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Enantioselective determination of tramadol and its main phase I metabolites in human plasma by high-performance liquid chromatography

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Abstract

A sensitive and relatively rapid reversed-phase HPLC method was applied to the enantiomeric separation of tramadol and its two main metabolites, *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) in plasma samples. Chromatography was performed on an AGP column containing α1-acid glycoprotein as chiral selector with a mobile phase of 30 mM diammonium hydrogen phosphate buffer–acetonitrile–triethylamine (98.9:1:0.1, v/v), adjusted to pH 7 by phosphoric acid, and a flow rate of 0.5 ml/min. The fluorescence of analytes was detected at excitation and emission wavelengths of 200 and 301 nm, respectively. The sample preparation was a simple extraction with ethyl acetate using fluconazol as internal standard (IS). The enantiomers of all analytes and IS peaks eluted within 32 min, without any endogenous interference. The calibration curves were linear ($r^2 > 0.993$) in the concentration range of 2–200, 2.5–100 and 2.5–75 ng/ml for tramadol, M1, and M2 enantiomers, respectively. The within- and between-day variation determined by the measurement of quality control samples at four tested concentrations, showed acceptable values. The lower limit of quantitation was 2 ng/ml for tramadol enantiomers and 2.5 ng/ml for M1 or M2 enantiomers. Mean recoveries of enantiomers from plasma samples were >81% for all analytes. The procedure was applied to assess the pharmacokinetics of the enantiomers of tramadol and its two main metabolites following oral administration of single 100-mg doses to healthy volunteers.

Keywords: Enantioselective assay; Tramadol; Metabolite; AGP column; Pharmacokinetics

1. Introduction

Tramadol (T) hydrochloride is a synthetic atypical opiod analgesic which contains two chiral centers and thus has four stereoisomers. *trans*-T, a racemate consisting of 1R, 2R-T [(+)-T], and 1S, 2S-T [(−)-T], is a centrally acting analgesic with efficacy and potency ranging between weak opioids and morphine [1]. Tramadol produces analgesia in short-term and long-term pain states by synergistically combining weak μ-opioid and monoaminergic (noradrenalin and serotonin) mediated mechanisms. This dual mechanism of action may be attributed to the different, but complementary and interactive, mechanisms of action of its enantiomers. The (+)-enantiomer exhibits a ten-fold higher analgesic activity due to a greater affinity for the μ-receptor and is a more effective inhibitor of serotonin reuptake, while the (−)-enantiomer is a more effective inhibitor of noradrenalin reuptake and increases noradrenaline release by auto receptor activation [2].

This analgesic is rapidly and extensively metabolized in the liver. The principal metabolic pathways, *O*- and *N*-desmethylation, involve cytochrome P-450 isoenzymes 2D6 and 2B6 and 3A4, respectively (Fig. 1). The primary metabolites *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) may be further metabolized to three additional secondary metabolites namely, *N*,*N*-didesmethyltramadol (M3), *N*,*N*,*O*-tridesmethyltramadol (M4) and *N*,*O*-didesmethyltramadol (M5). Of all these metabolites, only M1 is pharmacologically active. In phase II, the *O*-demethylated metabolites are conju-

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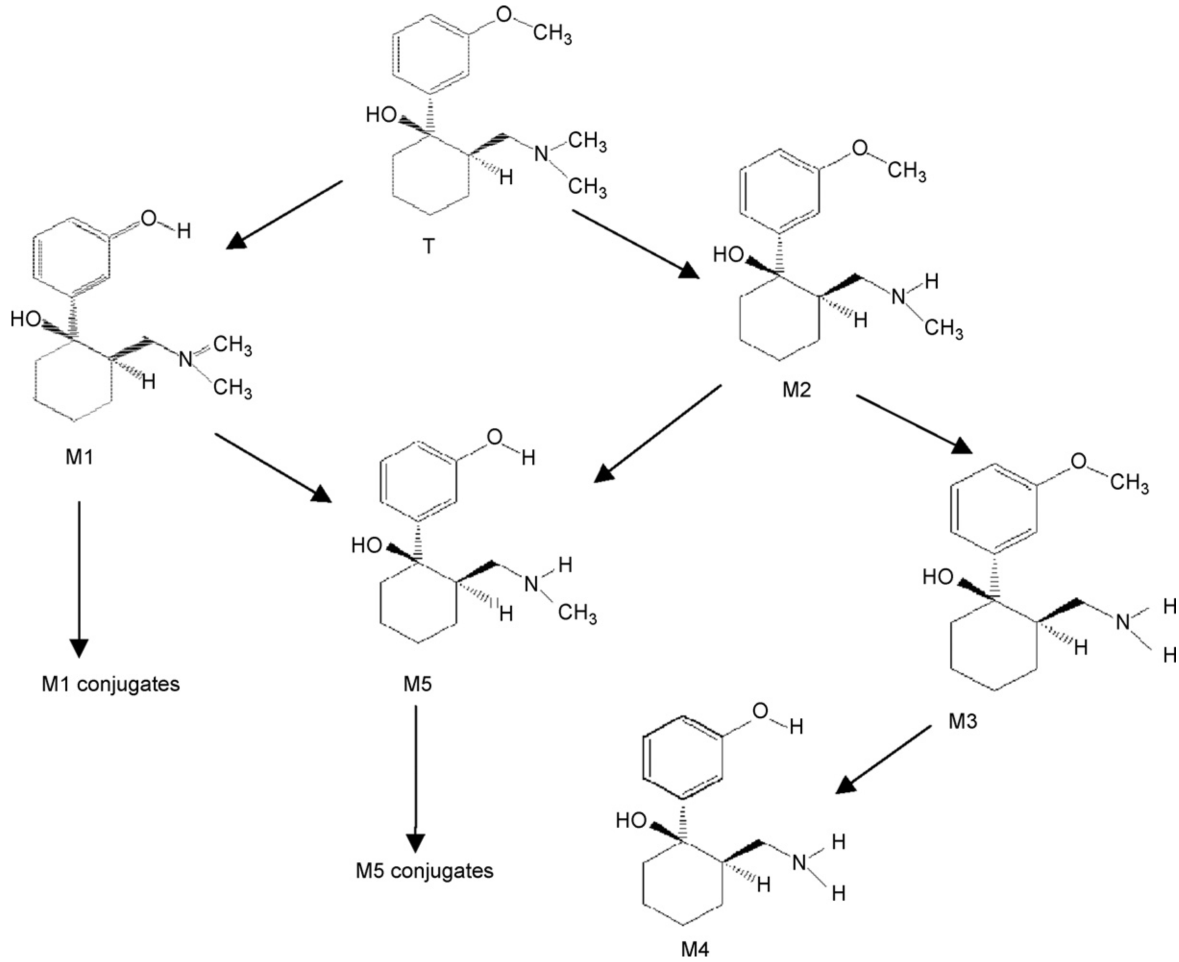

Fig. 1. Metabolic pathway of tramadol.

gated with glucuronic acid and sulfuric acid before excretion into urine [3].

Several in vitro and in vivo studies have shown that the pharmacokinetic of T and its main metabolites (M1 and M2) are stereoselective [4–10]. However, the sources of stereoselectivity in the pharmacokinetics of the T metabolites (e.g., stereoselectivity in protein binding, renal clearance and/or polymorphism of CYP2D6) have not yet been identified. Therefore, the development of stereospecific methods, capable of determination of the enantiomers of tramadol and its main metabolites in biological samples, is necessary in order to better understand the mechanisms of stereoselectivity in the kinetics of the drug and its metabolites.

Recently, a number of capillary zone electrophoresis (CE) methods have been developed for the stereospecific determination of T and its metabolites. Although CE has become a powerful technique as an alternative to chiral chromatographic methods, the reported methods are not sensitive enough for measurement of the plasma concentrations of the enantiomers of T and/or its metabolites in pharmacokinetic studies [7,11–14].

One of the most common analytical techniques in stereoselective pharmacokinetic studies is high-performance liquid chromatography using chiral stationary phases (CSP). Cellulose and amylase derivatives are the chiral selectors widely used in normal- [15–17] and reversed-phase chromatographic modes [8,18,19] to analyse tramadol and/or its metabolites using ultra-violet [17,18], fluorescence [8,19] or MS detection [15]. Among all these methods, only Campanero et al. reported a reversed-phase chromatographic method for simultaneous determination of tramadol, M1, and M2 enantiomers in human plasma. However, combining an achiral and chiral column resulted in relatively long run time of up to 70 min [8].

The other chiral selectors used in chiral chromatography are protein-based CSPs which have been widely used for the direct separation of drug enantiomers. Examples of such CSPs are the bovine serum albumin (BSA) and α1-acid glycoprotein (AGP). These proteins are very stable and tolerate pure organic solvents, high temperatures, and high and low pH. AGP is the chiral selector in CHIRAL-AGP columns which is usually used in reversed-phase mode. This column can be used for the resolution of a broad range of chiral compounds such as basic (primary, secondary, tertiary amines as well as quaternary ammonium), acidic (strong and weak acids), and nonprotolytic compounds (http://www.chromtech.com).

In this study, we developed and validated a new analytical method based on fluorescence detection coupled to CHIRAL-AGP column for selective and sensitive determination of the enantiomers of T and its main metabolites (M1 and M2) in plasma. The method employs a relatively small volume of organic modifier in the mobile phase and has a short run time. The method was applied to stereoselective pharmacokinetic study of T and its main phase I metabolites in healthy volunteers.

2. Experimental

2.1. Materials

The racemic forms, (+)-enantiomer of *trans*-T, and enantiomers of -(−) and -(+) *O*-desmethyltramadol (M1) and *N*-desmethyltramadol) (M2), as hydrochloride salts, were generously supplied by Grünenthal (Stolberg, Germany). Fluconazole (purity > 99%) (internal standard, IS) was provided by Pars-Daru Co. (Tehran, Iran). HPLC-grade acetonitrile and analytical grade ethyl acetate, phosphoric acid (85%), triethylamine and diammonium hydrogen phosphate were supplied by Merck (Darmstadt, Germany). Water used in all experiments was of Direct-Q® quality (Millipore, France).

2.2. Standard solutions

2.2.1. Solutions used for enantiomer identification

In order to determine the elution order of each enantiomer, the stock solutions of (+)-T and enantiomers of metabolites (M1 and M2) were prepared separately by dissolving the appropriate amount of each compound in methanol to give a concentration of 100 μg/ml. Intermediate stock standards of 10 and 1 μg/ml were then prepared using water as a solvent.

2.2.2. Solutions used for calibration and method validation

Stock solutions of racemic tramadol and metabolites containing 1 mg/ml of compounds were prepared in methanol and stored at 4 °C. Standard solutions of all compounds were prepared by appropriate dilution in water of the stock solution.

Mixed calibration pools of racemic tramadol, M1 and M2 were prepared by diluting appropriate volumes of each standard solution with plasma to achieve concentration ranges of 2–200, 2.5–100, and 2.5–75 ng/ml for the enantiomers of T, M1, and M2, respectively.

Pools of quality control samples were prepared by spiking human plasma with the standard solutions of all compounds. The enantiomeric concentrations in the quality control plasma samples were 5, 25, 75 and 150 ng/ml for T, 5, 15, 50 and 100 ng/ml for M1, and 5, 15, 50 and 75 ng/ml for M2.

Stock solution of IS, containing 1 mg/ml fluconazole in methanol, was prepared weekly and stored at 4 °C. Further dilution of the stock solution in water to obtain final IS concentration of 200 ng/ml was made daily.

2.3. Apparatus

Chromatographic experiments were performed in a Knauer high-performance liquid chromatography (Berlin, Germany), equipped with a low-pressure gradient HPLC pump, a fluorescence detector, a Rheodyne injector with a 100-μl loop and an online degasser. The excitation and emission wavelength were 200 and 301 nm, respectively. The data acquisition was carried out using ChromGate chromatography software (Knauer, Berlin, Germany).

2.4. Chromatographic conditions

The analytical separation was performed by isocratic separation at room temperature (22 °C) using a CHIRAL-AGP analytical column (5 μm, 150 mm × 4.0 mm i.d., Chromtech, Congleton, UK) in combination with Chiral-AGP guard column (5 μm, 10 mm × 4.0 mm i.d.). The flow rate of the mobile phase, consisting of 30 mM diammonium hydrogen phosphate buffer–acetonitrile–triethylamine (98.9:1:0.1, v/v) adjusted to pH 7 by phosphoric acid, was set at 0.5 ml/min.

2.5. Sample preparation

Five hundred microliters plasma sample was transferred into a 15-ml glass tube and 50 μl of each IS solution and NaOH (2 M) was added. The samples were shaken with 6 ml of ethyl acetate for 15 min. The organic layer was separated after centrifugation (2 min), evaporated under a gentle stream of air and reconstituted in 120 μl of HCl (5 mM). A 100 μl aliquot of sample was then injected to the HPLC system.

2.6. Optimization of the chromatographic conditions

In order to determine the optimum chromatographic conditions, the effects of pH, buffers, organic modifiers, and additives of the mobile phase on the quality of the separations of the enantiomers of tramadol and its main metabolites were investigated.

2.7. Application of the method

Three volunteers were included in this study. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences and written informed consent was obtained from the volunteers. Volunteers were not allowed to take any other medication for 2 weeks before and throughout the study. The volunteers received single 100-mg Tradolan tablets (Lannach, Austria) orally after an overnight fast. Intake of food was delayed for 3 h after medication. Peripheral venous blood samples were taken from each volunteer at predetermined intervals and plasma samples were stored at −20 °C until analysis.

3. Results

3.1. Method validation

3.1.1. Selectivity and resolution

Chromatograms of plasma samples, depicting the separation and detection of the enantiomers of T and its main metabolites, are presented in Fig. 2. Selectivity was indicated by absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of control human plasma and plasma spiked with compounds. Under the stated conditions, IS, (+)-M1, (+)-T, (+)-M2,(−)-M2, (−)-T, and (−)-M1 were eluted

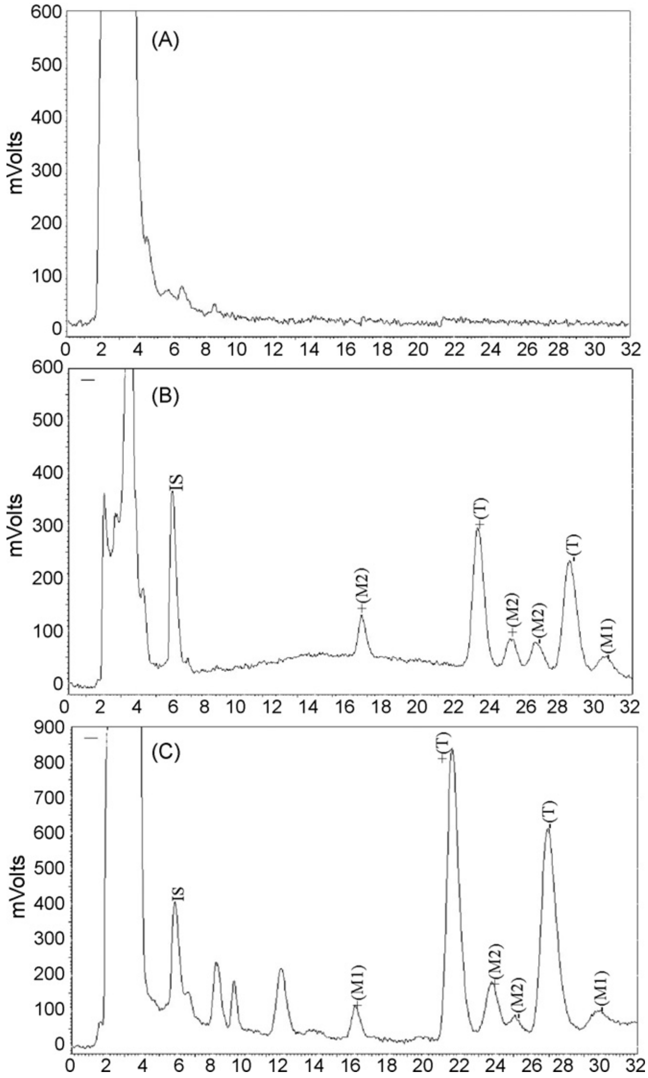

Fig. 2. Chromatograms of (A) a blank human plasma, (B) a blank human plasma spiked with (+)-M1 (15 ng/ml), (+)-T (50 ng/ml), (+)-M2 (10 ng/ml), (−)-M2 (10 ng/ml), (−)-T (50 ng/ml), (−)-M1 (15 ng/ml) and fluconazole (IS); and (C) plasma sample taken from a volunteer 2.5 h after a single oral dose (100 mg) of racemic tramadol, containing (+)-M1 (21.4 ng/ml), (+)-T (167 ng/ml), (+)-M2 (22.6 ng/ml), (−)-M2 (5.1 ng/ml), (−)-T (148.7 ng/ml) and (−)-M1 (13.3 ng/ml).

at approximately 6.7, 16.2, 21.6, 23.9, 25.2, 27.3, and 30.3 min, respectively. The method showed acceptable resolution for all analytes. As could be seen in Fig. 2, the resolutions between (+)-T:(+)-M2, (+)-M2:(−)-M2, (−)-M2: (−)-T and (−)-T: (−)-M1 were 1.7, 1.3, 1.5 and 1.4, respectively.

3.1.2. Linearity

Table 1 presents the linearity data from the analysis of the tramadol, M1, and M2 enantiomers. Each concentration level of the calibration curve is based on data from three separate runs. Linear regression analysis for the individual enantiomers of all compounds was performed by plotting the peak area ratios versus the respective plasma concentrations. The linearity analysis showed determination coefficient of greater than 0.993 for all compounds (Table 1).

Table 1
Linearity data from the stereoselective determination of tramadol T, M1 and M2

Linearity	(+)-T	(−)-T	(+)-M1	(−)-M1	(+)-M2	(−)-M2
[a] r^2	0.9997	0.9995	0.9993	0.9991	0.9944	0.9930
Slope	0.0249	0.0233	0.0102	0.0080	0.0176	0.0173
Intercept	−0.0260	+0.0099	−0.0094	+0.0003	−0.0274	−0.0031

[a] r^2 = Determination coefficient.

3.1.3. Accuracy and precision

Between- and within-day accuracy and precisions of the method were determined for each compound according to the FDA guidance for bioanalytical method validation [20]. Five replicate spiked samples were assayed between- and within-day at four different concentrations of each analyte in plasma. Accuracy was calculated as deviation of the mean from the nominal concentration. Precisions were expressed as the relative standard deviation of each calculated concentration. The between- and within-day assay validation data are reported in Table 2. Precision and accuracy studies in plasma showed an acceptable R.S.D. values (≤14.2%) and accuracy (82.5–105%) for both between- and within-day studies ($n = 5$).

Table 2
Between and within-day accuracy and precision and recovery of the HPLC method for the determination of enantiomers of T, M1, and M2 in plasma

Concentration (ng/ml)	Within-day		Between-day		Recovery	
	Accuracy	R.S.D.	Accuracy	R.S.D.	%	R.S.D.
(+)-T						
5	83.9	13.7	85.7	10.8	80.3	6.6
25	90.2	11.5	94.2	8.9	84.1	7.2
75	101.4	7.3	98.4	4.3	83.4	6.4
150	103.3	6.4	100.7	3.9	81.8	7.1
(−)-T						
5	88.1	12.8	92.2	9.7	78.7	6.1
25	90.5	12.7	93.6	8.6	80.1	7.2
75	103.1	10.4	100.1	7.0	82.4	8.4
150	101.4	9.6	98.2	6.7	81.4	7.8
(+) -M1						
5	82.5	14.0	84.6	12.8	89.2	9.1
15	86.7	13.7	85.1	10.6	81.1	7.9
50	104.0	11.8	98.6	8.1	84.3	7.8
100	92.9	10.1	95.7	4.7	87.8	7.6
(−) -M1						
5	80.4	14.2	83.7	14.1	84.6	12.6
15	85.4	13.7	84.9	8.7	79.9	10.6
50	102.6	11.1	95.4	13.4	80.6	12.5
100	95.6	10.9	94.8	8.7	82.4	15.1
(+) -M2						
5	83.7	12.7	86.7	11.6	91.2	6.2
15	88.6	13.4	90.1	11.4	92.4	7.2
50	105.4	11.8	98.9	10.8	91.4	9.1
75	89.3	10.2	92.1	7.9	87.3	10.1
(−) -M2						
5	82.9	14.2	86.4	12.1	85.5	5.9
15	84.6	13.5	88.8	10.5	84.1	6.8
50	95.4	12.8	94.8	10.9	88.9	9.8
75	101.7	11.9	98.9	8.4	91.6	7.9

Table 3
Lower limit of quantification for T, M1, and M2 ($n = 5$)

	(+)-T	(−)-T	(+)-M1	(−)-M1	(+)-M2	(−)-M2
Concentration (ng/ml)	2	2	2.5	2.5	2.5	2.5
Between-day R.S.D. (%)	8.8	7.6	11.3	16.5	10.1	11.3
Accuracy (%)	89.6	87.4	86.9	83.4	87.6	84.4

3.1.4. *Lower limit of quantification*

The lower limit of quantification (LLOQ) was defined as the lowest analyte concentration, which can be determined with an accuracy and precision <20% [20]. The LLOQ values for the enantiomers of all analytes are reported in Table 3.

3.1.5. *Recovery*

The extraction recovery of tramadol and its metabolites was calculated for the QC samples by comparing the data obtained by the direct injection of standard aqueous solutions to those obtained after the whole extraction procedure.

The one-step extraction procedure was quite rapid. Ethyl acetate is a very popular extracting solvent because of its high polarity and volatility. This solvent provides sufficient recovery for all compounds. The absolute recoveries of all analytes are shown in Table 2.

3.1.6. *Stability*

No significant degradation or chiral inversion of T, M1, or M2 enantiomers was observed in frozen plasma samples (−20 °C) over 3 months, and in plasma samples and stock solutions for at least 24 h at 4 °C.

3.2. *Application of the method*

The described assay procedure was applied successfully to the pharmacokinetic study of the enantiomers of tramadol and its metabolites in three volunteers. Mean plasma concentration–time courses of T, M1, and M2 enantiomers following a single oral dose of racemic T are shown in Fig. 3. The pharmacokinetic parameters of all analytes derived by non-compartmental analysis are summarized in Table 4.

Table 4
Pharmacokinetic parameters (mean ± S.D.) of the enantiomers of T, M1, and M2 after oral administration of 100 mg of racemic tramadol to healthy volunteers ($n = 3$)

	T_{max} (h)	C_{max} (ng/ml)	AUC (0–∞) (ng h/ml)	$t_{1/2}$ (h)
(+)-T	1.7 ± 0.3	162.1 ± 36.7	1133.6 ± 225.8	6.7 ± 0.6
(−)-T	1.8 ± 0.3	151.4 ± 30.3	1168 ± 87.2	6.0 ± 0.6
(+)-M1	2.3 ± 0.3	26.8 ± 12.8	398.1 ± 55.8	9.6 ± 1.9
(−)-M1	2.3 ± 0.3	37.8 ± 10.5	452.0 ± 129.7	7.3 ± 0.9
(+)-M2	2.8 ± 0.6	17.3 ± 12.0	255.5 ± 104.0	11.1 ± 2.4
(−)-M2	2.5 ± 0.5	5.8 ± 3.1	55.4 ± 28.3	8.2 ± 3.5

C_{max}: maximum concentration in plasma; AUC: area under the plasma concentration–time curve, $t_{1/2}$: half-life.

4. Discussion

The enantiomers of the chiral compounds are often differentiated by biological systems, resulting in different pharmacological, pharmacokinetic or toxicological effects. This has generated tremendous interest in the explanation of stereospecific pharmacokinetics, metabolism and clinical pharmacology of chiral drug molecules. Therefore, to investigate these issues, different analytical approaches have been employed

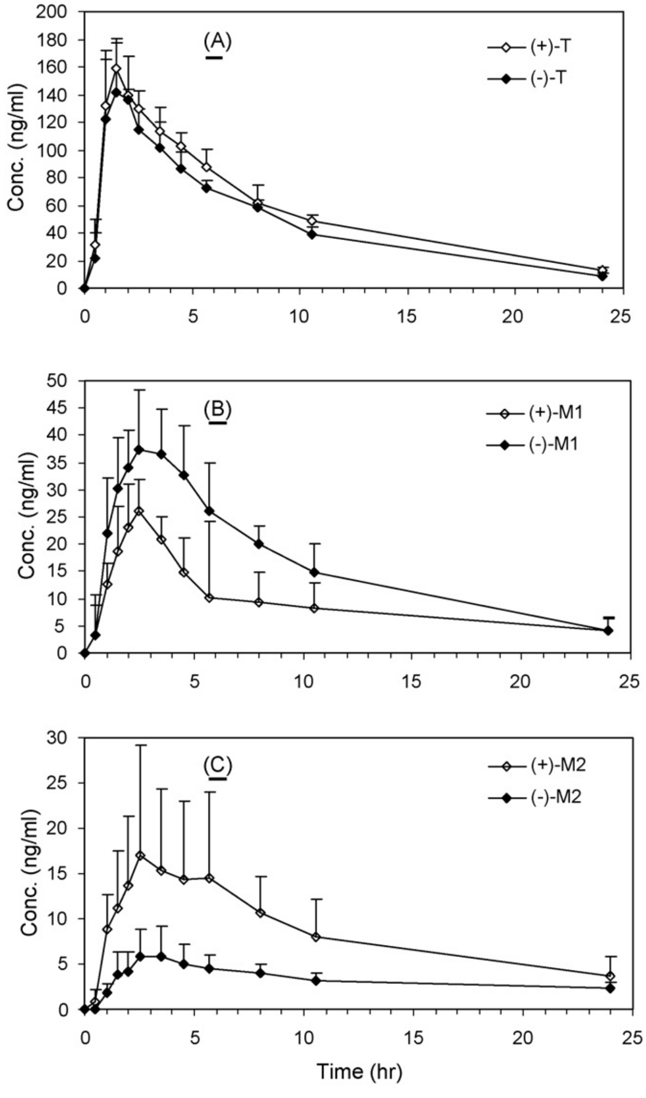

Fig. 3. Mean plasma concentration–time profiles of T (A), M1 (B), and M2 (C) enantiomers in three healthy volunteers after the oral administration of 100 mg of tramadol. Error bars represent standard deviation values.

in the stereoselective determination of chiral compounds such as tramadol and/or its main metabolites in biological samples.

In the last decade, CE was used for the stereoselective determination of tramadol and its metabolites [7,11–14]. Although using CE in stereoselective analysis represents high resolution power with regard to other commonly used methods, it has relatively low-sensitivity due to the short optical path-length for some analytes such as tramadol and its metabolites, which have a weakly absorbing chromophore in their molecules. Because of the sensitivity issues with regard to CE, most of the stereospecific analytical methods for the quantitation of tramadol and/or its metabolites in biological samples use HPLC with Chiralcel OD-R or Chiralpak AD as a chiral stationary phase operating in a reversed- or normal-phase mode, respectively [15–19].

More recently Mehvar et al. reported a sensitive method with a relatively short run time for stereospecific analysis of T and its *O*-demethylated (M1) and *N*,*O*-demethylated (M5) metabolites in human plasma on a Chiralpack AD column in normal-phase mode. However, normal-phase chromatography required the use of high volumes of toxic and expensive organic solvents such as hexane, heptane or isohexane. Additionally, the method did not quantitate the enantiomers of M2 metabolite, which is a major metabolite of T [16].

The reversed-phase chromatography was first used by Ceccato et al. [15] for analysis of tramadol enantiomers in human plasma. Nevertheless, the method was not validated for the metabolites of tramadol. Campanero et al. adapted this method by connecting an achiral column before the chiral column to analyse tramadol and M1 in human plasma. However, only a partial separation of the M2 enantiomers was observed under this chromatographic condition [19]. Afterward they extended their method to the simultaneous measurement of T, M1, and M2 enantiomers by using a longer achiral column at a low temperature (5 °C). However, connecting an achiral and chiral column resulted in relatively long run times of up to 70 min [19].

In the present study, a complete enantiomeric separation of tramadol and its main metabolites was obtained in room temperature using the protein-based AGP column with aqueous phosphate buffer.

To optimize our method, the manufacturer's instruction for the separation of hydrophilic amines (i.e. tramadol and its metabolites) was used as the initial experimental condition (i.e. 10 mM phosphate buffer pH 7.0:isopropanol (5:95)). At first step, the racemic mixture of each analyte was investigated. No retention of all analytes was observed under the above condition. A decrease in organic modifier concentration to 3% resulted in an acceptable retention of all analytes. In this condition, the enantiomers of M1 metabolite were baseline resolved, while no significant resolution was achieved for tramadol and M2 metabolite. A more decrease in organic modifier portion improved the separation of both tramadol and M1 peaks. However, enantiomers of M2 metabolite still were not separated. Substitution of ethanol and methanol instead of isopropanol as organic modifier (in the same concentrations) led to increase the retention times of all analytes (up to 50 min) with no significant enhancement in resolution of M2 metabolite. A comparison between methanol, ethanol and isopropanol suggested that a more hydrophobic part in the modifier structure shortens the retention of analytes. Nevertheless, the power of hydroxyl group to form a hydrogen binding could also affect the enantio-separation of M2. As a consequence excluding all hydroxylic modifiers (i.e. alcohols) and its substitution with acetonitrile resulted in considerable improvement in resolution of M2 enantiomers while maintaining the enantiomeric separation of other analytes. This finding indicates that hydrogen bonding to the secondary amine group of the M2 metabolite is essential for chiral selectivity. Still, tramadol and M1, having a methyl group in that position, did indeed show good chiral recognition also with alcohol modifiers. In a study by Öhman et al. using AGP column for enantiomeric separation of reboxetine and its main metabolite *O*-desethylreboxetine, the importance of hydrogen binding to the phenolic group of the metabolite (not the parent drug) was also emphasized [21]. They explained this by utilizing different binding sites and retention mechanisms for parent drug and its metabolite recalling the Enquist and Hermansson phenomenon for characterization at least two binding sites, a high- and a low-affinity site for the chiral drug terodiline in the AGP protein [22]. A change in conformation of the AGP as a consequence of using acetonitrile instead of isopropanol (hydroxylated modifier) and hence enhancement in setereoselective separation of methadone has also been suggested [23].

At the second step, the simultaneous chiral separation of all analytes was concerned. Therefore, the effects of pH, buffer concentration and additives of the mobile phase on the quality of the chiral separations of the mixture were further investigated. Since the isoelectric point (p*I*) of α-glycoprotein is reported to be 2.7 in phosphate buffer [24], an increase in the pH of mobile phase above p*I* value up to highest safe pH margin of AGP column (pH 7), may cause increment in negatively charged groups on the immobilized protein; whereas a basic solute like tramadol ($pK_a = 8.3$) is positively charged in this pH range, resulting in higher retention and chiral recognition of all the analytes. Therefore, no improvement in chiral separation is expected by decreasing the pH of mobile phase. A decrease in pH of mobile phase from 7 to 5 resulted in decrease in overall run time from 30 to less than 20 min with complete loss of M2 enantiomeric resolution.

The importance of the composition, concentration and ionic strength of buffer component of the mobile phase was also examined by using different salts such as mono- and dibasic sodium or potassium phosphate and diammonium hydrogen phosphate. Although no significant improvement in enantio-separation of M2 metabolite was observed, the separation of the tramadol and M2 metabolite was effectively enhanced using diammonium hydrogen phosphate as buffer in the mobile phase with maximum separation at concentration of 30 mM.

Generally, the resolution and enantioselectivity, especially for M2 metabolite, improved when basic mobile phase additives such as diethylamine, triethylamine or *N*,*N*-dimethylisopropylamine were included in the mobile phase. The best resolution between enantiomers was, however, obtained when triethylamine was utilized. The effect of triethylamine on the enantio-separation of all analytes was investigated in the concentration range of 0.01–0.1 M. The best results were obtained when triethylamine concentration was 0.1 M. The impact of the concentration of TEA on the fluorescence of analytes was not significant possibly due to constant pH provided by buffer in mobile phase. Finally, an acceptable separation, within a reasonable chromatographic run time, was obtained with a mobile phase composition of 30 mM diammonium hydrogen phosphate buffer–acetonitrile–triethylamine (97.9:2:0.1, v/v) adjusted to pH of 7 with phosphoric acid.

In comparison with previously published methods [8,17–19], which have used 20–30% organic solvent and/or relatively high concentrations of expensive chaotropic salts such as sodium perchlorate in the mobile phase, our developed method is environmentally friendly since less than 2% of organic solvent is used in the mobile phase. The method is also able to achieve excellent resolution for T, M1 and M2 in a relatively short run time ($\leq$32 min) by the use of a 15 cm AGP column.

In our study, all analytes are readily extracted into ethyl acetate at basic pH according to the liquid–liquid extraction procedure developed in our laboratory for the non-stereoselective determination of T, M1 and M2 in human plasma [25]. In order to increase the sensitivity of the analysis, a slight modification of this procedure has been carried out.

The plasma concentration–time profiles (Fig. 3) and the pharmacokinetic parameters of T, M1 and M2 enantiomers, estimated by the non-compartmental approach (Table 4) are in good agreement with those reported before, using an identical dosage regimen of 100 mg tramadol [8].

5. Conclusion

In conclusion, a sensitive, accurate, and precise bioanalytical method for simultaneous determination of the individual enantiomers of tramadol and its main metabolites M1 and M2 in human plasma was developed. The assay is based on a simple liquid–liquid extraction of plasma samples and chiral high-performance liquid chromatography, using an aqueous mobile phase with minimal (1%) organic solvent. The developed procedure may be recommended for pharmacokinetic studies as well as for therapeutic drug monitoring.

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