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Development and Validation of an Enantioselective HPLC–UV Method for Stereospecific Therapeutic Drug Monitoring of Methadone Enantiomers in Iranian MMT Patients: A Pilot Study

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Abstract

Methadone used in maintenance therapy is a racemic mixture of R- and S-enantiomers with distinct pharmacological and safety profiles. R-methadone mediates therapeutic effects via μ-opioid receptor activation, whereas S-methadone inhibits hERG potassium channels and may contribute to QT prolongation. Iran hosts the largest national methadone maintenance therapy (MMT) population (>800,000 patients), yet enantioselective therapeutic drug monitoring (TDM) is not available. To develop, validate, and clinically apply a simple and cost-effective HPLC–UV method for enantioselective quantification of methadone in plasma of Iranian MMT patients. Plasma samples from 20 clinically stable MMT patients (60-120 mg/day) were analyzed. Enantiomers were separated on a Chiral-AGP column (100 × 4 mm, 5 μm) using isocratic acetonitrile:0.01 M phosphate buffer (15:85, pH 6.8) at 1.2 mL/min with UV detection at 210 nm. Validation followed ICH Q2(R1) guidelines. Liquid–liquid extraction with n-hexane and imipramine as internal standard was applied. Calibration was linear (12.5-1000 ng/mL; $r^2 \geq 0.999$). LOQ was 10 ng/mL. Intra- and inter-day precision were <3%, accuracy within ±3%, recovery 91–94%, and matrix effects 95–102% (CV <4.5%). Trough concentrations showed high variability: total methadone 452±185 ng/mL; R- 226±92 ng/mL; S- 181±74 ng/mL; R:S ratio 0.6-1.9. Only 45% of patients were within the therapeutic R-methadone range (100-250 ng/mL), while 10% exhibited potentially elevated S-methadone levels. This validated, low-cost (~$4/sample) method enables stereospecific methadone monitoring. Marked interindividual variability highlights the clinical value of enantioselective TDM to optimize efficacy and minimize cardiovascular risk in MMT programs.

Keywords: Methadone Enantiomers; HPLC–UV; Therapeutic Drug Monitoring; Chiral Analysis; Methadone Maintenance Therapy; Stereoselective Pharmacokinetics.

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1. Introduction

Therapeutic drug monitoring (TDM) is an established clinical strategy for optimizing pharmacotherapy in drugs characterized by narrow therapeutic indices and pronounced interindividual pharmacokinetic variability(1). Methadone, a synthetic opioid widely used in opioid maintenance therapy (OMT), represents a paradigmatic example of a medication for which TDM may substantially improve both efficacy and safety outcomes. Despite its proven effectiveness in reducing opioid-related morbidity and mortality, up to 30–40%

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of patients receiving methadone maintenance therapy (MMT) continue to experience inadequate symptom control, persistent cravings, or dose-related adverse effects, highlighting the need for improved individualized dosing strategies (2, 3).

Methadone is administered clinically as a racemic mixture of two enantiomers, (R)- and (S)-methadone, which exhibit markedly distinct pharmacodynamic and safety profiles. (R)-methadone displays a substantially higher affinity for the μ-opioid receptor—reported to be 8- to 50-fold greater than that of the (S)-enantiomer—and is primarily responsible for the therapeutic effects of methadone, including suppression of withdrawal symptoms and opioid craving (4). In contrast, (S)-methadone exhibits minimal opioid activity but has been strongly implicated in the inhibition of cardiac hERG potassium channels, leading to QT interval prolongation and an increased risk of torsades de pointes and other potentially fatal arrhythmias (5, 6).

In addition to pharmacodynamic differences, the two methadone enantiomers undergo stereoselective metabolism. (R)-methadone is metabolized predominantly by CYP2B6 and CYP2D6, whereas (S)-methadone is mainly cleared via CYP3A4 (7, 8). Genetic polymorphisms further exacerbate interindividual variability in methadone exposure. Notably, the CYP2B6*6 allele—associated with reduced enzymatic activity—has a reported prevalence of approximately 30–35% in the Iranian population and has been linked to altered enantiomeric disposition and increased plasma concentrations of methadone enantiomers, thereby elevating the risk of adverse cardiovascular outcomes (9-11).

Given these stereoselective differences, reliance on total plasma methadone concentrations provides limited clinical insight and may obscure clinically relevant imbalances between therapeutic and potentially harmful enantiomer exposure. In contrast, enantiomer-specific plasma concentrations—particularly trough levels of (R)-methadone—have been shown to correlate more closely with treatment retention and suppression of withdrawal symptoms, with a proposed therapeutic range of approximately 100–250 ng/mL. Conversely, elevated concentrations of (S)-methadone have been associated with QT interval prolongation and increased cardiovascular risk (5, 12, 13).

Iran currently hosts the largest population of patients receiving MMT worldwide, with more than 800,000 individuals enrolled. Despite this unprecedented scale, enantioselective TDM of methadone is not routinely available in Iran. Existing clinical laboratories rely predominantly on non-chiral high-performance liquid chromatography (HPLC) methods, which are incapable of differentiating between methadone enantiomers and often exhibit relatively high limits of quantification (50–100 ng/mL) (14, 15). Consequently, clinicians lack the necessary analytical tools to detect insufficient exposure to (R)-methadone or potentially hazardous accumulation of (S)-methadone, both of which may contribute to treatment failure and adverse events in MMT patients.

Although several enantioselective analytical methods for methadone determination have been reported—primarily based on liquid chromatography coupled with mass spectrometric detection—these approaches are often costly, technically complex, and dependent on advanced instrumentation and specialized expertise (16-18). In many low-resource settings, including Iran, widespread implementation of LC–MS/MS-based enantioselective TDM is limited by financial, infrastructural, and human resource constraints. This gap underscores the urgent need for validated, cost-effective, and accessible enantioselective analytical methods suitable for routine clinical application.

Accordingly, the present pilot study aimed to develop, validate, and clinically apply a simple and economical enantioselective HPLC-UV method for the quantification of (R)- and (S)-methadone in plasma samples from Iranian patients receiving MMT. The method was validated in accordance with ICH Q2(R1) and FDA bioanalytical guidelines(19) and subsequently applied to assess enantiomer-specific plasma concentrations in a cohort of stable MMT patients. This study represents, to our knowledge, the first implementation of an enantioselective methadone TDM platform

in Iran and provides foundational evidence to support individualized methadone dosing strategies and improved patient safety in under-resourced healthcare settings.

2. Materials and Methods

2.1. Study Design and Participants

This pilot study comprised both analytical method validation and its clinical application and was conducted at the Addiction Treatment Clinic affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. A total of 20 patients receiving methadone maintenance therapy (MMT) were enrolled. Participants were clinically stable and had been receiving a fixed daily oral dose of methadone ranging from 60 to 120 mg for at least three months prior to enrollment. The study population included 17 males and 3 females, with an age range of 22–65 years.

Patients were excluded if they were receiving concomitant medications known to strongly induce or inhibit cytochrome P450 enzymes, had significant hepatic or renal impairment, presented with acute illness at the time of sampling, or were pregnant. The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (Approval No. IR.SUMS.PHARM.REC.10107). Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki and national regulatory guidelines.

2.2. Chemicals and Reagents

Racemic methadone hydrochloride (≥99.5% purity) and imipramine hydrochloride, used as the internal standard (IS), were obtained from Temad Pharmaceutical Company (Tehran, Iran). HPLC-grade acetonitrile, methanol, n-hexane, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced using a Milli-Q purification system (Millipore, Bedford, MA, USA). All chemicals and solvents were of analytical or HPLC grade.

2.3. Instrumentation and Chromatographic Conditions

Chromatographic separation was performed using a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary pump and UV/Vis detector (SPD-20A). Enantiomeric separation was achieved on a Chiral-AGP analytical column (100 × 4.0 mm, 5 µm; ChromTech, Sweden), maintained at room temperature (25 °C). Fourier-transform infrared (FTIR) spectra were recorded using a VERTEX 70 spectrometer (Bruker>, Germany) in the wavenumber range of 4000–400 cm^{-1}. Samples were prepared using the (KBr pellet/ATR/thin film) method as appropriate. Spectral resolution was set at (e.g., 4 cm^{-1}), and (e.g., 16–32 scans) were accumulated for each spectrum.

Optical rotation measurements were carried out using an AP-300 digital polarimeter (ATAGO>, Japan). Measurements were performed at (e.g., 25 °C) using the sodium D-line (589 nm). Reported values represent the average of at least three independent measurements.

UV–Vis absorption spectra were obtained using a T80 spectrophotometer (PG Instruments>, UK) over the wavelength range of (e.g., 200–800 nm). Quartz cuvettes with a 1 cm path length were employed for all measurements.

Following method optimization, isocratic elution was carried out using a mobile phase consisting of acetonitrile and 0.01 M phosphate buffer (15:85, v/v; pH 6.8) at a flow rate of 1.1 mL/min. The injection volume was 20 µL, and UV detection was performed at 210 nm.

2.4. Sample Collection and Preparation

Blood samples were collected at trough levels, approximately 24 ± 1 h after the last oral methadone dose. Five milliliters of venous blood were drawn into citrate-containing tubes and centrifuged at 3,000 × g for 10 min

at 4 °C. Plasma was separated and stored at −20 °C until analysis.

Plasma samples were prepared using a liquid–liquid extraction (LLE) procedure. Briefly, 100 µL of imipramine solution (1.5 µg/mL) and 200 µL of 1 N potassium hydroxide were added to 1 mL of plasma and mixed thoroughly. Extraction was performed by adding 3 mL of n-hexane, followed by vigorous shaking for 10 min. After centrifugation at 3,000 × g for 10 min, the organic phase was transferred to a clean tube and evaporated under a gentle nitrogen stream at 40 °C. The residue was reconstituted in 100 µL of mobile phase, and 20 µL was injected into the HPLC system.

2.5. Identification of Methadone Enantiomers

Due to the unavailability of commercially pure methadone enantiomer standards, enantiomer identification was performed according to a previously described approach with minor modifications (20). Racemic methadone was initially analyzed to establish enantiomeric retention behavior on the chiral stationary phase.

Preparative chiral HPLC was subsequently used to isolate individual enantiomeric fractions, which were re-injected to confirm chromatographic purity(21-23). Optical rotation measurements were performed using polarimetry; the first eluting fraction exhibited negative optical rotation and was assigned as (R)-methadone, whereas the second fraction showed positive optical rotation and was assigned as (S)-methadone. Further confirmation was obtained by comparing Fourier-transform infrared (FTIR) and ultraviolet (UV) spectra of the isolated fractions with those of the racemic compound.

2.6. Method Validation

2.6.1. Selectivity

Selectivity was confirmed by analyzing blank plasma samples from at least six independent sources to ensure absence of endogenous interference at the retention times of the enantiomers and the internal standard (IS).

2.7. Linearity and LLOQ

Linearity was established using calibration curves over the validated concentration range. Correlation coefficients (r^2) were ≥0.99. The lower limit of quantification (LLOQ) was defined as the lowest concentration with accuracy and precision within ±20%.

2.8. Accuracy and Precision

Intra- and inter-day accuracy and precision were evaluated at low, medium, and high QC levels, with all values required to be within ±15%.

2.9. Recovery and Matrix Effect

Extraction recovery and matrix effect were assessed at three QC levels following liquid–liquid extraction (LLE). Results demonstrated consistent recovery and negligible matrix interference (CV% ≤15%).

2.10. Dilution Integrity

Dilution integrity was verified by analyzing samples above the upper limit of quantification (ULOQ) after dilution with blank plasma, maintaining accuracy and precision within ±15%.

2.11. Carry-over

Carry-over was evaluated by injecting blank samples immediately after the highest calibration standard. No significant residual interference was observed (<20% of LLOQ for analytes and <5% for IS).

2.12. Statistical Analysis

Descriptive statistical analyses were performed using SPSS software (version 18.0). Data are presented as mean ± standard deviation. Statistical significance was defined as $p \leq 0.05$ at a 95% confidence interval.

3. Results

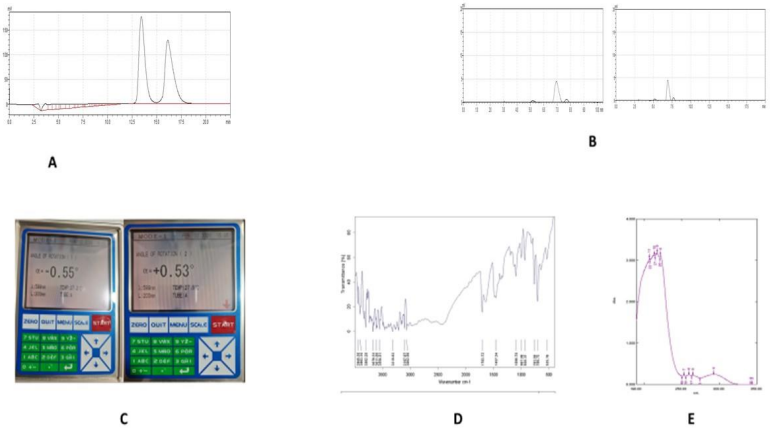

Figure 1. Identification and characterization of methadone enantiomers using preparative chiral HPLC, polarimetry, FTIR, and UV spectroscopy.A: Preparative HPLC chromatogram of the racemic drug showing two peaks for the R- and S-enantiomers.B: Reinjection of the collected fractions onto a chiral HPLC column resulting in single peaks for each enantiomer.C: Optical rotation measurements indicating negative rotation for the R-enantiomer and positive rotation for the S-enantiomer.D: FTIR spectrum of the isolated enantiomers, compared to the standard spectra.E: UV spectrum of the isolated enantiomers, confirming the structure of the separated enantiomers.

3.1. Identification and Characterization of Methadone Enantiomers

Figure 1 summarizes the stepwise strategy employed for the identification and characterization of methadone enantiomers in the absence of commercially available pure standards. Preparative chiral HPLC of racemic methadone resulted in two well-resolved fractions (Figure 1A). Re-injection of each collected fraction onto the analytical chiral column produced single, symmetrical peaks, confirming chromatographic purity (Figure 1B).

Optical rotation measurements demonstrated negative rotation for the first eluting fraction and positive rotation for the second, allowing assignment of the (R)- and (S)-enantiomers, respectively (Figure 1C). Further structural confirmation was obtained through FTIR and UV spectroscopic analyses, which showed concordance between the spectra of the isolated enantiomers and those of the racemic compound (Figures 1D and 1E). Collectively, these results confirmed the reliable identification of methadone enantiomers without the need for pure reference standards.

3.2. Analytical Performance and Method Validation

The analytical performance of the enantioselective HPLC-UV method is summarized in Figures 2 and 3. Separate calibration curves were constructed for (R)- and (S)-methadone over the concentration range of 12.5–1000 ng/mL. Excellent linearity was observed for both enantiomers, with correlation coefficients (r^2) ≥ 0.9991 and maximum deviations within ±5%.

The limits of detection (LOD) and quantification (LOQ) were determined to be 5 ng/mL and 10 ng/mL, respectively, for each enantiomer. Intra-day precision ranged from 1.1% to 2.8%, while inter-day precision across all quality control (QC) levels remained below 3.0%. Method accuracy was within ±3% at all QC concentrations, demonstrating high reproducibility and reliability.

Mean extraction recoveries were 92.4±3.1% for (R)-methadone and 91.8±3.4% for (S)-methadone, with an average recovery of 94.8±2.1% for the internal standard. Matrix effects were minimal, as evidenced by IS-normalized matrix factors ranging from 95% to 102% (CV < 4.5%).

Stability studies confirmed that both enantiomers remained stable (>95% of initial

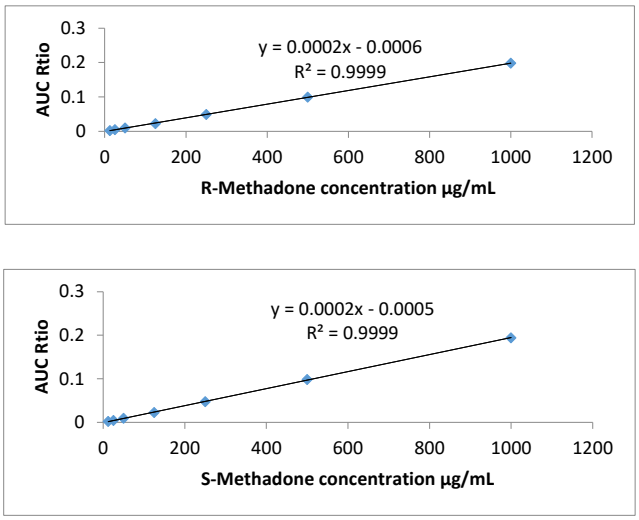

Figure 2. Calibration curves for (R)-methadone and (S)-methadone over the concentration range of 12.5–1000 ng/mL

concentration) under all tested conditions, including bench-top storage (24 h), autosampler storage (24 h), three freeze–thaw cycles, and long-term storage at −20 °C for 30 days. Dilution integrity was preserved following 1:5 and 1:10 dilutions, with accuracy between 97% and 101%. Carry-over following injection at the upper limit of quantification was negligible (<0.1%).

3.3. Chromatographic Characteristics

The optimized chromatographic conditions using the Chiral-AGP column provided clear baseline separation of methadone enantiomers. Mean retention times were approximately 7.9 min for (R)-methadone, 9.5 min for (S)-methadone, and 12.8 min for the internal standard. The resolution factor (Rs = 2.2) exceeded the minimum requirement for enantioselective analysis, confirming suit-

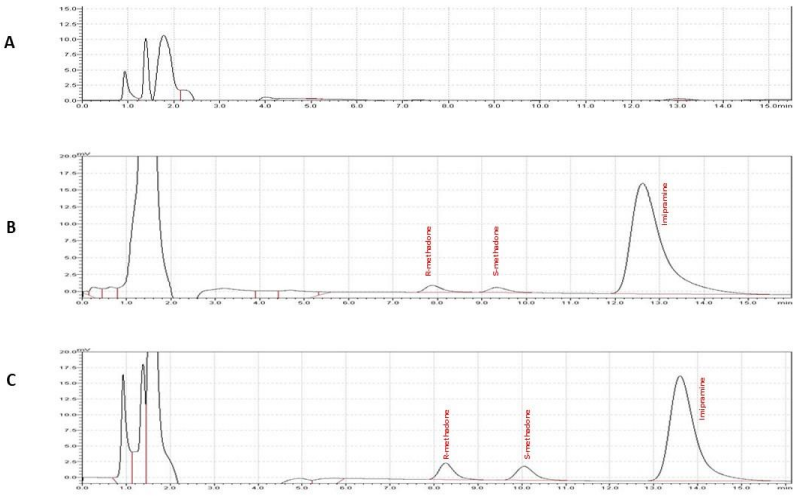

Figure 3. CRepresentative chromatograms obtained using the enantioselective HPLC-UV method: blank plasma (A) and patient samples under MMT program (B,C) illustrating baseline separation of (R)- and (S)-methadone. samples injected into the HPLC system under mobile phase containing phosphate buffer:acetonitryl in the ratio (85:15 v/v) and 2Mm DMOA at pH 6.8.The flow rate was set at 1.2 mL/min and the injection volume was 20 µl

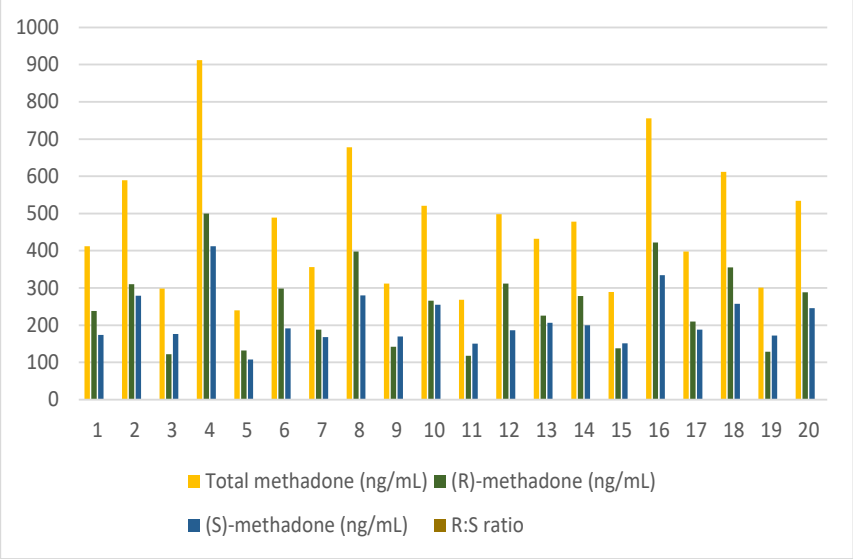

Figure 4. Individual (R/S) plasma concentration ratios of methadone enantiomers across the study population.

ability for routine quantitative application in patients under MMT program and No endogenous interferences were observed in blank plasma samples (Figure 3).

3.4. Clinical Application: Enantiomer-Specific Plasma Concentrations

Application of the validated method to plasma samples from 20 clinically stable MMT patients revealed substantial interindividual variability in methadone exposure (Table 1, Figure 4). Mean trough concentrations were 452±185 ng/mL for total methadone, 226±92 ng/mL for (R)-methadone, and 181±74 ng/mL for (S)-methadone. The mean (R/S) plasma concentration ratio was 1.25, ranging from 0.6 to 1.9 across participants (Figure 4).

When assessed stereospecifically, 45% of patients exhibited trough (R)-methadone concentrations within the proposed therapeutic range (100–250 ng/mL). In contrast, a subset of patients showed either subtherapeutic (R)-methadone concentrations or elevated enantiomer levels (>450 ng/mL), highlighting pronounced variability in enantiomer disposition despite comparable daily methadone doses.

Notably, several patients displayed (R/S) ratios below 1.0, indicating relatively higher exposure to (S)-methadone compared with (R)-methadone. These findings underscore the limitations of total methadone measurements and emphasize the value of enantiomer-specific analysis for individualized assessment in MMT.

4. Discussion

The present study describes the development, validation, and clinical application of an enantioselective HPLC–UV method for the quantification of (R)- and (S)-methadone in human plasma and represents the first validated stereospecific analytical approach applied to methadone maintenance therapy (MMT) patients in Iran. Given the global reliance on methadone as a cornerstone of opioid substitution therapy and Iran's extensive national MMT program, the availability of an affordable and reliable enantiomer-specific analytical method addresses an important unmet clinical and analytical need.

A key strength of the proposed method lies in its analytical performance, which is comparable to that of advanced LC–MS/MS methodologies(24-26) while relying on substantially more accessible instrumentation. The method demonstrated excellent linearity, precision, accuracy, recovery, and stability, with a limit of quantification of 10 ng/mL and a resolution factor (R_s = 2.2) that meets or

Table 1. Demographic characteristics, daily methadone dose, enantiomer-specific plasma concentrations, and (R/S) ratios in patients receiving methadone maintenance therapy.

Patient No.	(S)-methadone (ng/mL)	(R)-methadone (ng/mL)	R:S ratio
1	156	217	1.4
2	125	122	0.97
3	289	187	0.6
4	355	456	1.3
5	121	178	1.4
6	136	189	1.4
7	212	216	1.01
8	113	145	1.3
9	175	285	1.6
10	476	500	1.05
11	345	315	0.9
12	466	387	0.8
13	499	413	0.8
14	251	186	0.7
15	364	342	0.9
16	171	179	1.04
17	332	351	1.05
18	158	258	1.6
19	238	271	1.1
20	210	398	1.9

Note: Values in bold indicate subtherapeutic (R)-methadone (<100 ng/mL) or potentially toxic levels (>450 ng/mL per enantiomer).

exceeds previously reported chiral methadone assays, including mass spectrometry–based approaches (27). Earlier AGP-based chromatographic methods were limited by longer run times and reduced sensitivity (18); optimization of mobile phase composition and flow rate in the present study resulted in sharper peaks, improved reproducibility, and shorter analysis times, thereby enhancing suitability for routine clinical use.

The acquisition of enantiomerically pure compounds is a critical prerequisite for reliable pharmacological, toxicological, and clinical evaluation of chiral drug candidates. In cases where enantiopure reference standards are not commercially available, preparative chiral high-performance liquid chromatography (HPLC) represents a robust and widely accepted strategy for the isolation of individual enantiomers from racemic mixtures. Previous studies have demonstrated the effectiveness of preparative chiral HPLC in producing optically pure compounds suitable for subsequent analytical characterization and biological assessment (22, 23)

Chiral separations performed at both analytical and preparative scales have become integral to pharmaceutical research and development. As discussed by Gumustas et al. (2018)(21), preparative chiral chromatography not only enables accurate determination of enantiomeric purity but also provides sufficient quantities of isolated enantiomers for structural elucidation, stability studies, and clinical investigations. Furthermore, large-scale enantiomeric resolution using preparative chromatographic techniques has been shown to accelerate drug discovery workflows by supplying stereochemically defined compounds for early-stage biological screening

(28).

Given the stereochemical dependence of pharmacodynamic and pharmacokinetic properties, the use of preparative chiral HPLC in the present study was therefore justified to obtain highly pure individual enantiomers prior to further physicochemical and biological analyses. This approach ensures the reliability of the observed activity profiles and eliminates potential interference arising from the presence of the counter-enantiomer. Consequently, preparative chiral HPLC constitutes a scientifically sound and methodologically appropriate strategy when authentic enantiopure standards are unavailable.

Importantly, application of the validated method to plasma samples from MMT patients revealed substantial interindividual variability in enantiomer-specific methadone exposure despite comparable daily doses. The observed mean (R/S) plasma concentration ratio of 1.25 is consistent with ratios reported in studies involving Caucasian and Middle Eastern populations (2, 29), suggesting that the stereoselective disposition of methadone in Iranian patients follows patterns comparable to those observed in other ethnic groups. However, the wide range of (R)- and (S)-methadone concentrations observed across individuals underscores the limitations of total methadone measurements and highlights the clinical relevance of stereospecific analysis.

The pronounced variability in enantiomeric exposure is likely multifactorial and may, in part, reflect interindividual differences in methadone metabolism mediated by cytochrome P450 enzymes, particularly CYP2B6 and CYP3A4 (13, 30). Several pharmacogenetic studies have demonstrated that reduced-function CYP2B6 alleles preferentially impair the clearance of (S)-methadone, resulting in disproportionate accumulation of this enantiomer (8, 31). Given reports of a high prevalence of the CYP2B6*6 allele in the Iranian population, an increased exposure to (S)-methadone may be anticipated in a subset of patients, which has been associated in previous studies with an elevated risk of concentration-dependent adverse effects, including QT interval prolongation (32). While the present study was not designed to assess clinical outcomes, the enantiomer-specific concentration profiles observed here support the potential value of stereospecific therapeutic drug monitoring (TDM) in identifying patients at risk of unfavorable exposure patterns.

From a practical perspective, the low analytical cost of approximately USD 4 per sample represents a major advantage of the proposed HPLC–UV method. Although LC–MS/MS remains the analytical gold standard for chiral drug quantification, its limited availability in many low- and middle-income countries restricts widespread clinical implementation(33, 34). In contrast, the method presented here offers a feasible alternative for routine use in hospital and regional laboratories, enabling broader access to enantiomer-specific methadone monitoring. Such accessibility may facilitate individualized dose optimization, improve treatment retention, and provide an additional layer of quality assurance for methadone formulations distributed through national programs (35, 36).

The present study also provides the first stereospecific pharmacokinetic data for Iranian MMT patients, contributing novel regional data to the existing literature on methadone enantiomer disposition. While the sample size was modest, it is consistent with those employed in prior pharmacokinetic and bioanalytical studies investigating methadone enantiomers, where cohorts of approximately 10 to 30 subjects have been sufficient to characterize enantiomeric concentrations, ratios, and stereoselective pharmacokinetics when supported by robust analytical validation. For example, several investigations have reported stereoselective plasma profiles of R and S methadone in maintenance or clinical settings (Steady state pharmacokinetics of methadone enantiomers; Pharmacokinetics and pharma-

codynamics of methadone enantiomers in opioid dependent subjects; Stereoselective determination of methadone and metabolites in serum/ urine). Accordingly, the current findings offer reliable insight into enantiomer specific exposure patterns within this population. (20, 37, 38)

In conclusion, this study demonstrates that an enantioselective HPLC–UV method can provide analytically robust and clinically informative measurements of methadone enantiomers at a fraction of the cost of mass spectrometry–based techniques. Implementation of this approach could enable routine stereospecific TDM in Iran and similar resource-limited settings, supporting more individualized methadone therapy and potentially improving the safety and effectiveness of MMT programs.

5. Conclusion

In this study, a simple, cost-effective, and reliable enantioselective HPLC–UV method was successfully developed and fully validated for the simultaneous determination of R- and S-methadone in human plasma. The method met all international bioanalytical validation requirements and demonstrated adequate sensitivity, precision, accuracy, and selectivity for clinical application, while remaining accessible to laboratories lacking advanced mass spectrometric facilities.

Application of the validated method to plasma samples from Iranian patients receiving methadone maintenance treatment revealed marked interindividual variability in enantiomer-specific exposure. Notably, a substantial proportion of patients exhibited subtherapeutic R-methadone concentrations or disproportionately elevated S-methadone levels, highlighting potential risks of reduced therapeutic efficacy or increased cardiotoxicity that cannot be identified by measurement of total methadone alone.

These findings underscore the clinical relevance of enantiomer-specific therapeutic drug monitoring and support its potential role in optimizing methadone dosing strategies. Importantly, this work represents the first validated analytical platform for stereospecific methadone monitoring in Iran and provides foundational pharmacokinetic data for this population.

Further studies with larger sample sizes, inclusion of pharmacogenetic and electrocardiographic data, and longitudinal clinical follow-up are warranted to better define therapeutic ranges and to facilitate the integration of stereospecific monitoring into routine methadone maintenance programs.

Authors contributions

Conceptualization: H. Montaseri, F. Baha-al-Dini Bigi ZarandiMethodology, Supervision, Funding acquisition, Resources: H. Montaseri, F. Baha-al-Dini Bigi ZarandiInvestigation, Data curation, Formal analysis, Software, Writing – original draft: M. FarazValidation, Visualization, Project administration, Writing – review & editing: H. Montaseri, M. Faraz, F. Baha-al-Dini Bigi Zarandi

All authors have read and approved the final manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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