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Simultaneous stereoselective analysis of tramadol and its primary phase I metabolites in plasma by liquid chromatography Application to a pharmacokinetic study in humans

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Abstract

This paper describes a bioanalytical method involving a simple liquid–liquid extraction for the simultaneous HPLC determination of the enantiomers of tramadol, the active metabolite *O*-desmethyltramadol (M1), and the other main metabolite *N*-desmethyltramadol (M2) in biological samples. Chromatography was performed at 5 °C on a Chiracel OD-R column containing cellulose tris(3,5-dimethylphenylcarbamate) as chiral selector, preceded by a achiral end-capped C_8 column (LiChrospher 60-RP-selected B 5 μm, 250 mm × 4 mm). The mobile phase was a mixture of phosphate buffer containing sodium perchlorate (1 M) adjusted to pH 2.5–acetonitrile–*N*,*N*-dimethyloctylamine (74.8:25:0.2). The flow rate was 0.5 ml/min. Fluorescence detection (λ_{ex} 200 nm/λ_{em} 301 nm) was used. Fluconazol was selected as internal standard. The limit of quantitation of each enantiomer of tramadol and their metabolites was 0.5 ng/ml (sample size = 0.5 ml). The chiral conditions and the LC optimisation were investigated in order to select the most appropriate operating conditions. The method developed has also been validated. Mean recoveries above of 95% for each enantiomer were obtained. Calibration curves for tramadol enantiomers (range 1–500 ng/ml), M1 enantiomers (range 0.5–100 ng/ml), and M2 enantiomers (range 0.5–250 ng/ml) were linear with coefficients of correlation better than 0.996. Within-day variation determined on four different concentrations showed acceptable values. The relative standard deviation (R.S.D.) was determined to be less than 10%. This method was successfully used to investigate plasma concentration of enantiomers of tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol in a pharmacokinetic study.

Keywords: Enantiomer separation; Kinetic studies; Tramadol; Desmethyltramadol

1. Introduction

Tramadol hydrochloride {a 1:1 racemic mixture of (+)-tramadol {(1*R*, 2*R*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride} and (−)-tramadol {(1*S*, 2*S*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride} (Fig. 1A) is an orally active, clinically effective, centrally acting analgesic drug with an analgesic efficacy and a potency that ranges between weak opioids and morphine [1]. This drug can be administered orally, rectally, and parentally. This analgesic is rapid and extensively metabolized in the liver mainly in *O*-desmethyltramadol (M1) (Fig. 1A), and mono-*N*-desmethyltramadol (M2), in most species. These primary metabolites may be further metabolized to three additional secondary metabolites namely, *N*,*N*-didesmethyltramadol (M3), *N*,*N*,*O*-tridesmethyltramadol (M4), and *N*,*O*-desmethyltramadol (M5). All metabolites are further conjugated with glucuronic acid and sulfate before excretion in urine [2]. In man, however, tramadol metabolism appears to be rather slow and is affected by tramadol administration route [2]. It is interesting to note that both *O*-demethylation and *N*-demethylation catabolic tramadol reactions were found to be highly stereoselective in vitro [3,4]. Consequently, differences in the pharmacokinetics profile of the enantiomers of M1 and M2 can be expected when tramadol is administered to human subjects. This is currently under investigation in different laboratories [5]. With this objective different analytical approaches have been employed in the stereoselective determination of tramadol and/or its main metabolites in biological samples.

Recently, a number of capillary electrophoresis (CE) methods for the stereoselective determination of tramadol and its metabolites have also been developed [6–10]. In the last years, this analytical technique has revealed an

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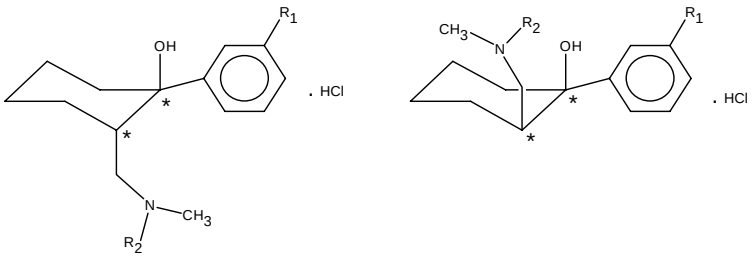
(A)



Tramadol	R_1: OCH_3	R_2: CH_3
O-desmethyltramadol	R_1: OH	R_2: CH_3
N-desmethyltramadol	R_1: OCH_3	R_2: H

(B)

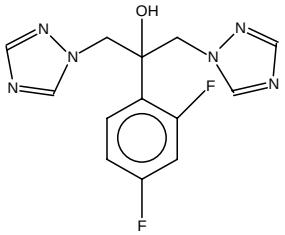


Fig. 1. Chemical structures of (A) tramadol and its main phase I metabolites *O*-desmethyltramadol and *N*-desmethyltramadol and (B) fluconazole.

enormous separation potential for the determination of drugs in biological fluids because of its high efficiency and flexibility and very high resolution. CE also exhibits excellent results in the field of optical isomer separation. It is interesting to note that CE enantioseparation is performed in the direct separation mode, by means of the addition of a chiral selector (generally neutral and charged cyclodextrins derivatives) to the background electrolyte. Although CE enantioseparation has some advantages with respect to other commonly used techniques, it is a relatively low-sensitivity analytical technique due to the short optical path-length afforded by the small internal diameters of the capillaries. As tramadol and its metabolites contain a weakly absorbing chromophore in their molecules, CE with on-column ultraviolet detection is inadequate for the determination of their concentrations in plasma samples. Other detection techniques, such as electrospray mass spectrometry [9] and laser-induced native fluorescence detection [10], has been recently used coupled to CE with these objective. However, the previously cited equipment is not commonly available in most analytical laboratories.

Other analytical method used so far for the determination of tramadol enantiomers and its main phase I metabolites in different biological fluids is the high-performance liquid chromatography with ultra-violet [11,12], fluorescence [12–14] and MS detection [15]. The enantiomeric separation of tramadol and its metabolites M1 and M2 was performed using chiral stationary phases (CSP). Cellulose and amylose derivatives were the chiral selectors that were widely used in these analytical methods. These chiral stationary phases were previously used, either in normal- [11,13,15] and reversed-phase chromatographic mode [12,13] for the determination of enantiomers in biological fluids obtained in pharmacokinetic studies.

Tramadol and its metabolites were profiled, characterised, identified and determined in different biomatrices, such as urine [7–10,11], plasma [9,12–15] and in the microsomal fraction of the hepatocytes [16]. A sample handing step is usually necessary prior to liquid chromatographic analysis of drugs in biological fluids in order to remove interfering compounds and to increase the selectivity and sensitivity of the analytical method. The sample preparation step is particularly important when an enantioselective analysis is performed using a chiral stationary phase [17]. In most methods reported for the determination of tramadol in biofluids, the sample clean-up of the biological sample was based on liquid–liquid extraction [8,9,11,13]. An alternative to this tedious and time-consuming extraction technique is the solid-phase extraction using disposable extraction cartridges and automated procedures [7,12,15].

Despite of the great number of chiral HPLC and CE developed methods to the date, only four methods [13–16] have been employed in stereoselective pharmacokinetic studies after oral or intravenous administration of tramadol in humans. In none of them *N*-desmethyltramadol was determined in biological samples obtained from patients or human healthy volunteers.

This communication describes a bioanalytical enantioselective method involving a simple liquid–liquid extraction procedure of plasma samples and subsequent determination of tramadol enantiomers and their main metabolites, *O*-desmethyltramadol and *N*-desmethyltramadol. Fluorescence coupled to chiral reversed-phase liquid chromatography was selected in order to obtain a selective and sensitive determination. The chiral liquid chromatographic conditions were investigated in order to select the most appropriate operating conditions. The developed and validated analytical method was applied to stereoselective pharmacokinetic studies of tramadol and its main phase I metabolites in humans.

2. Experimental

2.1. Chemicals, reagents and solutions

Tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol enantiomers were a gift from Grünenthal (purity >99%) (Stolberg, Germany). Fluconazole (purity >99%) (selected as internal standard) was provided by Pfizer (New York, USA). Acetonitrile, methanol, *tert*-butyl methyl ether, (HPLC grade), hydrochloric acid, phosphoric acid (85%), sodium hydrogenphosphate monohydrate, and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany). Sodim perchlorate and *N*,*N*-dimethyloctylamine were purchased by Aldrich (Madrid, Spain). Ultra-high quality water (obtained using a Milli-Q apparatus, Millipore, Bedford, MA, USA) was used for chromatography.

2.2. Standard solutions and samples

Stocks solutions of (+)-tramadol, (−)-tramadol, (+)-M1, (−)-M1, (+)-M2 and (−)-M2 with a concentration of 1 mg/ml, were prepared separately by dissolving 10 mg of each analyte in water. Intermediate stock standards of 100 μg/ml were prepared using 50% methanol in water as solvent.

Eight standard solutions of each enantiomer of tramadol and its main phase I metabolites (10–5000 ng/ml for (+)-tramadol, (−)-tramadol; 5–1000 ng/ml for (+)-M1, (−)-M1; 5–2500 ng/ml for (+)-M2, (−)-M2) were made by further dilution of the intermediate stock solution with appropriate volumes of water. The standard solution of fluconazole (1 μg/ml) was similarly prepared. Standard and stock solutions and of (+)-tramadol, (−)-tramadol, (+)-M1, (−)-M1, (+)-M2 and (−)-M2 and fluconazole were stored at −80 °C.

Mixed calibration pools of (+)-tramadol, (−)-tramadol, (+)-M1, (−)-M1, (+)-M2 and (−)-M2 were prepared by diluting appropriate volumes of each standard solution with plasma to achieve concentrations ranging from 1 to 500 ng/ml for tramadol enantiomers, 0.5–100 ng/ml for (+)-M1, (−)-M1, and 0.5–250 ng/ml for (+)-M2, (−)-M2.

Pools of quality control samples were prepared by spiking drug-free human plasma with the different working standard solutions of (+)-tramadol, (−)-tramadol, (+)-M1, (−)-M1, (+)-M2 and (−)-M2. The concentrations of each enantiomer of tramadol in these solutions were 1, 10, 30 and 300 ng/ml. The corresponding concentrations of (+)-M1 and (−)-M1 were 0.5, 1.5, 15 and 80 ng/ml. Finally, 0.5, 1.5, 15 and 75 ng/ml were the plasmatic concentrations of (+)-M2 and (−)-M2 in the quality control samples. Pools are measured into 1.2 ml aliquots in propylene tubes and frozen at −80 °C until use.

2.3. Sample preparation

Plasma samples (0.5 ml) were transferred to 13 mm × 100 mm conic tubes and spiked with the internal standard (50 μl of 1000 μg/ml of fluconazole). Then, 2 ml Tris buffer (pH 11, 0.01 M) was added to the tubes. After vortex-mixed well for 10 s, 6 ml of *tert*-butyl methyl ether was added to the sample tubes. The tubes were capped, vortex-mixed for 1 min, and centrifuged at 2000 × *g* and 1 °C for 10 min. Finally, the obtained supernatant was transferred to a clean tube and dried under vacuum pressure (Vortex evaporator, Labconco) at 40 °C for 15 min. The dried extracts were reconstituted in 250 μl of mobile phase, transferred to limited volume autosampler vials, capped and placed on the HPLC autosampler. A 100 μl-aliquot of the supernatant was injected onto HPLC column.

2.4. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1100 series LC coupled with a fluorescence diode array detector [excitation wavelength (λ_{ex}) 200 nm/emission wavelength (λ_{em}) 301 nm] (Hewlett-Packard, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA program. Separation was carried out at 5 ± 0.1 °C on a reversed-phase, 250 mm × 4 mm column packed with C_8, 5 μm silica reversed-phase particles (LiChrospher Select B) obtained from Merck. This achiral column was coupled to a chiral stationary phase. The CSP used for the determination of enantiomers was a Chiralcel OD-R column (250 mm × 4.6 mm i.d.) containing cellulose *tris*-(3,5-dimethylphenylcarbamate) coated on silica (10 μm) from Daicel (Tokyo, Japan). For the determination of suitable conditions for the enantioseparation of tramadol and its metabolites, different mobile phases were tested.

The mobile phase finally used was a mixture of phosphate buffer (0.05 M) containing sodium perchlorate (1 M) pH 2.5–acetonitrile–*N*,*N*-dimethyloctylamine (74.8:25:0.2). Separation was achieved by isocratic solvent elution at a flow-rate of 0.5 ml/min.

2.5. Quantitation

Each calibration curve consisted of eight calibration points [1–500 ng/ml for (+)-tramadol, (−)-tramadol; 0.5–100 ng/ml for (+)-M1, (−)-M1; 0.5–250 ng/ml for (+)-M2, (−)-M2]. Calibration curves were determined by least square linear regression analysis (weighting $1/X^2$). Peak area ratio of each enantiomer of tramadol, M1 or M2 and fluconazole versus the corresponding analyte concentration was plotted. The linearity of the method was statistically confirmed by comparing the slopes, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student's *t*-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones.

2.6. Validation

The method was validated by analysis of human plasma quality control samples prepared at four concentrations spanning the calibration range as the same form that has been previously indicated. Three samples of each quality control pool, and calibration samples were analyzed on six different days. On day 1, the number of samples each quality control pool was five. Precision and accuracy were determined. Precision of a method is expressed as the percentage of the relative standard deviation (R.S.D.) of replicate measurements. Accuracy was measured according to the following equation:

$$\text{Percentage difference from theoretical value} = \left[\frac{X - C_T}{C_T}\right] \times 100$$

where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The overall recovery for each enantiomer of tramadol and its main phase I metabolites was calculated by comparing the peak area ratios of spiked samples with those obtained by direct injections of the same amount of both compounds.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of each enantiomers of tramadol, M1, M2 and the internal standard fluconazole.

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. The limit of quantification (LOQ) was defined as the lowest drug concentration, which can be determined with an accuracy and precision <20%. In this work, the LOD and LOQ of the assay method was determined by analysis of the peak baseline noise in 10 blank samples.

The stability of enantiomers of tramadol an its main phase I metabolites in both frozen whole-blood samples (−80 °C) over 3 months, and in processed samples left at room temperature (20 ± 3 °C) over 24 h, was also studied.

2.7. Application of the method

The developed method has been applied to a pharmacokinetic study in which the concentrations of tramadol and M1 were measured in more than 900 plasma samples. The study was approved by the state authority and the institutional ethics committee. In this study, 24 healthy volunteers (12 males and 12 females) received a single oral dose of tramadol (100 mg). Venous blood samples of 5 ml volume were withdrawn from the cubital vein into heparinized tubes immediately prior to dosing and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 36, and 48 h after drug administration. The samples were centrifuged immediately at 3000 rpm for 10 min. The plasma was separated and stored in polypropylene tubes at −80 °C until analysis.

3. Results and discussion

3.1. Liquid chromatographic conditions

Today, chiral high-performance liquid chromatography has proven to be one of the best methods for the direct separation and analysis of enantiomers. In particular, the use of chiral stationary phases as chiral selectors provides us fast and accurate methods for chiral separation. However, sometimes choosing the right chiral stationary phase for the enantiseparation of chiral compound is difficult. Generally, most chiral separations achieved on chiral stationary phases were obtained based upon the accumulated trial-and-error knowledge of the analyst, intuition, and often simply by chance. All three components, analyte, chiral stationary phase, and mobile phase must be taken into consideration.

As reported in previous studies [11,13,15], the complete enatiomeric separation of tramadol and its main phase I metabolites was obtained on a amylose tris(3,5-dimethylphenylcarbamate) (Chiralpack AD) stationary phase used 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. In a previous paper we have developed and validated a chiral HPLC method that provides the simultaneous separation of tramadol and M1 enantiomers using in reversed phase mode a cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (Chiralcel OD-R). Nevertheless, only a partial separation of the M2 enantiomers was observed under these chromatographic conditions.

Chiralpack AD and Chiralcel OD-R are the most useful polysaccharide-based stationary phases in the organic,

biorganic and pharmaceutical fields [18]. Chemically, these CSPs show a higher order structure that provides multiple alternative chiral recognition sites. As shown in several previous studies, the N–H and C=O groups of polysaccharide are involved in intramolecular hydrogen-bonding between different carbamate fragments of the polysaccharide phenylcarbamate, given an helical space structure with polar carbamate groups nicely located inside, and hydrophobic aromatic groups outside. The enantiomeric separation is based on the formation of transistent diastereomeric complexes between the functional groups of chiral analytes and the remaining free N–H and C=O groups for the higher order structure of those polysaccharide phenylcarbamates. Usually, these diastereomeric complexes are formed by hydrogen bond, and dipole–dipole and π–π stacking interactions [19]. The chiral recognition abilities of phenylcarbamates of polysaccharides are greatly influenced by the substituents on the phenyl moieties, since the substituents modify the polarity of the carbamate groups. In Chiralpack AD stationary phases enantiomers effectively interact with the polar carbamate groups via hydrogen bonding with the NH and C=O groups and dipole–dipole interaction on the C=O [20], whereas the latter is no longer critical for chiral discrimination in Chiralcel OD-R stationary phases. In the last case, the separation system is governed by hydrophobic π–π interactions between the phenyl group of a chiral stationary phase and an aromatic group of the solute [21,22].

The choice of an appropriate eluent is the most important part of the method development and optimisation. In order to determine suitable analytical conditions the effect of several analytical parameters such as nature of organic modifiers (diethylamine, triethylamine and *N*,*N*-dimethyloctylamine were tested), concentration of sodium perchlorate in the mobile phase, type percentage of organic solvent, or temperature on the quality of the separations of the enantiomers of tramadol and its is main metabolites were investigated.

As was mentioned earlier, Ciralcel OD-R is a hydrophobic stationary phase without functional groups in the polysaccharide susceptible to interact with the charged analytes. Therefore, it is of primary importance to keep the analyte neutral when working with polysaccharide reversed-phase CSPs to achieve a good separation. Two approaches can be employed to fulfil this objective. One involves the suppression of dissociation of the analyte and the other involves ion pair formation of the analyte with a suitable chosen counter ion. In this work the physicochemical characteristics of tramadol and its main metabolites, weak basic compounds with pK_a values near to 9, make the first approach unapproachable. Therefore, it is preferable to develop separation conditions at acidic pH, under basic analytes are positively charged, and add a considerable amount of anions in the mobile phase to form an ion pair which can be considered to be electrically neutral. Some previous studies have shown that the addition of a chaotropic salt, such as sodium perchlorate, to the mobile phase was very useful with this objective when Chiralcel OD-R column was used as CSP. An increase in sodium perchlorate concentrations in the mobile phase led to a significant improvement in enantioselectivity and resolution with a moderate increase in the capacity ratios of tramadol, M1 and M2 enantiomers. Chaotropic salts also have the property to disrupt the water structure by breaking up the hydrogen bonds and other hydrophobic interactions [19]. Therefore, their addition to the mobile phase probably decreased the amount of the carbamate fragments involved in the intramolecular hydrogen bonds, increasing the interactions between the chiral analytes and the CSP. The latter concentration evaluated, 1.0 M, was selected as sodium perchlorate concentration in the mobile phase.

As the same form, the resolution and estereoselectivity showed an increase when an organic modifier was included in the mobile phase composition. Three organic modifiers were tested in order to enhance the separation of tramadol, M1 and M2 enantiomers: diethylamine, triethylamine and *N*,*N*-dimethyloctylamine. The best resolution between enantiomers was more easily obtained when *N*,*N*-dimethyloctylamine was utilised ($R_s > 2.5$). This is due to most of the commercially available derivatized cellulose and amylose CSPs are adsorbed into a macroporous silica gel support. Consequently, strong interactions between the accessible silanol groups of the packing material and the basic groups of tramadol and its metabolic derivatives, positively charged between pH 2.5 and 8, can be yield. Tailing peaks and longer retention times are then observed without these mobile phase components. Organic phase modifiers essentially deactivated the chiral stationary phase silanol free groups resulting in less peak tailing and therefore, better resolution and enantioselectivity.

The effect of pH and the presence of organic solvents on the enantioseparation were also investigated in presence of sodium perchlorate and *N*,*N*-dimethyloctylamine. As can be observed in previous works [12], the retention of enantiomers of tramadol and their metabolites was only slightly increased with pH without significant changes in enantioselectivity. An increasing effect on the retention factor was observed when the amount of acetonitrile in the mobile phase is reduced. Probably, this effect is due to an increase in hydrophobic interactions with the chiral stationary phase.

Variation of temperature also affects the enantioselectivity of the chiral stationary phase. As temperature decreases the retention and enantioselectivity were increased, obtaining the best values of enantioselectivity at 5 °C, the minimum temperature that can be supported by the chiral column. An decrease of temperature probably affect the multiple type of interactions occurring between the analyte and the polysaccharide CSPs generate conformational changes that leads to an improvement in selectivity and resolution [18].

A complete enantioseparation of tramadol enantiomers and its main phase I metabolites was obtained using the Chiralcel OD-R stationary phase with a mobile phase compound by a mixture of phosphate buffer (0.05 M) containing sodium perchlorate (1 M) pH 2.5-acetonitrile and *N*,*N*-dimethyloctylamine (74.8:25:0.2). However, the (+)

enantiomers of tramadol and M2 were coeluted under these chromatographic conditions. In order to increase the chromatographic resolution we have coupled to the chiral stationary phase an achiral reverse-phase. A Merck column containing LiChrospher RP-Select B (250 mm × 4 mm) was chosen. LiChrospher RP-Select B is a reversed-phase column packed with a monofunctional octylsilane phase bonded to extremely pure spherical porous silica particles with a 6 nm pore size. In this column, the silica material has been optimised in order to avoid any ionic interactions with basic substances. Therefore, basic compounds would be eluted as symmetric chromatographic peaks from these columns. Under these chromatographic conditions, the retention time of tramadol enantiomers were 51.0 and 57.1 min, respectively, while 53.9 and 62.6 min were the retention time of (+)-*N*-desmethyltramadol, (−)-*N*-desmethyltramadol. The order of elution of enantiomers was determined by separately injecting solutions of each anantiomer. As can be seen, in the chromatographic conditions described above (+)-tramadol and (+)-*N*-desmethyltramadol eluted before their respective (−)-enantiomers, with R_s value upper to 1.85. The R_s values between tramadol enantiomers, and between their respective primary metabolite M2 were 5.82 and 4.21, values that reflected the high degree of selectivity that show the chromatographic developed method. A complete enantioseparation of M1 was also obtained under these chromatographic conditions with retention times for (+)-*O*-desmethyltramadol and (−)-*O*-desmethyltramadol of 24.5 and 26.2 min. The symmetry factor of all peaks ranged from 0.8 to 0.97, values near to 1. Fig. 2 displays the chromatograms of plasma

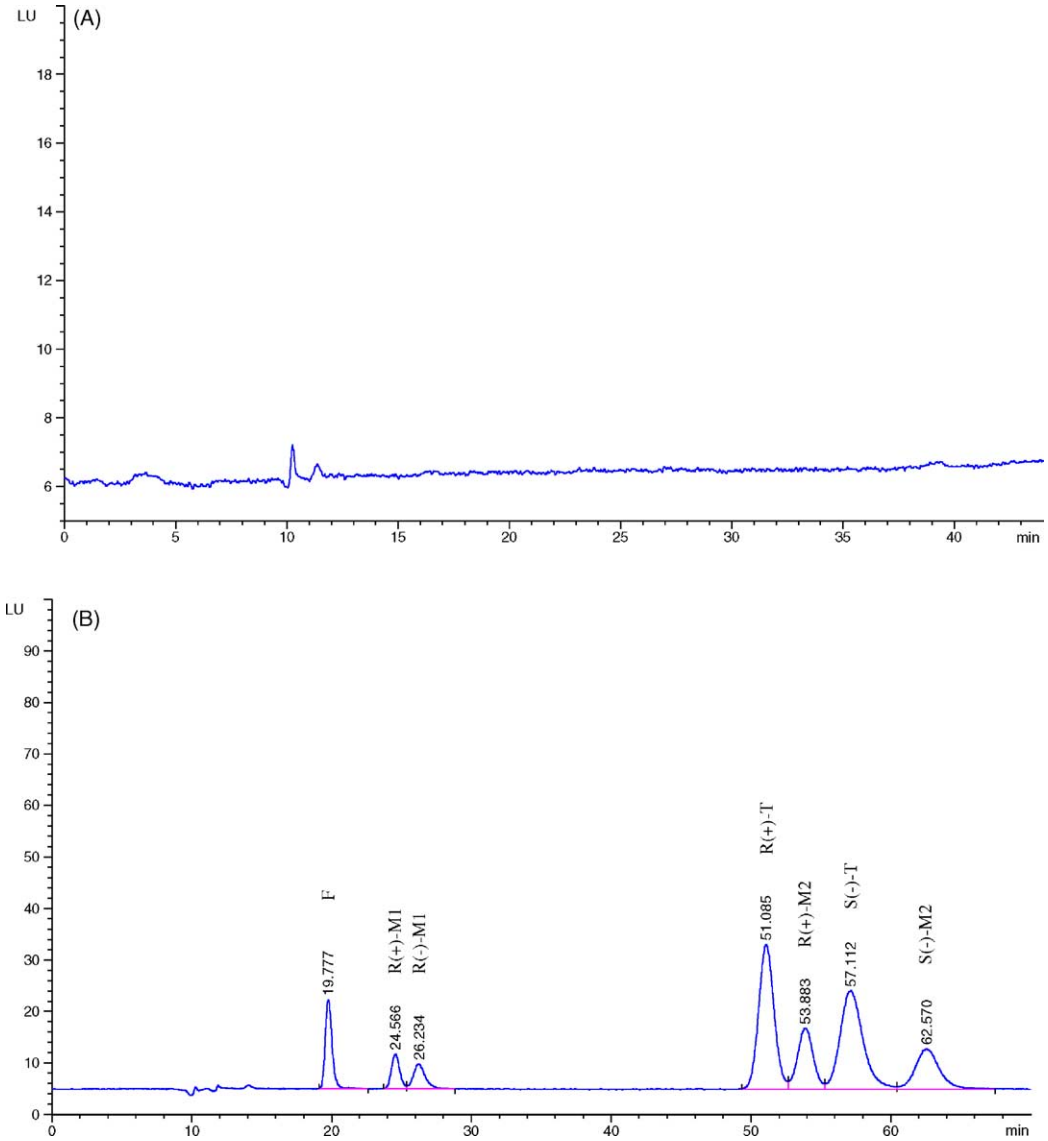


Fig. 2. Chromatograms of (A) blank human plasma, (B) blank human plasma spoked with *R*(+)-tramadol (100 ng/ml), *S*(−)-tramadol (100 ng/ml), *R*(+)-*O*-desmethyltramadol (10 ng/ml), *S*(−)-*O*-desmethyltramadol (10 ng/ml), *R*(+)-*N*-desmethyltramadol (10 ng/ml), *S*(−)-*N*-desmethyltramadol (10 ng/ml), and fluconazole (internal standard), and (C) resulting from the analysis human plasma samples obtained at 24 h (15.3 ng/ml *R*(+)-tramadol, 8.2 ng/ml *S*(−)-tramadol, 2.1 ng/ml *R*(+)-*O*-desmethyltramadol, 3.2 ng/ml *S*(−)-*O*-desmethyltramadol, 6.1 ng/ml *R*(+)-*N*-desmethyltramadol, 3.3 ng/ml *S*(−)-*O*-desmethyltramadol) from a subject who received a single oral dose of Adolonta (100 mg).

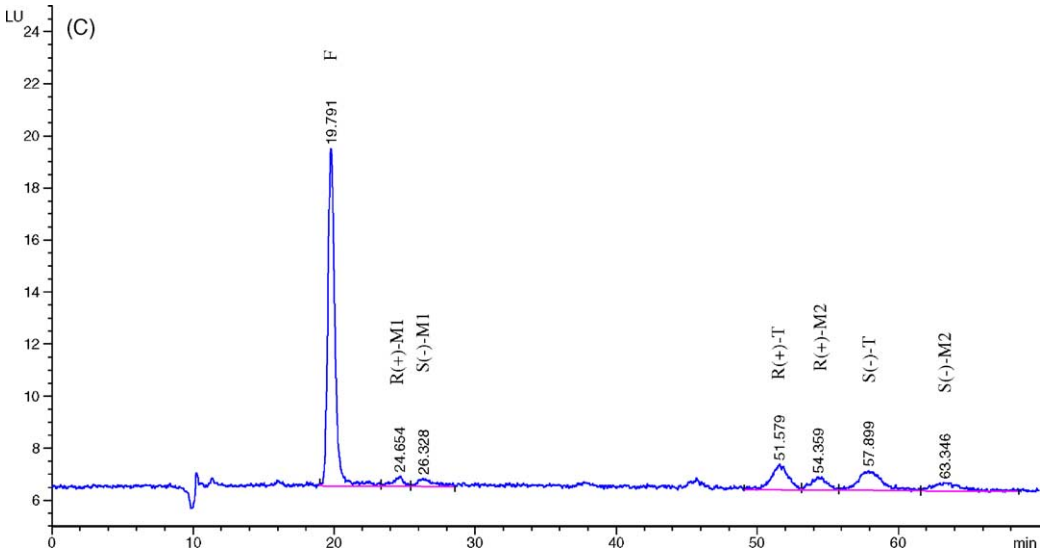


Fig. 2. (*Continued*).

extracts of a healthy volunteer given orally tramadol. Other tramadol metabolites, as *N*,*N*-didesmethyltramadol (M3), *N*,*N*,*O*-tridesmethyltramadol (M4), and *N*,*O*-desmethyltramadol (M5) were not detected in the conditions previously described, since they can only be extracted from plasma by more polar organic solvents than *tert*-butyl methyl ether. The stereospecifcity of the method was also investigated using plasma samples fortified with amounts of a single enantiomer of tramadol, M1 and M2. In all cases only <1% of an enantiomer was measured as the opposite enantiomer. In addition, no other potentially separable diasteromers of tramadol and its main phase I metabolites have been detected when standard solutions of a unique enantiomer were injected into the HPLC.

The absence of interfering endogenous components at the retention times of the enantiomers of tramadol, M1 and M2

Table 1
Standard calibration curves of tramadol enantiomers and their main phase I metabolites M1 and M2 in plasma samples

	r	*a*	*b*	*r*	*a*	*b*
	R(+)-Tramadol			*S*(−)-Tramadol		
n	6	6	6	6	6	6
Mean	0.9987	0.044	0.004	0.9980	0.043	0.006
S.D.	0.0003	0.003	0.005	0.0006	0.002	0.003
R.S.D. (%)	0.03	6.53		0.06	5.58	
	R(+)-M1			*S*(−)-M1		
n	6	6	6	6	6	6
Mean	0.9984	0.028	0.001	0.9980	0.028	0.004
S.D.	0.0013	0.001	0.003	0.0015	0.002	0.002
R.S.D. (%)	0.13	4.19		0.15	5.84	
	R(+)-M2			*S*(−)-M2		
n	6	6	6	6	6	6
Mean	0.9985	0.042	0.003	0.9974	0.044	0.005
S.D.	0.0009	0.002	0.003	0.0021	0.003	0.009
R.S.D. (%)	0.09	4.61		0.21	6.48	

r: Correlation coefficient; *a*: slope; *b*: intercept.

Table 2
Accuracy of the method, expressed as relative error in %, for the determination of enantiomers of tramadol, M1, and M2 in plasma

Accuracy ($n = 5$)		
Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Accuracy (%)
R(+)-Tramadol		
1	0.96 ± 0.08	−4.00
3	2.92 ± 0.18	−2.67
30	30.02 ± 1.05	0.07
300	299.25 ± 11.68	−0.25
S(−)-Tramadol		
1	0.98 ± 0.09	−2.00
3	3.04 ± 0.26	1.33
30	30.85 ± 1.27	2.83
300	297.52 ± 15.21	−0.83
R(+)-*O*-Desmethyltramadol		
0.5	0.48 ± 0.06	−4.00
1.5	1.53 ± 0.14	2.00
15	15.32 ± 1.06	2.13
75	74.28 ± 4.25	−0.96
S(−)-*O*-Desmethyltramadol		
0.5	0.44 ± 0.06	−12.00
1.5	1.58 ± 0.21	5.33
15	16.25 ± 1.58	8.33
75	76.05 ± 3.28	1.40
R(+)-*O*-Desmethyltramadol		
0.5	0.48 ± 0.06	−4.00
1.5	1.53 ± 0.14	2.00
15	15.32 ± 1.06	2.13
75	74.28 ± 4.25	−0.96
S(−)-*O*-Desmethyltramadol		
0.5	0.44 ± 0.06	−12.00
1.5	1.58 ± 0.21	5.33
15	16.25 ± 1.58	8.33
75	76.05 ± 3.28	1.40

in blank plasma extracts allows to show the higher selectivity degree of the developed chromatographic method. Mainly, the fluorescence properties of the analytes are responsible of this effect. Based on the excitation and emission fluorescence spectra of the analytes the detection was performed at λ_{ex} 200 and λ_{em} 301 nm, wavelengths to fluorescence response of endogenous plasma components are negligible.

3.2. Validation

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, stability, and applicability to pharmacokinetic studies. The response function between the response (y) and the corresponding concentration of tramadol, *O*-desmethyltramadol and *N*-desmethyltramadol enantiomers (x), was linear over different ranges in the samples. The regression equations and determination coefficients are presented in Table 1. For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves, and R.S.D.s were computed. The obtained values were lower than 3.9 and 6.7% for (+)- and *S*(−)-tramadol, 3.8 and 3.7% for (+)- and (−)-*O*-desmethyltramadol, and finally, 2.8 and 3.5% for (+)- and (−)-*N*-desmethyltramadol. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. The linearity of the analytical method was assessed by the development of a least square regression of the back-calculated concentrations versus the nominal ones for each enantiomer of tramadol, *O*-desmethyl tramadol and *N*-desmethyl tramadol. In all cases, the regression model provided a unit slope and intercept equal to 0 (Student's *t*-test). The extraction recoveries of each enantiomer in plasma over the respective entire concentration range were 94.5 ± 2.3 and 95.2 ± 2.6% for (+) and (−)-tramadol, 91.3 ± 2.0 and 90.3 ± 1.1% for (+) and (−)-*O*-desmethyltramadol, and 97.5 ± 3.0 and 96.4 ± 3.0% for (+) and (−)-*N*-desmethyltramadol. The recovery of internal standard, fluconazole, was quite similar (93.6 ± 2.1%).

Accuracy values were within acceptable limits for tramadol, M1 and M2 enantiomers (Table 2). The results for within-day and between-day precision for our sample are

Table 3
Between and within-day variability of the HPLC method for the determination of enantiomers of tramadol, M1, and M2 in plasma

Concentration added (ng/ml)	Within-day variability ($n = 5$)		Between-day variability ($n = 18$)	
	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)
R(+)-Tramadol				
1	0.96 ± 0.08	8.3	1.05 ± 0.12	11.7
3	2.92 ± 0.18	6.2	2.91 ± 0.23	7.9
30	30.02 ± 1.05	3.5	30.05 ± 1.48	4.9
300	299.25 ± 11.68	3.9	299.76 ± 18.93	6.3
S(−)-Tramadol				
1	0.98 ± 0.09	9.2	1.03 ± 0.12	11.9
3	3.04 ± 0.26	8.6	3.00 ± 0.28	9.4
30	30.85 ± 1.27	4.1	31.40 ± 2.59	4.7
300	297.52 ± 15.21	5.1	311.38 ± 19.37	6.2
R(+)-*O*-Desmethyltramadol				
0.5	0.48 ± 0.06	12.5	0.47 ± 0.07	14.9
1.5	1.53 ± 0.14	9.2	1.54 ± 0.09	6.0
15	15.32 ± 1.06	6.7	14.95 ± 1.05	7.1
75	74.28 ± 4.25	5.7	74.96 ± 2.50	3.3
S(−)-*O*-Desmethyltramadol				
0.5	0.44 ± 0.06	13.6	0.53 ± 0.07	13.2
1.5	1.58 ± 0.21	13.3	1.54 ± 0.08	5.4
15	16.25 ± 1.58	9.7	14.44 ± 1.19	8.2
75	76.05 ± 3.28	4.3	74.81 ± 2.08	2.8
R(+)-*N*-Desmethyltramadol				
0.5	0.53 ± 0.04	7.6	0.54 ± 0.07	14.6
1.5	1.47 ± 0.13	8.8	1.48 ± 0.11	7.6
15	14.25 ± 1.25	8.8	15.74 ± 0.58	3.7
75	77.84 ± 3.55	4.6	71.61 ± 5.61	7.8
S(−)-*N*-Desmethyltramadol				
0.5	0.47 ± 0.05	10.6	0.54 ± 0.07	13.0
1.5	1.55 ± 0.15	9.7	1.50 ± 0.09	5.8
15	15.65 ± 1.38	8.8	15.80 ± 1.16	7.4
75	76.25 ± 3.12	4.1	72.52 ± 4.85	6.7

Table 4
Pharmacokinetic parameters of the enantiomers of tramadol and their main phase I metabolites *O*-desmethyltramadol (M1) and *N*-desmethyltramadol (M2) after oral administration of 100 mg of Adolonta to 24 healthy volunteers

	C_{max} (mg/l)	t_{max} (h)	AUC (mg h l^{-1})	MRT (h)	$t_{1/2}$ (h)
(+)-Tramadol	0.12 ± 0.03	1.9 ± 0.7	1.16 ± 0.56	8.9 ± 2.3	5.4 ± 2.1
(−)-Tramadol	0.10 ± 0.03	2.1 ± 0.7	0.90 ± 0.36	8.1 ± 1.6	4.6 ± 1.6
(+)-M1	0.025 ± 0.02	3.4 ± 1.9	0.27 ± 0.12	10.8 ± 4.1	9.5 ± 5.2
(−)-M1	0.034 ± 0.01	2.7 ± 1.3	0.35 ± 0.11	10.4 ± 3.3	7.0 ± 2.0
(+)-M2	0.015 ± 0.17	4.1 ± 2.8	0.15 ± 0.49	10.3 ± 6.2	8.8 ± 7.2
(−)-M2	0.007 ± 0.01	3.1 ± 1.3	0.04 ± 0.13	5.7 ± 4.3	5.0 ± 4.1

C_{max}: maximum concentration in plasma after tramadol administration; AUC: area under the plasma concentration–time course, $t_{1/2}$: half-life of drug; MRT: mean residence time.

presented in Table 3 and the values were below to 9.4% for calibrators above LOQ. For LOQ the values obtained were below 14.9%. Then, the obtained values for the precision were also acceptable.

The LOD for the enantiomers of tramadol, M1 and M2 were found to be 0.18 and 0.16 ng/ml for (+)-tramadol, (−)-tramadol, 0.08 and 0.06 ng/ml for (+)-M1, (−)-M1, and 0.15 and 0.16 ng/ml for the (+) and (−) enantiomers of M2 ($S/N = 3$). The estimated LOQs was calculated as low as the lower values of each calibration range (0.5 ng/ml for M1 and M2 enantiomers, 1 ng/ml for tramadol eantiomers). These values were confirmed for each analite in our samples obtaining R.S.D. values below to 20%.

No significant degradation of tramadol and *O*-desmethyltramadol enantiomers were observed in frozen plasma samples (−80 °C) for at least 1 month, and in plasma samples and stock solutions over 24 h at 18 °C [15]. The stability of *N*-desmethyltramadol enantiomers in stock solutions and plasma samples at 18 °C, processed samples left at 4 ± 0.3 °C, and plasma samples storage at −80 °C was also studied for our laboratory. No significant degradation was also observed under these conditions at time stability has been evaluated (24 h for ambient temperature, 1 month for samples stored at 80 °C).

3.3. Application of the method

The applicability of this method has been demonstrated in vivo by the determination of the enantiomers of tramadol and its main phase I metabolites, *O*-desmethyltramadol and *N*-desmethyltramadol in plasma samples from healthy subjects receiving a single oral dose. Pharmacokinetic parameters estimated by the non-compartmental approach are listed in Table 4. The obtained values were comparable to those reported for the enantiomers of tramadol and *O*-desmethyltramadol enantiomers in a previous study [23]. The sensitivity of the analytical method was adequate for the determination of each enantiomer along four times of their respective mean half-life. The observed values of the pharmacokinetic parameters were comparable to those reported for sirolimus in previous studies. The mean maximum concentration of each enantiomer of tramadol was 88 times higher than the LOQ. For M1 and M2 enantiomers the values were 70 and 48 time higher, respectively.

4. Conclusions

A sensitive, accurate and precise bioanalytical method involving a simple liquid–liquid extraction of plasma samples and chiral high-performance liquid chromatographic determination of tramadol enantiomers and their principal metabolites was developed and validated to meet the requirements of the pharmacokinetic investigations of these chiral compounds. The procedure developed was applied to an eanatioselective pharmacokinetic study of tramadol and its metabolites *O*-desmethyltramadol and *N*-desmethyltramadol in human healthy volunteers.

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