Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 809 (2004) 313–321
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Stereoselective determination of methadone and the primary metabolite EDDP in human plasma by automated on-line extraction and liquid chromatography mass spectrometry

Dale Whittington[a], Pam Sheffels[a], Evan D. Kharasch[a,b,*]

[a] *Department of Anesthesiology, University of Washington, Box 356540, 1959 NE Pacific St. RR-442, Seattle, WA, USA*
[b] *Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA*

Received 19 May 2004; received in revised form 12 June 2004; accepted 25 June 2004

Available online 22 July 2004

Abstract

A sensitive stereoselective bioanalytical liquid chromatographic assay with mass spectrometric detection (LC–MS) was developed and validated for the on-line extraction and quantification of *R*- and *S*-methadone and the primary metabolite *R*- and *S*-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) from human plasma. Deproteinized plasma was injected directly onto a small C8 column, washed and then back-flushed using a column switching valve and a second pump onto an α_1-acid glycoprotein analytical column, and enantioselective separation achieved using a mobile phase gradient of methanol and ammonium formate. Analytes were validated over a range of 0.1–25 ng/ml *R*- and *S*-EDDP and 0.1–100 ng/ml *R*- and *S*-methadone, respectively. Unweighted standard curves were linear over this concentration range (regression coefficients >0.999). Quality control samples were evaluated at 1, 5, 12.5 ng/ml *R*- and *S*-EDDP and 1, 10, 50 ng/ml *R*- and *S*-methadone. Intra- and inter-day accuracy was >95%, and intra- and inter-day coefficients of variation were less than 10% for all analytes and concentrations. This assay represents the only method currently available which combines on-line extraction and achieves chiral separation of both methadone and EDDP from plasma, and offers improvements in sensitivity over existing methods.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Methadone; EDDP; On-line extraction

1. Introduction

Methadone (6-dimethylamino-4,4-diphenyl-3-heptone hydrochloride) is a synthetic opioid agonist widely used in the prevention of opiate abstinence syndrome and as an analgesic in patients with moderate and severe pain [1–4]. Methadone is the cornerstone of opiate addiction therapy, and methadone maintenance is a vital public health strategy for HIV/AIDS risk reduction [5]. Methadone is chiral, possessing a single asymmetric carbon atom, and is administered clinically in many countries as a racemic mixture of *R*(−) and *S*(+) enantiomers. The main metabolic pathway for methadone inactivation is *N*-demethylation to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is also chiral (Fig. 1).

There are stereoselective differences in methadone pharmacodynamics and pharmacokinetics [6,7] *R*-methadone has a higher μ-opiod receptor affinity, and the analgesic potency is up to 50 times greater than that of the *S*-enantiomer [8,9]. It has also been reported that *R*-methadone has a longer plasma elimination half-life than *S*-methadone [10,11] and that the enantiomers bind differently to human plasma proteins [12]. Due to interindividual variability in the pharmacokinetics of methadone, and potential for drug interactions [13,14], dose adjustments are often required to prevent withdrawal symptoms and manage pain. For therapeutic monitoring, as well as for investigations regarding methadone pharmacokinetics and drug interactions, there is considerable interest in analytical methods for the quantitation of methadone and EDDP.

* Corresponding author. Tel.: +1 206 543 4070; fax: +1 206 685 3079.
E-mail address: kharasch@u.washington.edu (E.D. Kharasch).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.041

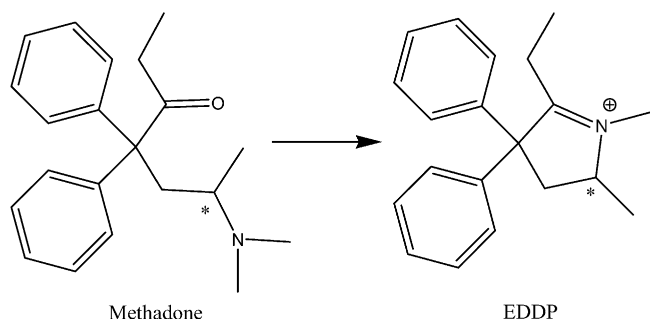

Fig. 1. The chemical structure of methadone and EDDP. The asterisk indicates the position of the asymmetric carbon in both compounds.

Several analytical procedures have been developed for the chiral analysis of methadone and/or EDDP. Capillary electrophoresis has been shown to obtain chiral separation of both methadone and EDDP in urine [15–17], however this method has been infrequently applied to plasma [16], perhaps due to the lack of sensitivity. Gas chromatography can achieve chiral methadone separation, however this was not applied to chiral EDDP separation, and an extensive derivatizing process was necessary for sample preparation [18]. Furthermore, the high temperature in gas chromatograph injectors causes decomposition of methadone to EDDP, creating an artifact, and confounds the quantification of both analytes [19]. Liquid chromatography, using detection originally with ultraviolet absorption [20–29] and subsequently with mass spectrometry (LC–MS) [30–34] has become the most common method to achieve stereoselective separation of methadone and EDDP. Stationary phases such as cellulose [25,33], cyclodextrin [22,24,25,28], and α_1-acid glycoprotein (AGP) [20,21,23,25–27,29–32,34], have been shown to successfully separate enantiomers of methadone, either alone [20–23,25,26,28,31–33] or simultaneously with EDDP [24,27,29,30,34], from serum or plasma [20–24,26,28,33], urine [24,27,29] saliva [32,34], hair [30], or sweat [31]. The use of these chiral stationary phases coupled with mass spectrometry provides the most sensitive and specific analytical methods. Nevertheless, of the methods published for simultaneous chiral separation and quantification of both methadone and EDDP, only one has been applied to the analysis of plasma [24], while the others have been applied to hair [30], urine [27,29], and saliva [34], and only two have used mass spectrometric detection [30,34]. The only method which has been used to analyze plasma used ultraviolet detection, and lacked sensitivity (10–20 ng/ml) [24]. Even the most sensitive LC–MS assay, used to quantify methadone and EDDP in saliva, had a limit of quantification of 5 ng/ml methadone [34]. Hence, there is presently no LC–MS assay for the simultaneous quantification of methadone and EDDP enantiomers in plasma.

Both liquid–liquid extraction [20–24,26–30] and more recently solid phase extraction (SPE) [26,35–40] have been typically used to isolate methadone and/or EDDP from various biological matrices prior to analysis. These sample preparation procedures are often costly and time consuming. The effectiveness of using on-line extraction for sample preparation, without protein precipitation prior to injection, has recently been demonstrated for methadone and EDDP analysis in plasma, however this method did not address the chiral separation of methadone or EDDP, and lacked sensitivity (limit of quantification 10–25 ng/ml) [41,42]. A protein precipitation step before on-line injection improved the limit of detection, however this method was achiral, analyzed saliva, and required tandem mass spectrometry [43]. Hence, there is presently no method for on-line extraction of both methadone and EDDP, amenable to sensitive, chiral analysis of plasma by LC–MS.

This paper presents the development and validation of a method for the simultaneous stereoselective determination of the enantiomers of methadone and EDDP in human plasma, using on-line extraction and high-pressure liquid chromatography mass spectrometry. The assay was sensitive (0.1 ng/ml for both enantiomers of methadone and EDDP) and robust.

2. Experimental

2.1. Materials

(±)-(6-Dimethyamino-4,4-diphenyl-heptan-3-one) hydrochloride (methadone) was purchased from Sigma (St. Louis, MO). (±)-6-Di(trideuteromethyl)amino-4,4-diphenyl-1-trideuteromethyl-3-heptanone (d9-methadone) was from Cerilliant (Austin, TX). (±)-2-Ethyl-1,5-dimethyl-3,3-diphenylpyrrolimium perchlorate (EDDP) and [ethyl-$2',2',2'-{}^2H_3$]-3,3-diphenyl-2-ethyl-5-methyl-1-pyrroline hydrochloride (d3-EDDP) were obtained from the National Institute of Drug Abuse. HPLC-grade methanol, zinc sulfate, glacial acetic acid, ammonium formate were from Fisher Scientific (Pittsburgh, PA). All stock drug solutions, buffers, and HPLC mobile phase were prepared using Milli-Q grade water (Millipore, Bedford, MA). Outdated human plasma was pooled from several donors.

2.2. Sample preparation

Plasma was deproteinated prior to LC–MS analysis. Subject plasma, calibration, or quality control samples (0.25 ml) were pipetted into a polypropylene 96-well (2.2 ml) plate. Internal standard mix (120 μl, consisting of 2.4 ng *RS*-d3-EDDP (1.2 ng of each enantiomer) and 12 ng *RS*-d9-methadone (6 ng of each enantiomer) in 0.4 M $ZnSO_4$, prepared daily from a concentrated stock) was added to each sample. Samples were vortexed for 5 min, the plate was placed at 4 °C for 10 min, then 480 μl of cold methanol (−18 °C) was added to each sample. The plate was vortexed again for 5 min and centrifuged at 12,000 rpm for 15 min to pellet proteins. The supernatant was removed and evaporated to dryness at 65 °C under nitrogen (TurboVap 96-well plate evaporator, Zymark, Hopkington MA). Samples were

reconstituted with 325 μl of 20 mM ammonium formate (adjusted to pH 5.7 with formic acid).

2.3. On-line extraction and chiral separation

The column switching system included an Agilent (Palo Alto, CA) 1100 series HPLC with two 1100 series binary solvent pumps (one for sample loading and washing, the other for chiral separation), a 96-well plate auto sampler with 500 μl injection loop, and a six-port switching valve. A Metaguard Polaris C8-A guard column (12.0 mm × 2.0 mm, 5 μm) (Varian, Torrance, CA) was used as an inline sample extraction column for methadone and EDDP. Chiral separation of methadone and EDDP was achieved using a Chiral-AGP analytical column (100 mm × 2.0 mm, 5 μm) with a Chiral-AGP (12 mm × 2.0 mm) guard column (ChromTech Ltd., Cheshire, UK).

Reconstituted deproteinated plasma (100 μl) was injected onto the Metaguard column and then washed with a mobile phase of 100% 20 mM ammonium formate (pH 5.7) at 5 ml/min. Simultaneously, the analytical column was conditioned with 20 mM ammonium formate (pH 5.7): methanol (9:1) at 0.22 ml/min. After 1 min, the valve was switched to back-flush the analytes from the Metaguard onto the analytical column with 20 mM ammonium formate (pH 5.7): methanol (9:1). After 4 min the valve was switched back to Position 1 and the methanol concentration in the analytical column eluent was increased to 25% over the next 1.5 min, maintained for 2.5 min, then further increased to 30% over 0.5 min and maintained for 6.5 min before decreasing back down to 10% to re-equilibrate the column for 5 min. Simultaneously, the Metaguard extraction column was washed by increasing the methanol concentration to 90% over the next 7 min at 0.5 ml/min and held at 90% for 2 min, then re-equilibrated to 100% 20 mM ammonium formate (pH 5.7) prior to the next injection. According to recommendations of the manufacturer, the analytical column was flushed with deionized water:isopropanol (75:25) after each sample set (typically 100 samples) to remove potential accumulation of interferences not removed by the on-line extraction process. Total run time per sample and equilibration was 21 min. Under these conditions, the retention times were 12.5 and 16.2 min for *R*- and *S*-EDDP, and 13.3 and 15.5 min for *R*- and *S*-methadone, respectively. Fig. 2 shows a typical chromatogram of a calibration control sample (1 ng/ml of each enantiomer of EDDP and methadone) and Fig. 3 shows a chromatogram of blank plasma containing internal standards. Enantiomeric resolution (R_s) was 2.0 and 3.8 for methadone and EDDP, respectively.

2.4. Mass spectrometry

The Agilent 1100 series mass spectrometer was operated in positive electrospray ionization mode. Parameters were: nitrogen drying gas at 10 L/min and 350 °C, nebulizer pressure 206.7 kPa, capillary voltage 3500 V, and fragmentor

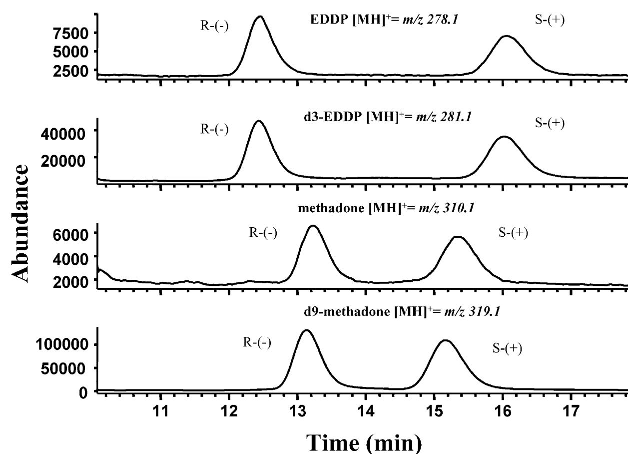

Fig. 2. Representative chromatogram of the low quality control sample (1 ng/ml of both enantiomers of EDDP and methadone) extracted using the on-line-extraction technique described in Section 2.3. Internal standard concentrations were 1.2 ng/sample of *R*- and *S*-d3 EDDP and 6 ng/sample of *R*- and *S*-d9 methadone.

70 V. All analytes were monitored in the same ion group: *m*/*z* 278.1 and 281.1 for EDDP and d3-EDDP, *m*/*z* 310.1 and *m*/*z* 319.1 for methadone and d9-methadone.

2.5. Calibration standards and quality control samples

Dilutions of stock solutions containing methadone, EDDP, and their corresponding deuterated internal standards were prepared from racemic mixtures in water and stored at −20 °C. Calibration curves were obtained by analyzing drug-free plasma to which was added *R*- and *S*-EDDP at 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 12.5, 25 ng/ml of each enantiomer, and *R*- and *S*-methadone at 0.5, 1, 2.5, 10, 25, 50, 100 ng/ml of each enantiomer. Quality control (QC) samples in plasma (1, 5, 12.5 ng/ml each *R*- and *S*-EDDP; 1, 10, 50 ng/ml each

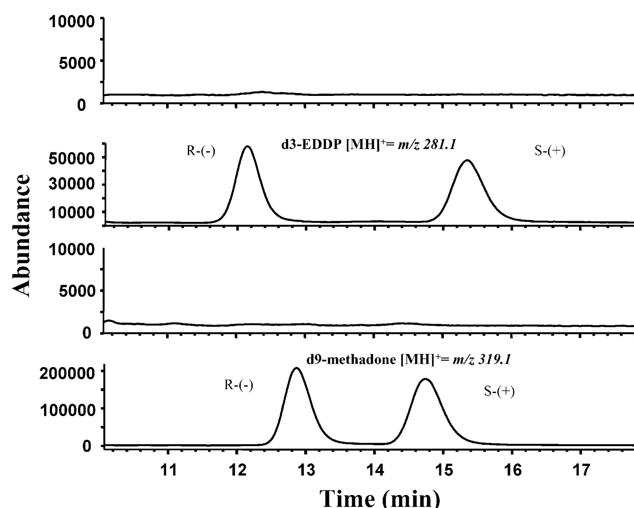

Fig. 3. Representative chromatogram of blank plasma with internal standards extracted using the technique described in Section 2.3.

R- and *S*-methadone) were prepared from separate dilutions of stocks than those used for the calibration curves. Calibration and QC samples were aliquotted and stored at −20 °C until extracted. Calibration and QC samples were analyzed daily with the analytical samples. Standard curves were constructed using linear regression. The acceptance standard for the calibration curves was a regression coefficient (r^2) >0.95 and back-calculated values of calibrations standards that deviated ≤15% from nominal and less than 20% at the limit of quantification.

2.6. Method validation

Accuracy and precision (coefficient of variation, %CV) were evaluated at three concentrations using QC samples for each analyte. The assay was considered acceptable if the variation and deviation were <20% at the low QC (including diluted samples) and <15% for medium and high QC samples for intra- and inter-day runs.

Recovery from the protein precipitation step was calculated by comparing the peak area of the analyte, added to and precipitated from plasma, compared to the same concentration in water. For this analysis, on-line extraction was not used. Recovery for the on-line extraction step was also determined by comparing peak areas of the analytes in water, extracted using the column switching procedure described above, to the same sample injected directly onto the chiral column. The assay was considered acceptable if recovery allowed sub-nanogram levels to be detected.

Specificity testing evaluated potential interference from other sample components. Since this method was designed for clinical studies to assess potential pharmacokinetic interactions between HIV protease inhibitors and methadone, several protease inhibitors were added to QC plasma samples to check for potential interference. Two sample sets of plasma were prepared at medium (5 ng/ml EDDP and 10 ng/ml methadone, each *R*- and *S*-) and high (12.5 ng/ml EDDP and 50 ng/ml methadone, each *R*- and *S*-) concentrations, to which was added 8 μg/ml indinavir, 5 μg/ml nelfinavir, and 10 μg/ml ritonavir. These antiretroviral drug concentrations were based on the maximum plasma concentration anticipated from previous studies [44]. Also tested was the possibility of interference from hemolyzed red blood cells in the plasma samples tested. Red blood cells (25 μl) were added to the sample prior to each extraction.

The limit of detection (LOD) was defined as a signal to noise ratio of 2:1. The limit of quantification (LOQ) was the lowest concentration on the standard curve with an acceptable level of variation (<20%) and a signal to noise ratio >10:1.

Stability of EDDP and methadone was assessed in several ways. Un-extracted QC plasma samples were subjected to three freeze/thaw cycles. Frozen QC samples were left at room temperature for 3 h then refrozen, and this process repeated on two consecutive days. The accuracy of this sample set was determined by comparison of untreated QC samples extracted and run in the same session. Reconstituted extracted samples were subjected to two conditions: 48 h at 4 °C and 48 h at room temperature. Robustness (capacity of the assay to remain unaffected by small deliberate changes) was determined by comparing results obtained from QC samples extracted with different lots of Varian Metaguard and ChromTech chiral AGP columns.

2.7. Method application

The method was applied to samples obtained from a clinical investigation of methadone disposition, which was approved by the University of Washington Institutional Review Board and performed after obtaining written informed consent of the research subject. The subject received 6 mg IV methadone HCl and 11.2 mg oral methadone HCl, and venous blood samples were obtained for 96 h. Plasma was stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Protein precipitation and extraction procedure

Methods used for treating biological samples prior to their introduction into a high-performance liquid chromatography system generally fall into three categories—direct injection, extraction and more recently, on-line sample extraction. For extraction methods, the analytes of interest are removed from the matrix, in this case plasma, using proper sorbent, solvents, and pH conditions, while leaving behind unwanted matrix components. The direct injection technique is the simplest and most rapid method for sample preparation. However, injections without some form of minimal clean up result in rapid increase in back pressure and deterioration of column performance. To eliminate this potential problem protein precipitation is commonly used for fast sample clean-up and disruption of protein–drug binding [45,46]. With the application of 96-well extraction plates, the number of samples prepared per unit time is greatly increased over liquid–liquid extraction methods. However, these solid phase extraction plates are expensive and greatly reduce the cost efficiency when large sample numbers are to be analyzed. If used effectively, protein precipitation can provide sufficient clean up at the fraction of the cost when compared to SPE devices currently on the market.

Multiple methods for protein precipitation were evaluated. Various organic solvents (methanol, acentonitrile, and isopropanol), and acids (trichloroacetic, phosphoric, and perchloric), were evaluated to determine the most effective protein precipitation method for plasma samples (evaluating both analytes recovery and removal of interferents). Samples precipitated with 10% (w/v) trichloroacetic acid showed decreases in the ion abundance of methadone and EDDP when the same sample was repeatedly injected over a 24 h period, suggesting continuous analyte degradation in samples not

immediately injected. The supernatant of samples treated with phosphoric acid was cloudy and a poor pellet was formed after centrifugation. Although 6% (w/v) perchloric acid was effective for precipitation of proteins and yielded a clear supernatant, insoluble perchlorate salts would continuously precipitate, even after centrifugation. This would be problematic if salts continued to precipitate and were introduced to the on-line extraction column or analytical column. Acetonitrile was evaluated for protein precipitation [33,43], however recovery was less than with zinc sulfate. In addition, any residual acetonitrile in the evaporated samples after precipitation resulted in incomplete analyte retention on the C8 inline extraction column, and hence decreased recovery. The best results were obtained using sequential addition of 0.4 M aqueous zinc sulfate and methanol, which provided a clear supernatant, a condensed pellet of protein, and minimal loss of methadone and EDDP. Step-wise addition of zinc sulfate, vortexing, cooling for 15 min, addition of cold methanol, vortexing, and refrigeration prior to centrifugation, provided the best results. The stepwise addition of zinc sulfate then methanol, with cooling, increased the recovery of both methadone and EDDP by over 40% compared to addition of premixed zinc sulfate and methanol without cooling.

A major challenge for this assay was to identify an on-line extraction column that was compatible with the mobile phase used for the chiral AGP column. Preliminary tests first established the mobile phase for the chiral AGP column (10% initial methanol). The on-line extraction column needed to retain both methadone and EDDP under nearly aqueous conditions, yet elute both analytes with a low enough percentage of organic solvent to permit enantiomeric separation on the chiral AGP column. Based on Christians et al. [47] an on-line extraction procedure was established and multiple extraction columns, typical HPLC guard columns or cartridges, were tested. Guard columns for evaluation were chosen based on stability in 100% aqueous, packing material, particle size, carbon loading, and technical recommendations. The majority of guard columns tested for on-line extraction demonstrated analyte binding affinity great enough that the percentage of organic solvent needed to elute methadone and EDDP from the packing material was too high to permit resolution on the subsequent analytical column. Zorbax Bonus-RP and Zorbax 300SB-C8 guard columns (both 2.1 mm × 12.5 mm, 5 um) (Agilent) needed greater than 30% methanol to elute methadone and EDDP. Strata-X (2.0 mm × 20 mm) (Phenomenex) poorly retained methadone and EDDP during loading, which greatly diminished recovery. Initial results using Zorbax XDB C8 (2.1 mm × 12.5 mm, 5 μm) (Agilent) or C18 (4.0 mm × 2.0 mm, 10 μm) (Poulter Scientific) were promising, but the peak areas of the analytes decreased more than 40% over extended injections (<60). Metaguard Polaris C8-A (2.0 mm × 12.5 mm, 5 μm) (Varian) retained both methadone and EDDP under 100% aqueous conditions, permitted the elution of both analytes using 10% methanol, and showed stable packing material. Recovery from the Metaguard column was >60 % for methadone enantiomers and >87% for EDDP enantiomers (Table 1).

3.2. Chromatography

Multiple columns were tested for simultaneous chiral separation of both methadone and EDDP, with an initial preference for a non-protein stationary phase. Eight columns were tested (Whelk-01, ULMO, DACH-DNB, Pirkle 1-J, β-GEM, α-Burke 2, Phenylglycine, Luecine) by a vendor (Regis Technologies, Inc, Morton Grove, IL). Only the α-Burke 2 was reported to separate methadone enantiomers and none were said to resolve EDDP enantiomers, and further attempts to optimize separation using a non-protein stationary phase were not pursued. Chiral methadone separation with an AGP column was known [21,25,32], so the ability to separate both methadone and EDDP enantiomers was evaluated using this stationary phase. Multiple buffered aqueous mobile phases were tested and a mobile phase was identified which could separate both enantiomers of methadone as well as both enantiomers of methadone EDDP (aqueous ammonium formate:methanol gradient). Mobile phase pH was an important factor for resolution of both analytes, but particularly for methadone. For example, a shift from pH 5.2 to 5.7 increased the resolution of methadone enantiomers from a 25% valley to full baseline separation.

3.3. Validation

Less than 30–40% of methadone was lost due to protein precipitation at low (1 ng/ml) concentrations, and less than 20% at higher concentrations. Less than 20% of EDDP was lost due to protein precipitation at all concentrations. Recovery from the on-line extraction process was excellent, exceeding 93% at low analyte concentrations, and essentially complete at higher concentrations (Table 1).

Precision and accuracy for intra- and inter-day quality control samples are summarized in Table 2. The coefficient of variation (CV) for both inter and intra-day determinations was <6% at the medium and high quality control concentrations and <9% at the low quality control concentrations, for both methadone and EDDP enantiomers. Accuracy was >95% for all analytes at all concentrations. Dilutions of high quality control samples were within 16% of expected concentrations with a CV <19% for both analytes and enantiomers (Table 3). Inter-day comparisons of calibration standards are provided in Table 4. The assay was linear up the highest concentration (25 ng/ml for *R*- and *S*-EDDP and 100 ng/ml for *R*- and *S*-methadone). The inter-day CV was ≤18% for all analytes. The accuracy for all calibration standards was within 7 and 14% of the expected values for both *R*- and *S*-methadone and 6 and 14% for both *R*- and *S*-EDDP. Linearity of all calibration curves was excellent ($r^2 > 0.999$), without weighting.

Table 1
Analyte recovery

	Protein precipitation recovery[a]			On-line extraction recovery[b]		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
(*R*)-Methadone (ng/ml)	1	10	50	1	10	50
Recovery (%)	60 ± 7	81 ± 5	89 ± 14	93 ± 11	102 ± 7	100 ± 4
(*S*)-Methadone (ng/ml)	1	10	50	1	10	50
Recovery (%)	71 ± 8	83 ± 4	92 ± 12	94 ± 14	101 ± 7	102 ± 5
(*R*)-EDDP (ng/ml)	1	5	12.5	1	5	12.5
Recovery (%)	95 ± 12	80 ± 7	89 ± 11	94 ± 7	106 ± 6	105 ± 3
(*S*)-EDDP (ng/ml)	1	5	12.5	1	5	12.5
Recovery (%)	87 ± 6	81 ± 6	90 ± 8	97 ± 1	104 ± 7	98 ± 4

All results are the mean ± S.D. ($n = 5$).

[a] Protein precipitation recovery was determined by comparing the peak area of the analyte, added to and precipitated from plasma, compared to the same concentration in water. For this analysis, on-line extraction was not used.

[b] On-line extraction recovery was determined by comparing peak areas of the analytes in water, extracted using the column switching procedure, to the same sample injected directly onto the chiral column.

Table 2
Accuracy and precision of quality control samples

	Intra-day ($n = 5$)			Inter-day ($n = 6$)		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
(*R*)-Methadone (ng/ml)	1	10	50	1	10	50
Mean ± S.D.	1.01 ± 0.09	10.3 ± 0.51	49.4 ± 1.5	0.97 ± 0.04	10.1 ± 0.42	49.8 ± 0.9
CV (%)	9	5	3	4	4	2
Accuracy (%)	101	103	99	97	102	100
(*S*)-Methadone (ng/ml)	1	10	50	1	10	50
Mean ± S.D.	0.99 ± 0.12	9.95 ± 0.51	50.8 ± 3.3	0.96 ± 0.03	9.96 ± 0.32	50.2 ± 1.2
CV (%)	8	5	6	3	3	2
Accuracy (%)	99	100	102	103	100	100
(*R*)-EDDP (ng/ml)	1	5	12.5	1	5	12.5
Mean ± S.D.	0.97 ± 0.03	5.07 ± 0.29	12.9 ± 0.28	0.99 ± 0.03	5.05 ± 0.09	12.6 ± 0.2
CV (%)	3	6	2	3	2	2
Accuracy (%)	97	101	103	99	101	101
(*S*)-EDDP (ng/ml)	1	5	12.5	1	5	12.5
Mean ± S.D.	0.95 ± 0.03	5.11 ± 0.25	12.9 ± 0.23	0.96 ± 0.03	4.95 ± 0.16	12.8 ± 0.5
CV (%)	3	5	2	4	3	4
Accuracy (%)	95	102	103	96	99	102

Table 3
Dilution (10-fold) evaluations

	Intra-day ($n = 5$) High QC	Inter-day ($n = 5$) High QC
(*R*)-Methadone (ng/ml)	5	5
Mean ± S.D.	5.34 ± 0.17	5.58 ± 1.1
CV (%)	3	19
Accuracy (%)	107	112
(*S*)-Methadone (ng/ml)	5	5
Mean ± S.D.	5.24 ± 0.31	5.51 ± 0.94
CV (%)	6	17
Accuracy (%)	105	110
(*R*)-EDDP (ng/ml)	1.25	1.25
Mean ± S.D.	1.45 ± 0.03	1.34 ± 0.09
CV (%)	2	7
Accuracy (%)	116	107
(*S*)-EDDP (ng/ml)	1.25	1.25
Mean ± S.D.	1.42 ± 0.03	1.33 ± 0.08
CV (%)	2	6
Accuracy (%)	114	106

The limit of quantification was 0.1 ng/ml for *R*- and *S*-EDDP and *R*- and *S*-methadone. Both EDDP and methadone were detectable at concentrations less than 0.1 ng/ml, however the accuracy was above the allowable 20% criterion. Stability was assessed by comparing newly extracted calibration curves and quality control samples with those that were extracted, reconstituted in mobile phase, and stored at room temperature for 48 h. There were no significant differences in the mean values between the sample sets. Quality control plasma samples ($n = 5$) underwent three freeze/thaw cycles and were then extracted and analyzed, with no effects on compound stability (Table 5). No interference with methadone or EDDP by added ritonavir, nelfinavir, indinavir, or hemolyzed red blood cells were observed (data not shown). The assay was also performed using different lots of chiral AGP columns and Metaguard columns and no difference in retention times and recovery were observed.

Table 4
Accuracy, precision (CV), and linearity of inter-day standards (n = 5)

	Analyte concentration (ng/ml)									Slope	r^2	Intercept
(*R*)-Methadone	0.10	0.25	0.50	1.00	2.50	10.0	25.0	50.0	100			
Mean ± S.D.	0.10 ± 0.01	0.24 ± 0.04	0.46 ± 0.04	1.07 ± 0.18	2.49 ± 0.17	10.2 ± 0.3	24.8 ± 1.0	49.9 ± 0.8	100 ± 0.6	0.0529 ± 0.00	0.9996 ± 0.00	0.020 ± 0.01
CV (%)	7	14	9	17	7	3	4	2	1	3	0.0	
Accuracy (%)	102	97	93	107	100	102	99	100	100			
(*S*)-Methadone	0.10	0.25	0.50	1.00	2.50	10.0	25.0	50.0	100			
Mean ± S.D.	0.11 ± 0.01	0.24 ± 0.03	0.45 ± 0.03	1.11 ± 0.07	2.46 ± 0.09	10.1 ± 0.2	25.1 ± 0.5	50.8 ± 1.3	99.5 ± 1.0	0.0500 ± 0.00	0.9993 ± 0.00	0.038 ± 0.02
CV (%)	6	13	3	6	4	2	2	3	1	8%	0.0%	
Accuracy (%)	114	96	90	111	98	101	100	102	100			
(*R*)-EDDP	0.10	0.25	0.50	1.00	2.50	5.00	7.50	12.5	25.0			
Mean ± S.D.	0.11 ± 0.02	0.24 ± 0.03	0.53 ± 0.03	0.99 ± 0.04	2.34 ± 0.04	5.15 ± 0.14	7.60 ± 0.21	12.6 ± 0.4	24.9 ± 0.2	0.2567 ± 0.01	0.9993 ± 0.00	0.018 ± 0.02
CV (%)	17	14	6	4	2	3	3	3	1	5%	0.1%	
Accuracy (%)	105	97	105	99	94	103	101	101	100			
(*S*)-EDDP	0.10	0.25	0.50	1.00	2.50	5.00	7.50	12.5	25.0			
Mean ± S.D.	0.11 ± 0.02	0.25 ± 0.03	0.52 ± 0.03	0.98 ± 0.02	2.39 ± 0.03	5.14 ± 0.15	7.56 ± 0.15	12.4 ± 0.2	25.0 ± 0.1	0.2661 ± 0.01	0.9997 ± 0.00	0.013 ± 0.01
CV (%)	18	11	5	2	1	3	2	1	0.4	4%	0.02%	
Accuracy (%)	114	101	103	97	96	103	101	100	100			

Fig. 4 presents results from a subject who was dosed with 6 mg of IV methadone and 11 mg oral methadone. Plasma samples were analyzed with the method validated in this paper.

3.4. Additional considerations

This assay was designed for pharmacokinetic studies in which subjects were taking no other opiates or opioids. It was not designed for therapeutic monitoring. Interference from other opiates was therefore not evaluated.

An LC–MS method for the analysis of methadone and EDDP enantiomers was recently published by Rosas et al. [34], although several important differences merit address. The purpose of the assays and their limits of quantification are very different. The method of Rosas et al. was designed for therapeutic drug monitoring in patients and high analyte concentrations, while the present method was designed for pharmacokinetic studies and high sensitivity. The present limit of quantification was substantially lower (0.1 ng/ml for methadone and EDDP enantiomers) compared with 5 ng/ml methadone and 0.5 ng/ml EDDP enantiomers [34]. There were differences in the sample preparation processes in the two assays, due the matrix difference (saliva versus plasma). Saliva analysis used direct injection into the HPLC. Saliva is a much cleaner matrix. For example, saliva is 98% water and typically contains <100 mg/dL protein, while plasma contains 5500–8000 mg/dL protein. Substantial development effort in sample prep was needed because of the more complex plasma matrix, resulting in the on-line extraction method. There were also differences in the analytical HPLC column used, and hence the separation methodology. Rosas et al. [34] used a 4 mm AGP column from Advanced Separation Technologies (Whippany, NJ) while we used a 2 mm AGP column from ChromTech Ltd. (Cheshire UK). Although both were chiral AGP columns, they had markedly different retention properties. Rosas et al. [34] used a mobile phase of 18% acetonitrile in 10 mM ammonium acetate (pH 7.0). We used a gradient of 10–25% methanol and 20 mM ammonium formate (pH 5.7). Acetonitrile concentrations exceeding 5% could not achieve chiral separation on the ChromTech AGP column. In addition, Rosas et al. [34] found little or no separation (at pH 7.0) for EDDP (α = 1) and methadone ($\alpha \sim 1.2$) when using 1-propanol, 2-propanol, or methanol. In contrast, we found optimal separation with methanol (gradient to 25%). Effects of pH were also different. At low pH (5–6), Rosas et al. [34] did not achieve baseline separation for EDDP, resolution for both methadone and EDDP increased with increasing pH, and pH 7.0 was ultimately used. Conversely, with our AGP column, methadone enantiomer separation decreased as pH increased, and pH 5.7 provided the best separation (methadone R_s = 2.0, EDDP R_s = 3.8). Use of on-line extraction necessitated certain solvent conditions. For example, acetonitrile concentrations exceeding 5% could not achieve chiral separation on the ChromTech AGP column, while those less than 5% were

Table 5
Stability evaluation

	Freeze/thaw ($n = 5$)		48 h at 4 °C ($n = 5$)		
	Medium QC	High QC	Low QC	Medium QC	High QC
(*R*)-Methadone (ng/ml)	10	50	1	10	50
Mean ± S.D.	10.0 ± 0.4	50.8 ± 1.4	0.95 ± 0.33	10.3 ± 0.3	50.9 ± 1.8
CV (%)	4	3	35	3	4
Accuracy (%)	100	102	95	103	102
(*S*)-Methadone (ng/ml)	10	50	1	10	50
Mean ± S.D.	9.82 ± 0.14	52.0 ± 1.9	0.97 ± 0.15	10.2 ± 0.3	49.9 ± 1.4
CV (%)	2	4	16	3	3
Accuracy (%)	98	104	97	102	100
(*R*)-EDDP (ng/ml)	5	12.5	1	5	12.5
Mean ± S.D.	5.09 ± 0.19	13.3 ± 0.2	0.91 ± 0.07	4.90 ± 0.44	12.4 ± 0.8
CV (%)	4	2	8	9	6
Accuracy (%)	102	107	91	98	99
(*S*)-EDDP (ng/ml)	5	12.5	1	5	12.5
Mean ± S.D.	5.00 ± 0.18	12.8 ± 0.3	0.90 ± 0.03	4.94 ± 0.27	12.8 ± 0.5
CV (%)	4	2	3	6	4
Accuracy (%)	100	103	90	99	102

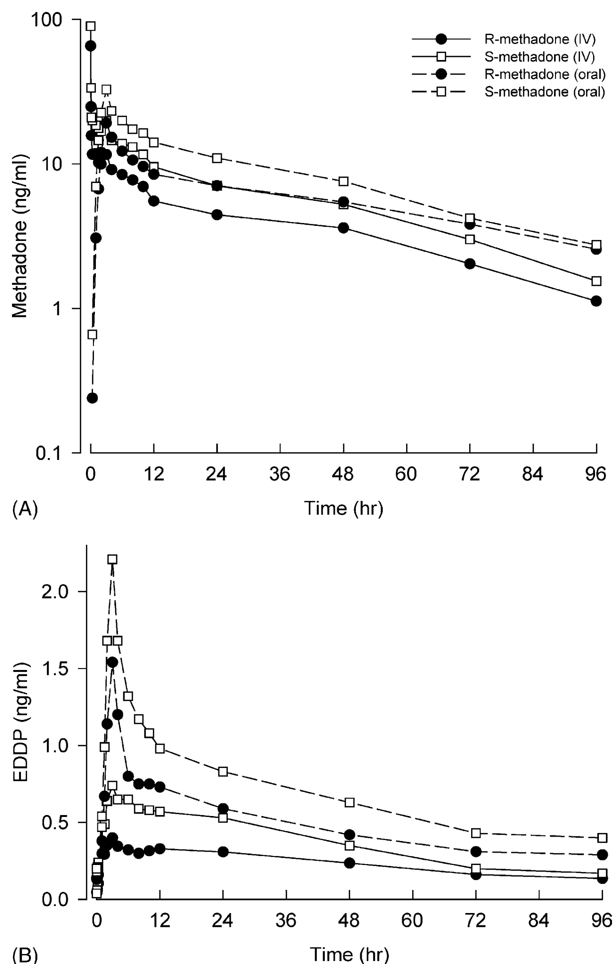

Fig. 4. Plasma methadone (A) and EDDP (B) enantiomer concentrations, analyzed with the on-line extraction assay, from a research subject who received 6 mg intravenous methadone (solid lines) and 11 mg oral methadone (dotted lines). *R*-enantiomers are shown with circles, *S*-enantiomers are shown with squares.

insufficient to elute the analytes from the on-line extraction column.

4. Conclusion

On-line extraction greatly reduced sample preparation time and the cost for solid phase extraction cartridges compared with our previous LC–MS assay for methadone and EDDP [39,40]. The present assay appears to be the only one to date that utilizes on-line extraction with column switching to isolate both methadone and EDDP enantiomers from plasma, using LC–MS detection. With the use of the chiral AGP column, baseline separation of both methadone and EDDP was achieved from a single injection. This appears to be the first LC–MS assay for the simultaneous quantification of methadone and EDDP enantiomers in plasma. Detection limits for EDDP are lower than other published methods (0.5 ng/ml [34]) and the assay is two to five times more sensitive than other LC–MS methods for methadone [21,34]. Total LC–MS run time for 100 samples is approximately 36 h.

In summary, a semi-automated LC–MS method for a single quadrupole mass spectrometry for the enantomeric separation and quantification in human plasma was designed and validated. The assay is sensitive, precise, accurate, and robust. The assay is well suited for chiral pharmacokinetic studies of methadone and its primary metabolite, EDDP.

Acknowledgements

We appreciate the helpful suggestions of Dr. Uwe Christians regarding the development and implementation of on-line extraction methods. Supported by NIH grants R01-DA14211 and K24-DA00417.

References

[1] M.J. Kreek, F.J. Vocci, J. Subst. Abuse Treat. 23 (2002) 93.
[2] R.P. Mattick, C. Breen, J. Kimber, M. Davoli, Cochrane Database Syst. Rev. 2 (2003) CD002209.
[3] C. Ripamonti, M. Bianchi, Hematol. Oncol. Clin. North Am. 16 (2002) 543.
[4] B. Gagnon, A. Almahrezi, G. Schreier, Pain Res. Manage. 8 (2003) 149.
[5] J.L. Sorensen, A.L. Copeland, Drug Alcohol. Depend. 59 (2000) 17.
[6] M.J. Garrido, I.F. Troconiz, J. Pharmacol. Toxicol. 42 (1999) 61.
[7] C.B. Eap, T. Buclin, P. Baumann, Clin. Pharmacokinet. 41 (2002) 1153.
[8] J.G. Gerber, R.J. Rhodes, Clin. Pharmacol. Ther. 69 (2001) 11.
[9] K. Kristensen, T. Blemmer, H.R. Angelo, L.L. Christrup, N.E. Drenck, S.N. Rasmussen, P. Sjogren, Ther. Drug Monit. 18 (1996) 221.
[10] M.J. Kreek, D.L. Hachey, P.D. Klein, Life Sci. 24 (1979) 925.
[11] G.D. Olsen, H.A. Wendel, J.D. Livermore, R.M. Leger, R.K. Lynn, N. Gerber, Clin. Pharmacol. Ther. 21 (1976) 147.
[12] C.B. Eap, C. Cuendet, P. Baumann, Clin. Pharmacol. Ther. 47 (1990) 338.
[13] K. Verebely, J. Volavka, S. Mulé, R. Resnick, Clin. Pharm. Ther. 18 (1975) 180.
[14] D.W. Boulton, P. Arnaud, C.L. Devane, Br. J. Clin. Pharmacol. 51 (2001) 350.
[15] M. Lanz, W. Thormann, Electrophoresis 17 (1996) 1945.
[16] M. Frost, H. Köhler, G. Blaschke, Electrophoresis 18 (1997) 1026.
[17] A. Ramseier, J. Caslavska, W. Thormann, Electrophoresis 20 (1999) 2726.
[18] K. Kristensen, H.R. Angelo, Chirality 4 (1992) 263.
[19] F.R. Galloway, N.F. Bellet, J. Anal. Toxicol. 23 (1999) 615.
[20] O. Beck, L.O. Boreus, P. Lafolie, G. Jacobson, J. Chromatogr. Biomed. Appl. 570 (1991) 198.
[21] N. Schmidt, K. Brune, G. Geisslinger, J. Chromatogr. 583 (1992) 195.
[22] R.L.G. Norris, P.J. Ravenscroft, S.M. Pond, J. Chromotogr. B 661 (1994) 346.
[23] K. Kristensen, H.R. Angelo, T. Blemmer, J. Chromatogr. A 666 (1994) 283.
[24] 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.
[25] S. Rudaz, J.L. Veuthey, Chirality 11 (1999) 319.
[26] S. Rudaz, D. Ortelli, M. Gex-Fabry, J.J. Deglon, L. Balant, J.L. Veuthey, Chirality 11 (1999) 487.
[27] H.R. Angelo, N. Beck, K. Kristensen, J. Chromatogr. B 724 (1999) 35.
[28] D.W. Boulton, C.L. Devane, Chirality 12 (2000) 681.
[29] D.J. Foster, A.A. Somogyi, F. Bochner, J. Chromatogr. B 744 (2000) 165.
[30] P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele, P. Mangin, J. Forensic Sci. 42 (1997) 291.
[31] P. Kintz, A. Tracqui, C. Marzullo, A. Darreye, F. Tremeau, P. Greth, B. Ludes, Ther. Drug Monit. 20 (1998) 35.
[32] D. Ortelli, S. Rudaz, A.F. Chevalley, A. Mino, J.J. Deglon, L. Balant, J.L. Veuthey, J. Chromatogr. A 871 (2000) 163.
[33] S. Souverain, C. Eap, J.L. Veuthey, S. Rudaz, Clin. Chem. Lab. Med. 41 (2003) 1615.
[34] M.E.R. Rosas, K.L. Preston, D.H. Epstein, E.T. Moolchan, I.W. Wainer, J. Chromatogr. B 796 (2003) 355.
[35] T.L. Pierce, A.G. Murray, W. Hope, J. Chromatogr. Sci. 30 (1992) 443.
[36] S. Rudaz, W. Haerdi, J.-L. Veuthey, Chromatographia 44 (1997) 283.
[37] G.A.A. Cooper, J.S. Oliver, J. Anal. Toxicol. 22 (1998) 389.
[38] Y.-F. Cheng, U.D. Neue, L.L. Woods, J. Chromatogr. B 729 (1999) 19.
[39] O. Dale, C. Hoffer, P. Sheffels, E.D. Kharasch, Clin. Pharmacol. Ther. 72 (2002) 536.
[40] E.D. Kharasch, C. Hoffer, D. Whittington, Br. J. Clin. Pharmacol. 57 (2004) 600.
[41] S. Souverain, S. Rudaz, D. Ortelli, E. Varesio, J.L. Veuthey, J. Chromatogr. B 784 (2003) 117.
[42] R. Dams, C.M. Murphy, W.E. Lambert, M.A. Huestis, Rapid Commun. Mass. Spectrom. 17 (2003) 1665.
[43] R. Dams, C.M. Murphy, R.E. Choo, W.E. Lambert, A.P. De Leenheer, M.A. Huestis, Anal. Chem. 75 (2003) 798.
[44] P. Newton, A. Simpson, S. Wanwimolruk, P. Maliakal, L. Villegas, D. Kuypers, N.J. White, Eur. J. Clin. Pharmacol. 57 (2001) 111.
[45] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, J. Chromatogr. B 785 (2003) 263.
[46] J. Blanchard, J. Chromatogr. 226 (1981) 455.
[47] U. Christians, W. Jacobsen, N. Serkova, L.Z. Benet, C. Vidal, K.F. Sewing, M.P. Manns, G.I. Kirchner, J. Chromatogr. B 748 (2000) 41.