chiral analytical column and precolumn connected in series with the chiral analytical column without sacrificing accuracy, precision or robustness.

Acknowledgements

The results were presented in part at the XIIIth International Congress of Pharmacology in Munich, Germany (26–31 July 1998). DJR Foster was a recipient of a Dawes Scholarship from the Royal Adelaide Hospital and an NH&MRC Dora Lush (Biomedical) Postgraduate Scholarship. The authors wish to thank the National Institute of Drug Abuse for supplying the drug compounds, K. Dyer for his assistance in recruiting subjects and collection of samples, and Professor J. White and the staff at the

Warinilla Clinic of the South Australian Methadone Maintenance Programme for their invaluable assistance in this project. The study was supported by the National Health and Medical Research Council of Australia.

References

[1] M. Farrell, J. Ward, R. Mattick et al., Br. Med. J. 309 (1994) 997–1001.
[2] K. Kristensen, C.B. Christensen, L.L. Christrup, Life Sci. 56 (1995) 45–50.
[3] H. Isbell, A.J. Eisenman, Fed. Proc. 7 (1948) 162.
[4] C. Iribarne, F. Berthou, S. Baird et al., Chem. Res. Toxicol. 9 (1996) 365–373.
[5] C. Iribarne, Y. Dreano, L.G. Bardou, J.F. Menez, F. Berthou, Toxicology 117 (1997) 13–23.
[6] D.E. Moody, M.E. Alburges, R.J. Parker, J.M. Collins, J.M. Strong, Drug Metab. Dispos. 25 (1997) 1347–1353.
[7] D.J.R. Foster, A.A. Somogyi, F. Bochner, Br. J. Clin. Pharmacol. 47 (1999) 403–412.
[8] E. Änggård, L.-M. Gunne, J. Homstrand, R.E. McMahon, C.G. Sandberg, H.R. Sullivan, Clin. Pharmacol. Ther. 17 (1975) 258–266.
[9] H.R. Sullivan, S.L. Due, J. Med. Chem. 16 (1973) 909–913.
[10] M.J. Kreek, F.A. Bencsath, F.H. Field, Biomed. Mass Spectrom. 7 (1980) 385–395.
[11] M.J. Kreek, F.A. Bencsath, A. Fanizza, F.H. Field, Biomed. Mass Spectrom. 10 (1983) 544–549.
[12] K. Verebely, J. Volavka, S. Mulé, R. Resnick, Clin. Pharmacol. Ther. 18 (1975) 180–190.
[13] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobsson, J. Chromatogr. B 570 (1991) 198–202.
[14] R.L. Norris, P.J. Ravenscroft, S.M. Pond, J. Chromatogr. B 661 (1994) 346–350.
[15] N. Schmidt, K. Brune, K.M. Williams, G. Geisslinger, Chirality 6 (1994) 492–495.
[16] K. Kristensen, H.R. Angelo, T. Blemmer, J. Chromatogr. A 666 (1994) 283–287.
[17] C.B. Eap, T. Finkbeiner, M. Gastpar, N. Scherbaum, K. Powell, P. Baumann, Eur J. Clin. Pharmacol. 50 (1996) 385–389.
[18] S. Rudaz, J.L. Veuthey, J. Pharm. Biomed. Anal. 14 (1996) 1271–1279.
[19] S. Rudaz, J.L. Veuthey, Chirality 11 (1999) 319–325.
[20] S. Rudaz, D. Ortelli, M. Gex-Fabry, J.J. Déglon, L. Balant, J.L. Veuthey, Chirality 11 (1999) 487–494.
[21] M. Frost, H. Kohler, G. Blaschke, Electrophoresis 18 (1997) 1026–1034.
[22] M. Lanz, W. Thormann, Electrophoresis 17 (1996) 1945–1949.
[23] A. Ramseier, J. Caslavska, W. Thormann, Electrophoresis 20 (1999) 2726–2738.
[24] M. Lanz, J. Caslavska, W. Thormann, Electrophoresis 19 (1998) 1081–1090.
[25] P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele, P. Mangin, J. Forensic. Sci. 42 (1997) 291–295.
[26] C. Pham-Huy, N. Chikhichorfi, H. Galons et al., J. Chromatogr. B. 700 (1997) 155–163.
[27] H.R. Angelo, N. Beck, K. Kristensen, J. Chromatogr. B 724 (1999) 35–40.
[28] J. Ludbrook, Clin. Exp. Pharmacol. Physiol. 24 (1997) 193–203.
[29] R.A. Brace, Am. J. Physiol. 233 (1977) R94–99.
[30] R.C. Baselt, M.H. Bickel, Biochem. Pharmacol. 22 (1973) 3117–3120.